ABSTRACT

Title of Dissertation: BACTERIAL GROWTH AND INACTIVATION IN HETEROGENEOUS ENVIRONMENT

Shawn Tsai, Doctor of Philosophy, 2023

Dissertation directed by: Dr. Rohan V. Tikekar, Associate Professor, Department of Nutrition and Food Science, UMCP

This dissertation explores the growth and inactivation of *Salmonella enterica* Serovar Typhimurium in oil-in-water emulsion systems, with a particular focus on the effects of emulsifier type, oil fraction, temperature, molecular weight, and surface charge. First, investigation was conducted on the effects of emulsifier type (Tween 20, Tween 80, Triton X-100) and oil content (20%, 40%, 60%) on growth and thermal inactivation of *S*. Typhimurium in emulsions. The results indicated that while emulsifiers did not affect the bacterial growth rate or lag phase, the presence of high oil content (60%) prolonged the lag phase in emulsions. In addition, Tween 80 and Triton X-100 emulsifier solutions exhibited protective effects against thermal inactivation. Next chapter was focused on evaluating the impact of temperature on growth (7, 22, 37°C) and inactivation (55, 58, 60°C) kinetics of *S*. Typhimurium

in emulsion systems with same emulsifier and oil fraction as previous chapter. The results indicated that temperature had a significant impact on bacterial kinetics, with increasing temperatures leading to faster growth and inactivation rates.

Next, the effect of emulsifier molecular weight and surface charge on the growth and thermal inactivation of *S*. Typhimurium in emulsions was examined. To control the molecular weight with similar structure, whey protein was selected for experimentation. By adjusting the pH, it was possible to change the surface charge in whey protein. Results indicated that whey protein hydrolysate (WPH) with a lower molecular weight did not exhibit a lag phase in *Salmonella* growth. However, whey protein isolate (WPI) with higher molecular weight demonstrated no difference in the lag phase when compared to bacterial growth in TSB. Similar effects were observed with a positively charged emulsifier (WPI^+). The findings suggest that the molecular weight of emulsifiers has a more significant impact on bacterial growth than their surface charge. Regarding the evaluation of inactivation, emulsifier solutions exhibited no significant difference compared to TSB, while emulsions stabilized by WPH and WPI^+ showed some protective effects on *S*. Typhimurium. This observation can be attributed to the ability of positively charged emulsifiers to interact with the bacterial membrane, providing protective effects during thermal treatment.

Lastly, to gain a comprehensive understanding of the mechanism concerned with the impact of emulsifier and oil inclusion on bacterial growth and inactivation behavior, *S*. Typhimurium was cultured in different emulsion-related environments and evaluated for nine stress-related genes (*rpoE, rpoH,* *otsB, proV, fadA, fabA, dnaK, ibpA, ompC*) after 20 hours of incubation at 37°C and after thermal treatment at 55°C for 45 min. It was found that *ibpA* was upregulated in all emulsifier environments, regardless of the presence of oil, indicating that IbpA was synthesized in emulsifier environments. Moreover, increased expression of *fabA* was also observed in Triton X-100 stabilized 60% emulsion, indicating poor heat resistance due to increased membrane fluidity. In the combination of gene expression data, our results showed that emulsifier solutions without oil exhibited a greater number of regulatory mechanisms compared to those containing oil, indicating that the presence of oil did not provide as much protection after thermal treatment. Based on these findings, the stress-related mechanism was constructed by the expression of those selected genes.

Overall, this dissertation provides valuable insights into the factors influencing bacterial growth and inactivation in oil-in-water emulsion systems, as well as bacterial stress response in these systems. These findings provide important insights into the growth and inactivation behavior of *Salmonella enterica* Serovar Typhimurium in oil-in-water emulsion systems and the stress response mechanisms involved. Understanding these factors is crucial for developing effective control measures to ensure food safety and prevent foodborne illness outbreaks caused by this pathogen. This information can be used to optimize the formulation and processing of emulsion-based food products to minimize the risk of bacterial contamination and ensure their safety for consumption.

BACTERIAL GROWTH AND INACTIVATION

IN HETEROGENEOUS ENVIRONMENT

By

Shawn Tsai

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Advisory Committee:
Dr. Rohan V. Tikekar, Chair
Dr. Jianghong Meng
Dr. Abani K. Pradhan
Dr. Shirley A. Micallef
Dr. Mostafa Ghanem

Dedication

I would like to dedicate this work to my adviser, Dr. Rohan V. Tikekar. His expertise, guidance, and support have been invaluable throughout my academic journey. His mentorship has challenged me to grow both academically and personally, and I am grateful for his contributions to my professional development.

I would also like to dedicate this dissertation to my family: Y.S. Tsai, Yu-Mei Chang, and Chi-Chiung Tsai. Their sacrifices, guidance, and love have been my foundation, and I would not be where I am today without them. Their support has been unwavering, and I am forever grateful for their presence in my life.

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List of Abbreviations

o/w: oil-in-water

w/o: water-in-oil

TSB: Tryptic soy broth

TSA: Tryptic soy agar

HLB: Hydrophilic–lipophilic balance

WPI: Whey protein isolate

WPH: Whey protein hydrolysate

pI: Isoelectric point

WPI^+: Whey protein isolate with pH < pI

qPCR: Quantitative polymerase chain reaction

HSPs: Heat shock proteins

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1 Chapter 1: Literature review and statement of problems

1.1 Emulsions in food and associated issues with food safety

Food emulsions are a complex mixture of water and oil that can be found in many foods, including milk, salad dressings, mayonnaise, and whipped cream (Anakhov, Gumerov, Richtering, Pich, & Potemkin, 2020; Cambiella, Benito, Pazos, Coca, Ratoi, & Spikes, 2006; H. Singh & Gallier, 2017; Ushikubo & Cunha, 2014). It is common to use emulsions in food products because of the improvement of sensory properties, such as taste, texture, and appearance of final product (C. Chung & McClements, 2014; McClements, 2004). However, it is important to be aware that some types of emulsions can pose a threat to human health if they are not processed properly or stored at the correct temperature (Gilbert, Gallagher, Eads, & Elmore, 1986; Jung, Jang, & Matthews, 2014; Marangon, Martins, Leite, Santos, Nitschke, & Plepis, 2017; Nielsen & Zeuthen, 1985; Parker, Brocklehurst, Gunning, Coleman, & Robins, 1995). One of the biggest concerns associated with emulsions is the growth of foodborne pathogens (Brocklehurst, Parker, Gunning, Coleman, & Robins, 1995; Chang, Wilking, Kim, Shum, & Weitz, 2015; W. R. Jarvis & Highsmith, 1984; Parker et al., 1995; Smittle, 2000; Vasavada, 1988) (Table 1-1). For example, *Salmonella* can be spread through contaminated emulsion and can cause intense outbreak of foodborne illness (H. Lee & Yoon, 2021; O'Bryan, Ricke, & Marcy, 2022; Pivnick, Engelhard, & Thompson, 1954). The food industry has developed several methods to minimize the risk of contamination by pathogenic bacteria (Ehuwa, Jaiswal, & Jaiswal, 2021; Flynn et al., 2019; Machado-

Moreira, Richards, Brennan, Abram, & Burgess, 2019; Mostafidi, Sanjabi, Shirkhan, & Zahedi, 2020) (Table 1-2). One of the examples involves pasteurizing the product before it is sold to the public (F. V. M. Silva & Gibbs, 2012). This process kills most harmful organisms that have been introduced during the manufacturing process (F. Silva & Gibbs, 2009; F. V. M. Silva et al., 2012). Another example is to include ingredients or additives that act as natural or chemical preservatives in the product (Davidson, Taylor, & Schmidt, 2012; Jamali, Assadpour, Feng, & Jafari, 2021; Yu, Chin, & Paik, 2021). These preservatives prevent the growth of bacteria by increasing the permeability of cell membrane, producing bacterial metabolites, and interrupting the protein synthesis (Bajpai, Sharma, & Baek, 2013; N. K. Lee & Paik, 2016; Rathod et al., 2021). A combination of these techniques can be used to ensure that the product is potentially safe to eat (S. Singh & Shalini, 2016). However, this is not always sufficient to prevent the spread of pathogens (Aaliya et al., 2021). Some emulsifiers and the existence of oil might increase the chance of bacteria becoming heat resistant, allowing them to survive the pasteurization process (Juneja & Eblen, 2000; Podolak, Lucore, & Harris, 2017). Moreover, the breakdown of some emulsifiers could result in the growth of potentially harmful bacteria (C. K. Nielsen, J. Kjems, T. Mygind, T. Snabe, & R. L. Meyer, 2016). Therefore, an understanding of how emulsifiers and oil work in emulsion system, and how to choose the best pasteurizing option for industrial needs, is crucial to ensure food safety.

Table 1-1. Summary of common pathogens related to the contamination in emulsified foods.

Pathogen	**Contaminated sources related to emulsion**	**Symptoms**	**Reference**
Salmonella spp.	Raw eggs, which are often used in the preparation of mayonnaise, hollandaise sauce, or other emulsified sauces	Diarrhea, abdominal cramps, fever, nausea, vomiting, chills, headache	(Doorduyn, Van Den Brandhof, Van Duynhoven, Wannet, & Van Pelt, 2006; Ehuwa et al., 2021)
Listeria monocytogenes	Variety of food, including emulsions, such as meat emulsion or fish-based emulsion, since it can survive and growth in low temperature, low pH, and high salt concentration environments	Diarrhea, fever, headache, muscle ache, loss of balance, convulsions	(Ooi & Lorber, 2005; Quintavalla & Campanini, 1991)
Staphylococcus aureus	Mayonnaise, salad dressing, milk, and many other food products are susceptible to contamination during food processing, particularly from unwashed hands or wounds of food handlers	Nausea, vomiting, stomach cramps	(Jørgensen, Mørk, & Rørvik, 2005)
E. coli	Commonly associated with ground beef or ground pork, and other emulsified products, such as salad dressing and mayonnaise	Diarrhea, stomach cramps, fever	(Cassin, Lammerding, Todd, Ross, & McColl, 1998)

Table 1-2. Brief summary of common techniques for minimizing the risk of foodborne pathogens.

Method	**Description**	**Advantages**	**Limitations**	**Reference**
Good Manufacturing Practices (GMPs)	A set of guidelines that help ensure the food product is safe and consistent. This including keeping the facility clean and sanitary, monitoring temperature and humidity, and training employees on proper hygiene practices	Help ensure that food is produced safely and consistently	Relies on employees to follow guidelines, may not be effective against all pathogens	(Blanchfield, 2005)
Hazard Analysis and Critical Control Points (HACCP)	A systematic approach to identifying and controlling hazards that can cause foodborne illness. It involves identifying potential hazards at each stage of food production, implementing controls to prevent or reduce those hazards, and monitoring the effectiveness of those controls	Systematic approach to identifying and controlling hazards, can be customized to specific production processes	Requires significant resources and expertise to implement and maintain	(de Oliveira, da Cruz, Tavolaro, & Corassin, 2016)
Pasteurization	Heating food to a specific temperature for a certain amount of time to kill bacteria and other pathogens, can be categorized into HTST, LTLT, and UHT	Effective at killing bacteria and other pathogens, does not significantly alter the taste or nutritional content of the food	Not effective against all pathogens, can reduce the shelf life and quality of some foods	(Azizi-Lalabadi, Moghaddam, & Jafari, 2023)
Irradiation	Exposing food to a controlled amount of radiation to kill bacteria and other pathogens, the dose of radiation can be corresponding to the pasteurization level	Effective at killing bacteria and other pathogens, does not	Expensive, require specialized equipment and expertise	(Diehl, 2002)

		significantly alter the taste or nutritional content of the food		
Chemical sanitizer	Chemical compounds that can be used to kill bacteria and other pathogens on surfaces and equipment in the food processing plant	Effective at killing bacteria and other pathogens on surfaces and equipment	Not effective against all pathogens, can be toxic if not used properly	(H. Kim, Moon, Kim, & Ryu, 2019)
High pressure processing (HPP)	Applying high pressure to food to inactivate pathogens, this technique needs special material for packaging of the product	Effective at inactivating bacteria and other pathogens, does not significantly alter the taste or nutritional content of the food	Expensive, require specialized equipment	(H.-W. Huang, Wu, Lu, Shyu, & Wang, 2017)
UV radiation	UV radiation is commonly used to disinfect surfaces and equipment in the food processing plant	Effective at disinfecting surfaces and equipment, does not require chemicals	May not penetrate certain surfaces or materials, not effective against all pathogens	(Bintsis, Litopoulou-Tzanetaki, & Robinson, 2000)
Ozonation	Ozone gas can be used to disinfect surfaces and equipment in the food processing plant	Effective at disinfecting surfaces and equipment, does not leave chemical residues	expensive, require specialized equipment	(Guzel-Seydim, Greene, &

				Seydim, 2004)
Packaging	Modified atmosphere packaging (MAP), controlled atmosphere packaging (CAP) and vacuum packaging (VP) can be used to extend the shelf life of foods, maintain the quality, and reduce the risk of contamination by pathogens	Can extend the shelf life of foods and reduce the risk of contamination, does not require specialized equipment	Not effective against all pathogens, can be expensive	(Farber, 1991)

1.1.1 Salmonella as a pathogen in food industry and emulsion

Salmonella is a genus of Gram-negative bacteria that can cause foodborne illness in humans (Ehuwa et al., 2021; Kaavya et al., 2021; Monte, Lincopan, Fedorka-Cray, & Landgraf, 2019). In the US, about 1 million cases of *Salmonella* infection are reported annually (Sher, Mustafa, Grady, Gardiner, & Saeed, 2021; Tobolowsky, Cui, Hoekstra, & Bruce, 2022). On a broad scale, *Salmonella* can cause health problems in humans, including diarrhea, abdominal pain, and fever (Coburn, Grassl, & Finlay, 2007). Severe cases can result in hospitalization or death (Kariuki, Gordon, Feasey, & Parry, 2015). About 26,500 of *Salmonella* infections result in hospitalizations and about 420 people die because of infection each year (Punchihewage-Don, Hawkins, Adnan, Hashem, & Parveen, 2022). People who are most at risk for developing severe symptoms include young children, the elderly, pregnant women, and those with weakened immune systems (Mollo et al., 2021; Stanaway et al., 2019). On the other hand, the spread of *Salmonella* is a common problem in the food industry because it can be found in both plant and animal products (Guerrero, Bayas-Rea, Erazo, & Zapata Mena, 2021; Keerthirathne, Ross, Fallowfield, & Whiley, 2016). Meats, dairy, fruits, vegetables, and other food products can all become contaminated with *Salmonella* during production or processing (Escartin, Ayala, & Lozano, 1989; Franz & van Bruggen, 2008; Mani-López, García, & López-Malo, 2012; McClelland & Pinder, 1994; Szpinak, Ganz, & Yaron, 2022). Due to the risks posed by *Salmonella* contamination, it is critically important for food processors to take steps to reduce the likelihood of outbreaks.

In the food industry, emulsion systems are commonly used to produce various types of food products (L. Bai, Huan, Rojas, & McClements, 2021; Tan & McClements, 2021). These systems are composed of a liquid carrier and two or more immiscible phases (McClements & Jafari, 2018). Emulsions can be divided into two types, oil-in-water (o/w) and water-in-oil (w/o) (McClements, 2018). Both types of emulsions are used in the food manufacturing industry because of their excellent emulsion stability and good sensory properties (Guzey & McClements, 2006). For example, mayonnaise is a well-known emulsion that is produced using an o/w emulsion system (Depree & Savage, 2001). W/o emulsions are mainly used for food products containing large quantities of oil such as margarine, ice cream, sauces, and peanut butter (Kokini & Aken, 2006). However, it is important to keep in mind that both o/w and w/o emulsions can be contaminated by foodborne pathogens including *Salmonella*, *Escherichia coli* O157:H7, and *Listeria monocytogenes* (J. Lee et al., 2017; Omotani et al., 2021; Pivnick et al., 1954). This contamination may occur during the production process or through cross-contamination with other foods (Carrasco, Morales-Rueda, & García-Gimeno, 2012; Ehuwa et al., 2021). According to a report published by CDC, there were a total of 25 *Salmonella* outbreaks associated with o/w or w/o emulsions reported between 2007 and 2022 (Table 1-3).

Table 1-3. *Salmonella* outbreak related to emulsions between 2007-2022.

Food	**Strain**	**Year**	**Emulsion type**	**Total patients**	**Hospitalization**	**Death**
Peanut butter	*Salmonella* Tennessee	2007	w/o	425	71	0
Peanut butter	*Salmonella* Typhimurium	2009	w/o	714	171	9
Salami	*Salmonella* Montevideo	2010	w/o	203	52	0
Turkey burger	*Salmonella* Hadar	2011	w/o	12	3	0
Ground turkey	*Salmonella* Heidelberg	2011	w/o	94	37	1
Ground beef	*Salmonella* Typhimurium	2011	w/o	17	8	0
Ground tuna	*Salmonella* Bareilly, *Salmonella* Nchanga	2012	w/o	425	55	0
Ground beef	*Salmonella* Enteritidis	2012	w/o	46	12	0
Peanut butter	*Salmonella* Bredeney	2012	w/o	42	12	0
Ground beef	*Salmonella* Typhimurium	2013	w/o	22	11	0
Tahini sesame paste	*Salmonella* Montevideo, *Salmonella* Mbandaka	2013	w/o, o/w	16	1	1
Nut butter	*Salmonella* Braenderup	2014	w/o	6	1	0

Nut butter	*Salmonella* Java	2015	w/o	13	0	0
Shake & meal products	*Salmonella* Virchow	2016	w/o, o/w	33	6	0
Chicken salad	*Salmonella* Typhimurium	2018	o/w	265	94	1
Pasta salad	*Salmonella* Sandiego, *Salmonella enterica*	2018	o/w	101	25	0
Ground beef	*Salmonella* Newport	2018	w/o	403	117	0
Tahini	*Salmonella* Concord	2018	w/o, o/w	8	0	0
Ground turkey	*Salmonella* Schwarzengrund	2019	w/o	7	1	0
Tahini	*Salmonella* Concord	2019	w/o, o/w	15	2	0
Ground beef	*Salmonella* Dublin	2019	w/o	13	9	1
Ground turkey	*Salmonella* Hadar	2021	w/o	33	4	0
Italian-style meats	*Salmonella* Infantis, *Salmonella* Typhimurium	2021	w/o	40	12	0
Salami sticks	*Salmonella* spp.	2021	w/o	34	7	0
Peanut butter	*Salmonella* Senftenberg	2022	w/o	21	4	0

1.1.2 *Effect of emulsifier and oil on bacterial growth*

A food emulsion is a mixture of two or more immiscible liquids that are dispersed in the presence of a surfactant (McClements, 2010). Emulsifiers are surface-active molecules that help to stabilize the emulsion and act as agents of contact between the liquid phases (McClements & Jafari, 2018; Ozturk & McClements, 2016). There are many different types of emulsifiers that can be used to formulate emulsion systems, including phospholipids, polysaccharides, proteins, and synthesis emulsifiers (Evans, Ratcliffe, & Williams, 2013; Kralova & Sjöblom, 2009; Lam & Nickerson, 2013; McClements & Decker, 2018; Ozturk et al., 2016)(Table 1-4). One of the most common types of emulsifiers used in the food industry is lecithin (M. Wang, Yan, Zhou, Fan, Liu, & Li, 2021). When dissolved in water, it forms a milky solution and acts as a powerful emulsifier because it helps to stabilize and protect lipid membranes from contacting with other droplets (Hasenhuettl, 1997). In the presence of an emulsifier, the two phases of a blended mixture combine to form a single uniform phase in which the components are distributed evenly throughout the medium. This helps to prevent the formation of foam and reduces the appearance of visible separation lines between the two phases of the mixture (Ozturk et al., 2016). Besides that, emulsifiers may also present some effect on bacterial growth (Naimi, Viennois, Gewirtz, & Chassaing, 2021; Christina K. Nielsen, Jørgen Kjems, Tina Mygind, Torben Snabe, & Rikke L. Meyer, 2016). The type of emulsifier and its concentration have a great impact on the growth conditions of the microorganisms in a food emulsion system (Naimi et al., 2021). Therefore, it is important to understand how the properties of a given emulsifier affect the growth of bacteria in these systems to develop effective strategies for improving food safety and controlling contamination levels.

There has been limited research concerned with the effect of oil on bacterial growth in emulsion systems. Buranasuksombat et al. (2011) mentioned that oil had no effect on bacterial growth in emulsion system unless the antimicrobial agents were included in the oil phase. A study published in 2019 also found that increase of fat content not only had no effect on bacteria growth but also accelerate the trend that bacteria grew around the oil droplets (Verheyen, Xu, Govaert, Baka, Skåra, & Van Impe, 2019). However, the protective effect from oil was discovered by Castro et al. (2009). The results showed that while the oil phase was increasing, the effectiveness of antimicrobial compounds became lower. However, in the growth of a type of yeast, *Zygosaccharomyces bailii*, increasing oil fraction caused decreased specific growth rate and asymptotic value of *Z. bailii* from modified Gompertz model (Zalazar, Gliemmo, & Campos, 2016). During thermal processing or desiccation, higher content of oil had an impact on bacteria inactivation, enhancing their thermal resistance, so that the outbreak of *Salmonella* was increased (Ren, 2021; R. Yang, Xu, Lombardo, Ganjyal, & Tang, 2020). In general, the increase of oil content has little impact on bacterial growth but presents protective effect in the emulsion system.

Table 1-4. Common emulsifier used in food industry.

Emulsifier	**Source**	**Type**	**Advantages**	**Limitations**	**Examples in Food Industry**
Lecithin	Soybeans, Egg yolks	Natural phospholipid	Non-toxic, versatile, enhances nutritional value, can form transparent emulsions	Limited stability at high temperatures and low pH, high cost	Mayonnaise, salad dressings, baked goods, chocolates
Xanthan gum	Bacteria fermentation	Natural gum	Stable at wide range of pH and temperatures, enhances texture and mouthfeel, gluten-free	Limited emulsifying capacity, may cause gastrointestinal distress in large amounts	Salad dressings, sauces, frozen desserts
Guar gum	Legume seeds	Natural gum	Stable at wide range of pH and temperatures, enhances texture and mouthfeel, gluten-free	Limited emulsifying capacity, may cause gastrointestinal distress in large amounts	Baked goods, dairy products, sauces
Gum Arabic	Acacia tree sap	Natural gum	Stable at wide range of pH and temperatures, enhances texture and mouthfeel, non-toxic	Limited emulsifying capacity, may cause gastrointestinal distress in large amounts	Soft drinks, confectionery, coatings

Carrageenan	Seaweed	Natural gum	Stable at wide range of pH and temperatures, enhances texture and mouthfeel, can form transparent emulsions	Limited emulsifying capacity, may cause gastrointestinal distress in large amounts	Dairy products, deli meats, desserts
Casein	Milk	Natural protein	High emulsifying capacity, enhances nutritional value	Limited stability at low pH, may cause allergies	Creamers, whipped toppings, processed cheese
Whey protein	Milk	Natural protein	High emulsifying capacity, enhances nutritional value	Limited stability at low pH, may cause allergies	Salad dressings, ice cream, baked goods
Monoglycerides	Synthesis	Synthetic emulsifier	Enhances texture and mouthfeel, improves shelf-life	Limited emulsifying capacity, may cause gastrointestinal distress	Baked goods, peanut butter, confectionery
Tween 20	Synthesis	Synthetic emulsifier	Stable at high temperatures, enhances texture and mouthfeel, improves shelf-life	Limited emulsifying capacity, may cause gastrointestinal distress	Ice cream, whipped toppings, salad dressings

Tween 60	Synthesis	Synthetic emulsifier	Stable at high temperatures, enhances texture and mouthfeel, improves shelf-life	Limited emulsifying capacity, may cause gastrointestinal distress	Cake mixes, frosting, peanut butter
Tween 80	Synthesis	Synthetic emulsifier	Stable at high temperatures and low pH, enhances texture and mouthfeel, improves shelf-life	Limited emulsifying capacity, may cause gastrointestinal distress	Ice cream, whipped toppings, salad dressings
Triton X-100	Synthesis	Synthetic emulsifier	Stable at high temperatures and low pH, enhances texture and mouthfeel, improves shelf-life	Limited emulsifying capacity, may cause gastrointestinal distress	Salad dressings, sauces, marinades

1.2 Thermal treatment

Thermal treatment is a powerful and versatile tool for the control of bacteria in food products (Dash et al., 2022). It is an effective means of controlling the growth of bacteria and promoting the elimination of undesirable microbial populations (Chiozzi, Agriopoulou, & Varzakas, 2022). According to the World Health Organization (WHO), appropriate heat treatment is one of the most effective ways to control foodborne disease and prevent food spoilage (Todd, 2020). It is important that products are properly treated throughout the production process to ensure that they are free from harmful bacteria and pathogens. The effectiveness of thermal treatments depends on the temperature and duration of exposure. At high temperatures, bacterial cells undergo denaturation, cell death, and release endotoxins (Deak, 2014). This process not only destroys the bacteria but may also cause some structural changes in the cell, which may make the cell more susceptible to attack by other organisms in the environment (Guo, Mujumdar, & Zhang, 2019). Conversely, exposure to lower temperatures may cause the formation of protective substances within the bacteria cell that help it resist damage by heat and other external influences (Farber & Brown, 1990; Humphrey, 2004).

1.2.1 Current development of thermal treatment in food industry

Thermal treatment is an important step in food processing, as it helps to preserve food products by reducing the formation of spoilage microorganisms and pathogens (Deak, 2014). Several different methods of thermal treatments are available in food industry (Table 1-5). The most common two are pasteurization and sterilization. Both techniques are described in detail below:

Pasteurization is the process of heating food to a specific temperature for a specific time to kill bacteria and other microorganisms that may cause illness and spoilage (Milly, Toledo, Harrison, & Armstead, 2007; Peng, Tang, Barrett, Sablani, Anderson, & Powers, 2017). There are typically three methods for pasteurization based on the heating temperature and the duration of time, HTST, LTLT, and UHT (Y. Li et al., 2021). High-temperature short-time (HTST) pasteurization involves heating the food product to between 72°C for a minimum of 15 seconds (Terpstra et al., 2007). Low-temperature long-time (LTLT) pasteurization, or commercial batch pasteurization, involves heating the food product to 63°C for at least 30 minutes (Davidson & Critzer, 2012). Ultra-high temperature (UHT) pasteurization involves heating the food product to above 135°C for 2-5 seconds (Pearce, Smythe, Crawford, Oakley, Hathaway, & Shepherd, 2012). All three methods are used to maintain food safety by preventing the growth of bacteria and pathogens that can cause illnesses such as *Salmonella* and *Listeria*. However, they also have different advantages and limitations. For example, HTST pasteurization is the most common and commercial method for pasteurization, while some nutrients might be destroyed after treatment (Escuder-Vieco et al., 2018). LTLT pasteurization can keep the activity of many biologically compounds, such as enzymes or hormones, however, some heat resistant microorganisms might be still alive (Escuder-Vieco et al., 2018). UHT pasteurization has a longer shelf life than the other two methods of pasteurization, but flavors and nutrients of product might be loss (Chavan, Chavan, Khedkar, & Jana, 2011). In addition, not all microorganisms can be eliminated by these methods and some spores of certain microorganisms and the bacteria with heat resistance may remain alive and capable of growing again under favorable conditions (Tomasula et al., 2011). Therefore, it is important to always use pasteurization methods in combination with some other appropriate method for the elimination of spores (Mangalassary, Han, Rieck, Acton, & Dawson, 2008).

Sterilization is the process of reducing the level of microbial contamination by eliminating all biological forms of life (X. Li & Farid, 2016). There are several methods for sterilization which include dry heat, steam autoclaving, and radiation (Chiozzi et al., 2022; Rogers, 2012). Dry heat is the least preferred method as it may require high temperatures and prolonged heating times to achieve full inactivation of microorganisms (Darmady, Hughes, Jones, Prince, & Tuke, 1961). Steam autoclaving is a more commonly used method as it requires lower temperatures and shorter heating times and produces fewer undesired by-products during processing (Ansari & Datta, 2003). Radiation is the most preferred method as it is highly effective, fast, and produces very few byproducts (Darmady, Hughes, Burt, Freeman, & Powell, 1961). It is also environmentally friendly because there are no chemicals used during the process and there is no waste material produced at the end of sterilization (Grolichova, Dvorak, & Musilova, 2004). However, there are some limitations associated with the use of radiation. Specifically, it is not suitable for use in open containers because it can damage the materials used for the container (Z. Huang, Li, Si, & Yao, 2021). Some types of food are also not suitable for radiation sterilization as they may be damaged by the high heat produced by radiation and may become discolored or taste bitter. Examples of foods that cannot be irradiated include rice, nuts and seeds, tomatoes, dried fruits and vegetables, meat, poultry, and fish (Hossein Ahari, Seyed Mahyar, & Hadi, 2012). Additionally, public concern is another limitation associated with the extensive use of radiation (Harrell, Djonov, Fellabaum, & Volarevic, 2018). Many consumers are concerned about the safety of using radiation in food production and some have suggested that there are alternatives to using radiation such as cooking at high temperatures for an extended time to ensure that all bacteria are killed (Harrell et al., 2018). However, those concerns had been explained by Lagunas-Solar (1995). Additionally, despite these limitations, the use of radiation is still considered to be the most effective method of eliminating all types of microorganisms.

When applied properly, these techniques improve food safety by destroying harmful bacteria and making it less likely that they will contaminate the food product. However, thermal treatment is not suitable for all foods because of potential impacts on product texture, taste, color, and other sensory properties. Regarding the food with high content of fat, such as emulsion, the protective effect by oil might be presented to decrease the efficiency of those thermal treatments.

Table 1-5. Summary of thermal treatment method in food product, which is modified from the IFT publication.

Method	Description	Examples	Advantages	Limitations
Cooking	Increasing temperature to eliminate spoilage microorganisms and pathogens, inactivate enzymes to preserve food	eggs, meat, vegetables	Improving food safety, extended shelf life, improve the edibility of some foods	Potential loss of heat, sensitive nutrients, color change
Pasteurization	Heating food at specific temperature in specific time duration	milk, juice, sauces	Improving food safety, extended shelf life	Potential loss of heat, sensitive nutrients, modified protein structure
Drying	Using air with or without heated, or light to reduce water content to preserve food	meat, milk powder, grains	Improving food safety, extended shelf life	Potential loss of heat, sensitive nutrients, change color and texture, increase sweetness by water loss
Direct stream heating	Heating liquid food by injection of heated stream to achieve the specific temperature	milk, cheese, beverages	Improving food safety, extended shelf life	Potential loss of heat, sensitive nutrients, increase moisture content in products

Indirect stream heating	Rapid heating food above boiling point of water for a very short time	beverages, milk, pastes	Improving food safety, extended shelf life	Potential loss of heat, sensitive nutrients, produce burnt flavor
Frying	Rapid heating food in hot oil, typically at 300-500F	potatoes, fried vegetables, fried meat	Improving food safety, extended shelf life, change texture and flavors	Increase calories content, generate carcinogenic components, potential loss of heat sensitive nutrients
Baking	Heating food using batch or oven at specific temperature and time to achieve the desired temperature within the product	breads, cakes, potatoes	Improving food safety, extended shelf life, browning color on the surface	Potential loss of heat, sensitive nutrients, modified protein structure
Grilling	Heating food over open flame (350-600F) for specific time to achieve the desired temperature within the product	meat, potatoes, vegetables	Improving food safety, extended shelf life, change texture and flavors	Potential loss of heat, sensitive nutrients, modified protein structure, generate carcinogenic components
Smoking	Traditionally used to preserve meat products by smoke (190-250F) and salt, not only dehydrate products, but also generate special flavors	meat, cheese	Improving food safety, extended shelf life, change texture and flavors	Potential loss of heat, sensitive nutrients, generate carcinogenic components,

				might generate unpleasant flavor
Radio frequency heating	Heating food by radio waves, with the vibration of water molecular to achieve the effect of pasteurization	meat, potatoes, vegetables	Improving food safety, extended shelf life	Potential loss of heat, sensitive nutrients
Infrared heating	Only heating the food surface by infrared radio waves	meat, potatoes, vegetables	Improving food safety, extended shelf life	Potential loss of heat, sensitive nutrients, slow heating process, only heating the surface of food
Canning	Heating food over 250F for a set of time to destroy microorganisms and spores, especially the spores of *Clostridium botulinum*	jams, jellies, fishes	Improving food safety extended shelf life Influence the texture	Potential loss of heat, sensitive nutrients, change flavor and texture a lot
Extrusion	Heating blended food mixture to a specific temperature and extrude by extrusion head with pressure, the pathogens and microorganisms would be eliminated by heat and pressure	cereals, pastas	Improving food safety, extended shelf life, influence the texture, easily rehydration	Potential loss of heat, sensitive nutrients, change texture, limitation of application

1.2.2 Difficulty of thermal treatment to emulsion

The thermal treatment of emulsions has been a major focus of research over the past several decades due to its many important applications in the food and beverage industries (Tcholakova, Denkov, Sidzhakova, & Campbell, 2006). One of the main disadvantages of thermal treatment is its ability to change the physical structure of an emulsion, resulting in a flocculated of oil droplets that are dispersed throughout the aqueous phase (Tangsuphoom & Coupland, 2009). This leads to creaming and droplet coalescence, which greatly reduces the stability of the emulsion, and may cause negative effects on flavor and texture (Xiang et al., 2022). Several factors can influence the kinetics of this process, including the type of emulsifier used, the temperature and duration of the heat treatment, and the concentration of emulsifier and oils used in the formulation (Akbari & Nour, 2018; Bermúdez-Aguirre & Corradini, 2012; Boyd, Parkinson, & Sherman, 1972). On the other hand, many researchers have tried to improve the properties of the thermally treated emulsions by adjusting these process parameters with the goal of maximizing their stability and functionality. One option is to replace heat treatment with other methods, such as high-pressure homogenization (Gharibzahedi et al., 2019).

Another problem is the inactivation of bacteria in emulsion system under heat treatment. Thermal treatment has been used to effectively kill microorganisms in food products and prevent them from growing or multiplying, which can have a substantial impact on the shelf life of the product (Deak, 2014). However, the type of emulsifier and oil content of the emulsion can also influence the extent to which this process is effective in inactivating these pathogens (Mohammad & Taha Daod, 2021b; Ren, 2021; Sedaghat Doost, Dewettinck, Devlieghere, & Van der Meeren, 2018; R. Zhang, Zhang, Zhang, Decker, & McClements, 2015). In emulsions containing 50% oil, *Salmonella* was protected and had a resistance to thermal treatment (R. Yang et al., 2020). Therefore, a higher thermal treatment temperature or time is required for effective inactivation in emulsion system.

1.2.3 Current progressing on thermal treatment in emulsion

Emulsion is a heterogeneous environment that poses challenges for bacterial inactivation. As such, recent studies have focused on developing thermal treatment techniques that can effectively inactivate target microorganisms. Table 1-6 summarizes the results of several recent studies that have made significant progress in developing thermal treatment techniques for emulsions. For instance, Szpinak et al. (2022) targeted S. Typhimurium in w/o and o/w tahini emulsions using a temperature range of 70-90°C, while Ma et al. (2009) used a temperature range of 71-90°C to inactivate Salmonella spp. in w/o peanut butter emulsions. These studies offer valuable insights into the current progress of thermal treatments in emulsions and provide a foundation for future research in this field. Despite the progress made, most of the studies have focused on water-in-oil (w/o) emulsions, with only one study on oil-in-water (o/w) emulsion. Therefore, there is a need to conduct further research to understand how bacteria behave under different conditions. This could be done through the evaluation of stress-related gene expression of bacteria after thermal treatment, it would provide a more comprehensive understanding of mechanisms behind bacterial inactivation in emulsions and could provide the development of more effective thermal treatment to different emulsion types and oil fractions.

Table 1-6. Summary of thermal treatments in emulsions discussed in previous studies.

Product	**Emulsion type**	**Oil fraction**	**Target microorganism**	**Temperature**	**Reference**
tahini	w/o, o/w	59%	*S*. Typhimurium	70-90°C	(Szpinak et al., 2022)
peanut butter	w/o	NM	*Salmonella* spp.	71-90°C	(Ma, Zhang, Gerner-Smidt, Mantripragada, Ezeoke, & Doyle, 2009)
peanut butter peanut butter spread	w/o	50% 33%	*Salmonella* spp.	70-90°C	(C. Li, Huang, & Chen, 2014)
raw milk	o/w	NM	*Salmonella* spp.	60-74°C	(D'Aoust et al., 1987)
ground chicken breast	w/o	NM	*Salmonella* spp. *L. monocytogenes*	60-70°C	(Murphy, Marks, Johnson, & Johnson, 1999)
ground beef	w/o	19.1% 4.8%	*Salmonella* spp. *E. Coli*	55-66°C	(Smith, Maurer, Orta-Ramirez, Ryser, & Smith, 2001)
ground turkey thigh	w/o	4.9%	*Salmonella* spp.	55-63°C	(Stasiewicz, Marks, Orta-Ramirez, & Smith, 2008)

ground pork	w/o	2.5%	*Salmonella* spp.	55-63°C	(Stasiewicz et al., 2008)

w/o: water-in-oil emulsion

o/w: oil-in-water emulsion

NM: Not Mentioned

1.2.4 <u>Stress-related mechanism during thermal treatment in Salmonella</u>

The thermal treatment of food is a common practice used in many industrial and commercial establishments to reduce the number of pathogens and spoilage microorganisms, including *Salmonella*. However, it has been observed that the inactivation of *Salmonella* does not always occur as expected, leading to the presence of viable cells of the product after thermal treatment (N. A. Jarvis et al., 2016). The exact mechanisms behind this phenomenon are not fully understood and many factors can influence the outcome of thermal treatments.

Heat shock protein (HSP) is a group of proteins that confer protection against a broad range of environmental stresses and perturbations by triggering adaptive responses including heat shock response, cell death, metabolic activity, stress responses, and protection of genetic information (Maleki, Khosravi, Nasser, Taghinejad, & Azizian, 2016). Over-expression of HSP can lead to the production of proteins that assist in the survival or growth of the bacteria under various adverse conditions, especially when bacterial cells are stressed by heat (Soleimani, Zulkifli, Hair-Bejo, Omar, & Raha, 2012). *rpoE* and *rpoH* are two genes that control the expression of different HSPs (Dawoud et al., 2017). It has been shown that *rpoE* is involved in the expression of several cytoplasmic HSPs (Ramírez Santos, Solís Guzmán, & Gómez Eichelmann, 2001). On the other hand, *rpoH* is responsible for the production of HSPs (T. Yura, Nagai, & Mori, 1993). Inactivation of the *rpoE* gene can inhibit the growth of *Salmonella* and reduce the survival rate of cells in unfavorable environmental conditions such as heat shock or oxidative stress (Humphreys, Stevenson, Bacon, Weinhardt, & Roberts, 1999).

Virulence is another factor that can affect the survival of *Salmonella* after thermal treatment (Ibarra & Steele-Mortimer, 2009). Virulence is the ability of a microorganism to cause disease in a host organism (Groisman & Saier, 1990). Under heat treatment, some of the bacterial factors involved in virulence, such as the flagella, outer-membrane proteins, or lipopolysaccharides, are denatured and degraded by elevated temperatures. This degradation can impair the ability of the bacteria to adhere to the host tissues and cause an infection (Xu, Abdelhamid Ahmed, Sabag-Daigle, Ahmer Brian, & Yousef Ahmed, 2022). Therefore, the bacterial factors involved in virulence are important for the survival of *Salmonella* at high temperatures and can significantly affect the outcome of the thermal treatment process (Sirsat, Burkholder, Muthaiyan, Dowd, Bhunia, & Ricke, 2011). *ompC* is a gene that is involved in the pathogenesis of *Salmonella* and is part of the outer membrane protein complex (OMP) that acts as the main virulence factor in *Salmonella* (Q. Li et al., 2021). This gene has a protective role against stress conditions and contributes to the adaptation of the bacteria to the adverse conditions of the environment (Uddin, Jeon, & Ahn, 2018). In addition, the *ompC* gene encodes for a protein called OmpC, which contributes to the adhesion of *Salmonella* to host cells, making it a very important gene for virulence (Q. Li et al., 2021). Under thermal treatment, the decrease of *ompC* gene changes the cell permeability, increases the resistance of *Salmonella* to the heat treatment and contributes to the survival of the bacteria in the environment (Uddin et al., 2018). The mechanism of stress-related genes will be discussed more in chapter 5.

1.3 Common models for bacteria growth and inactivation

There are many models used to describe the growth and inactivation of bacteria. Each model is based on a particular assumption about how bacteria grow and interact with their environment (Table 1-7). The simplest growth model assumes that bacteria grow according to the three phase linear model (Garthright, 1997). This model states that bacteria will be constant for a certain period, and then the number will grow exponentially for some amount of time, and then become flat. This model has been used extensively because of its simplicity but is limited to express the behavior of bacteria in one formula (R. L. Buchanan, Whiting, & Damert, 1997).

Gompertz model is an empirical sigmoidal model used in predicting bacterial growth (R. L. Buchanan et al., 1997). This model has been widely applied to describe the growth of various organisms such as plants, fish, tumors, and bacteria (Chatterjee, Chatterjee, Majumdar, & Chakrabarti, 2015; Katsanevakis & Maravelias, 2008; Vaghi et al., 2020; Yamauchi, Nakazono, Inukai, & Tsutsumi, 2020). It is second only to the logistic model in terms of its fitting capacity for growth data, which will be discussed later (Tjørve & Tjørve, 2017). Despite its popularity, one significant limitation of the Gompertz model is the complexity of interpreting its parameters (Tjørve et al., 2017). (Tjørve et al., 2017). The model requires several parameters to be estimated, and the interpretation of these parameters can be challenging. The parameter estimates may also vary significantly based on the data used, making it challenging to compare and interpret results across different studies.

The Logistic model is another commonly used model for predicting bacterial growth, which assumes that the growth of bacteria is a function of a ratio, and the population of bacteria will not be limited by nutrients or space (Horowitz, Normand Mark, Corradini Maria, & Peleg, 2010). This model has been successfully applied to describe bacterial growth in various studies. For instance, Presser et al. (1998) used the logistic model to predict the probability of growth of *E. coli* as a function of environmental factors, such as pH, temperature, water activity, and lactic acid concentration. Following this study, López-Malo et al. (2000) developed a regression equation to estimate the probability of growth of *Saccharomyces cerevisiae*, considering pH, water activity, and the concentration of potassium sorbate. In recent years, scholars have attempted to modify the logistic model. In 2004, Fujikawa et al. proposed a new logistic model that includes an additional term in the differential equation to account for the very low rate of growth during a lag phase. This model accurately described *E. coli* and *S*. Typhimurium growth curves at various dynamic temperatures and other bacterial growth curves reported by other publications (Fujikawa, 2010; Fujikawa & Kano, 2009; P. Li & Ai, 2022).

The Baranyi model is another commonly used model to describe bacterial growth. One of its strengths is its ability to handle time-varying environmental conditions, making it suitable for predicting product shelf life and assessing risks in food production cycles (Foegeding, 1997; McMeekin & Ross, 1996). However, this model's complexity and sensitivity to parameter estimate errors limit its practicality (Grijspeerdt & Vanrolleghem, 1999). Overall, selecting the appropriate model depends on the particular application and the available data, and it is crucial to use the most suitable model to precisely describe and predict bacterial behavior.

To predict bacterial inactivation, various models are commonly used. One of the simplest models is the First order kinetic model, which was proposed by Chick (1908). This model assumes that bacterial inactivation follows a linear or log-linear survival curve. In the food industry, this model is applied to calculate the D-value, which indicates the time required to achieve a one-log reduction in target bacteria (Barrile & Cone, 1970). However, this model has its limitations as the complexity of food systems makes it unlikely that bacterial inactivation would follow such a simple model (Cerf, 1977; Corradini & Peleg, 2009). Therefore, non-linear inactivation models have been proposed.

Modified Gompertz model and logistic model are two non-linear models commonly used to predict the inactivation kinetics of bacteria (Xiong, Xie, Edmondson, & Sheard, 1999). While Gompertz and Modified Gompertz models are initially used to describe bacterial growth, in 1991, Bhaduri attempted to fit inactivation data with these models and successfully described the thermal inactivation curve of *L. monocytogenes* treated in liver sausage. This improved the prediction of microbial thermal resistance compared to first order kinetics model (Bhaduri et al., 1991). Additionally, in 2006, Gil et al. applied the Modified Gompertz model to fit inactivation curves under kinetic temperature environments and found shoulders, linear segments, and tails in the modeling curves (Gil, Brandão, & Silva, 2006). However, parameters used in Gompertz model do not have a direct link to microbial inactivation kinetics, which can lead to problems when interpreting the results (J. Baranyi, Roberts, & McClure, 1993). On the other hand, logistic model assumes that the inactivation of microorganisms can be divided into three sub-divisions: (a) bacteria that are resistant to the treatment, (b) microorganisms that repair themselves and survive after treatment, and (c) cells that are sensitive to the treatment (Y. Bai et al., 2020). In 1994, Little et al. successfully applied logistic model to describe the inactivation curve of *Yersinia enterocolitica* with different pH and temperature (Little, Adams, Anderson, & Cole, 1994). Chen (2007) also reported that for modeling food pathogens in milk, logistic model better describes and predicts the inactivation curve than modified Gompertz model and first order kinetics model (H. Chen, 2007).

The Weibull model is another commonly used inactivation model that offers greater flexibility and comprehensiveness (Buzrul, 2022). According to the equation of the Weibull model, the inactivation curve can take on linear ($\beta = 1$), concave ($\beta < 1$), or convex ($\beta > 1$) shapes, with the β value describing the relationship between bacteria and stress (van Boekel, 2002). Further discussion on the Weibull model will be presented in Chapter 2. An additional advantage of the Weibull model is that the parameters obtained from this model can be used as an alternative to the conventional D-value (Buzrul, 2022). This model has been applied in over 1000 references across a wide range of environmental factors, including high hydrostatic pressure inactivation, thermal treatment, intense pulsed light, and UV

LED light treatment (Cheng et al., 2020; Dilek Avsaroglu, Buzrul, Alpas, Akcelik, & Bozoglu, 2006; Hwang, Seo, Jeong, Cheigh, & Chung, 2019; Murthy, Xie, & Jiang, 2004; van Boekel, 2002). In summary, the Weibull model's convenience, comprehensiveness, and flexibility have made it the most popular model for describing bacterial inactivation curves in recent publications.

Although the models for bacteria growth and inactivation have been developed for decades, more complex models are being developed that incorporate new information about the complex nature of bacteria and the dynamic nature of the environment in which bacteria are exposed. Some of the more advanced models that are currently being developed include models that use more parameters to predict the inactivation process and will help scientists to understand how different environmental factors impact bacteria behavior.

Table 1-7. Predictive model for bacteria growth and inactivation.

--- Growth curve ---		
Model name	Formula	Reference
Three phase linear model	Lag phase: For $t \leqq t_{LAG}$, $N_t = N_0$	(Einarsson, 1994)
	Exponential phase: For $t_{LAG} < t < t_{MAX}$, $N_t = N_0 + \mu(t-t_{LAG})$	
	Stationary phase: For $t \geqq t_{MAX}$, $N_t = N_{MAX}$	
Gompertz model	$logN_t = logN_0 + Ce^{(-e^{(\mu e\left(\frac{t_{MAX}-t}{C+1}\right)})})}$	(Gibson, Bratchell, & Roberts, 1988)
Logistic model	$logN_t = logN_0 + \frac{C}{1+e^{\frac{4\mu(t_{LAG}-t)}{C+2}}}$	(Fujikawa, Kai, & Morozumi, 2004)
Baranyi model	$logN_t = logN_0 + \mu A - \ln\left(1 + \frac{e^{\mu A}-1}{e^{C}}\right)$ $\mathrm{A} = t + \frac{1}{\mu}\ln\left(e^{-\mu t} + e^{-\mu t_{LAG}} - e^{-\mu(t+t_{LAG})}\right)$	(József Baranyi & Roberts, 1994)
N_t: bacterial number at time t, N_0: bacterial number at time 0, μ: maximum growth rate, t_{LAG}: duration of lag phase, t_{MAX}: final bacterial number, C: log bacterial number increase from t_0 to t_{MAX}		
--- Inactivation curve ---		

Modified Gompertz model	$logN_t = logN_0 + ae^{-e^{(-\mu(t-t_{MAX}))}}$	(Xiong, Xie, Edmondson, Linton, & Sheard, 1999)
Logistic model	$logN_t = logN_0 + \frac{a}{(1+e^{d-ct})}$	(Dementavicius, Lukseviciute, Gómez-López, & Luksiene, 2016)
Weibull model	$log\, N_t = \left(\frac{-1}{2.303}\right)\left(\frac{t}{\alpha}\right)^{\beta}$	(van Boekel, 2002)
N_t: bacterial number at time t, N_0: bacterial number at time 0, μ: maximum growth rate, t_{MAX}: final bacterial number, a: log bacterial number decrease from t_0 to t_{MAX}, α: shape parameter, β: scale parameter		

1.4 Statement of problem

Based on the literature review, the issues related to food safety in emulsion based systems that remain under addressed are below:

a) To prevent the outbreak of *Salmonella*, it is extremely important to understand *Salmonella* growth and inactivation in different food systems. Except for fresh meat or produce, emulsion is another large category of foods that can be infected by *Salmonella* easily. Although the research on *Salmonella* growth and inactivation in emulsion system has been conducted in recent years, most of the studies were concentrated in low water activity w/o emulsion, such as peanut butter and ground beef. In contrast to w/o emulsions, the study of bacterial growth and inactivation in o/w emulsions has received relatively less attention in the scientific literature. Milk is one of the o/w emulsions taking into consideration for *Salmonella* growth and inactivation. As heterogeneous environments, such as emulsions, become more prevalent in our daily lives, it is increasingly essential to gain a better understanding of *Salmonella* growth and inactivation in such systems.

b) Although the growth and inactivation of *Salmonella* in some w/o emulsion systems have been studied for decades, the effects of the type of emulsifiers and the fraction of oil content in emulsion remains unknown. Synthetic emulsifiers, such as Tween 20 and Tween 80, were extensively applied in the food industry for manufacturing emulsions. For instance, liquid eggs, mayonnaise, desserts, processed meat and other food products were well-known products that have used synthetic emulsifiers. The bacterial strains, which are mainly responsible for food-borne illnesses have been identified as *Salmonella enterica* and *S*. Typhimurium. There is a strong link between food-borne salmonellosis and the consumption of contaminated o/w emulsion, such as soft cheese, milk, ice cream and other non-dairy foods. However, little information was reported on the effect of these emulsifiers on bacterial growth and inactivation. In addition, the effect of oil fraction has been studied up to 50%. However, certain emulsion-based formulations can contain much higher amount of oil phase. For instance, some emulsified sauces contain more than 60% oil. Thus, it is crucial to investigate the impact of emulsifiers and oil content on *Salmonella* growth and inactivation in emulsion systems, especially given the prevalence of emulsions in the food industry and the potential health risk associated with consumption of contaminated food products.

c) While bacterial inactivation in emulsions is affected by various parameters, a thorough understanding of the underlying molecular mechanisms necessitates investigation at the RNA level. Recent research has extracted and sequenced RNA to ascertain the mechanisms of inactivation, with results indicating that the inactivation of *Salmonella* is induced by elevated temperatures and state changes of membrane proteins caused by emulsifiers. The impact of higher oil fractions in emulsions, however, remains controversial, with some studies reporting membrane damage and others suggesting a protective effect under thermal treatment. Gene expression is therefore required to clarify the effects of higher oil fractions on bacterial inactivation, as well as to investigate the potential benefits or harms of using emulsifiers in food production. This information could contribute to the development of more effective strategies to prevent the growth and spread of foodborne pathogens, including *Salmonella*, and ultimately enhance food safety.

1.5 Specific objectives

a) Understand the effect of type of emulsifier and fraction of oil content on bacteria growth and inactivation in heterogeneous environment, oil-in-water (o/w) emulsion system.

   Hypothesis: By controlling the type of emulsifiers and oil fraction in oil-in-water emulsion, bacteria growth and inactivation would be affected by different type of emulsifiers and oil fraction.

b) Understand the effect of temperature on bacteria growth and inactivation in heterogeneous environment, o/w emulsion system.

   Hypothesis: Lower growth rate would be resulted from lower temperature. On the other hand, higher temperature would increase the inactivation efficiency. The effects would be dependent on the emulsifier type and oil fraction as well.

c) Understand the effect of molecular weight and surface charge of emulsifier on bacteria growth and inactivation in heterogeneous environment, o/w emulsion system.

   Hypothesis: By controlling the molecular weight and surface charge of emulsifier in oil-in-water emulsion, bacteria growth and inactivation rate can be altered.

d) Understand the mechanism behind the effect of emulsifier and oil content on bacteria inactivation in heterogeneous environment, o/w emulsion system.

Hypothesis: The expression of stress-related RNA in bacteria would be dependent on th type of emulsifier used to make the emulsion and the presence of oil phase within emulsion. The consequences of varying expression profile can partially explain the effects of emulsifier type and oil fraction on bacteria growth and inactivation.

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2 Chapter 2 The effect of emulsifier type and oil fraction on *Salmonella enterica* Serovar Typhimurium growth and thermal inactivation in oil-in-water emulsion

Publication status

Under review

Abstract

Current scientific literature has revealed that high water activity oil-in-water emulsions can promote the survival and growth of *Salmonella* Typhimurium. Nevertheless, the precise effects of emulsifier type and oil content on bacterial growth and inactivation are yet to be fully comprehended and necessitate further scientific investigation. In this study, emulsions were prepared using different emulsifiers (Tween 20, Tween 80, and Triton X-100) and different oil fractions (20%, 40%, and 60% (v/v)). TSB (control), emulsifier solutions, and emulsions were inoculated with *S*. Typhimurium and incubated at 37°C for up to 24 hours. The growth and inactivation of *S*. Typhimurium was enumerated by plate counting. The results showed that presence of individual emulsifiers had no effect on growth rate and lag phase of bacteria, the presence of high amount of oil (60%) significantly increased the duration of lag phase but not the growth rate, especially in Tween 80 and Triton X-100 stabilized emulsions. Moreover, the protection against inactivation at 55°C was found in Tween 80 and Triton X-100 solutions, while the presence of oil had no protective effect. The oleic acid in Tween 80 and lower HLB value of Triton X-100 may help maintain membrane integrity and improve the resistance of bacteria to heat inactivation. This study investigates the effect of emulsifier type and oil fraction on *S.* Typhimurium growth and inactivation in emulsion.

2.1 Introduction

Foodborne illness is a significant global concern (Bennett, Sodha, Ayers, Lynch, Gould, & Tauxe, 2018; Callejón, Rodríguez-Naranjo, Ubeda, Hornedo-Ortega, Garcia-Parrilla, & Troncoso, 2015; Scallan et al., 2011). *Salmonella* is a pathogen that causes a high number of foodborne illnesses in the United States (Finstad, O'Bryan, Marcy, Crandall, & Ricke, 2012). To prevent *Salmonella* outbreaks, it is critical to understand their growth and inactivation in various food systems. Aside from fresh meat and produce, emulsion is another prominent category affected with *Salmonella*. *Salmonella* can survive and grow in oil-in-water emulsions with 2-3 log CFU/mL growth after 2 days incubation at 2% oil content emulsion at temperatures below 30°C (Fabian & Pivnick, 1953; Pivnick et al., 1954). In water-in-oil emulsion with low water activity, such as peanut butter and ground beef, *Salmonella* can not only grow but also show increased thermal resistance (Shachar & Yaron, 2006; Smith et al., 2001). On the other hand, milk is one of the few o/w emulsions studied for *Salmonella* growth and inactivation. *Salmonella* growth showed no difference from 0-15% of fat content in emulsion system (Warren, 1998). In contrast, tailing effect was observed under 55°C thermal treatments, especially when the fat content was increased in the milk formula (Warren, 1998). Because oil-in-water emulsion is a large food category, there is an opportunity to understand the factors that affect *Salmonella* growth and inactivation in emulsion system.

The influence of emulsion parameters on bacteria growth and inactivation is not fully known. Emulsifier type is one of the essential factors that needs further investigation. Food emulsifiers are classified as anionic, cationic, non-ionic (ionic), high molecular weight, low molecular weight (size), and high hydrophobicity, low hydrophobicity (HLB value) (McClements & Jafari, 2018; Ozturk et al., 2016; R. Zhang et al., 2015). Although the application of different emulsifiers has been studied extensively, the correlation between emulsifier type and bacteria growth and inactivation is not well articulated. On the other hand, as the oil fraction in emulsion system changes, it is plausible that the growth and inactivation rate of *Salmonella* changes too. As a result, it is critical to assess the impact of emulsifiers and oil concentration in oil-in-water emulsion food systems on *Salmonella* growth and inactivation.

The goal of this study is to see how different emulsifiers (Tween 20, Tween 80, and Triton X-100) and oil concentration affect *Salmonella* growth and inactivation in an oil-in-water emulsion system. The Gompertz and Logistic models, as well as the Weibull model, are used to observe the growth and inactivation effects, respectively.

2.2 Materials and Methods

2.2.1 Materials

Salmonella enterica serotype Typhimurium (CVM98) was cultured from the stock collection of the department of Nutrition and Food Science at University of Maryland, College Park. Tryptic soy agar (TSA) and Tryptic soy broth (TSB) were purchased from BD Biosciences (236920, 211825; Franklin Lakes, NJ). Phosphate Buffer Saline (PBS) tablets were ordered from Fisher Scientific (BP2944-100; Hampton, NH). Buffered peptone water (BPW) was obtained from Thermo Scientific Remel Agar (R452672; Waltham, MA). Emulsifiers Tween 20, Tween 80, and Triton X-100 were obtained from Fisher Scientific (BP337-500, BP338-500; Hampton, NH), and Acros Organics (21568-2500; Pittsburgh, PA), respectively. Vegetable oil was purchased from local grocery store and stored at room temperature in dark.

2.2.2 Sample preparation

2.0% (v/v) of Tween 20, Tween 80 or Triton X-100 was added into different volume of TSB (10, 8, 6, 4 mL) and uniformly dispersed. To prepare 0, 20, 40, 60% (v/v) oil-in-water emulsion, specific amount of vegetable oil was added into TSB containing emulsifiers (0, 2, 4, 6 mL, respectively) to obtain total 10 mL of solutions. Solutions were then homogenized via ultrasonic processor (FB505; Fisher Scientific, Hampton, NH; 200 W, 3 min) to prepare stable emulsions.

2.2.3 Physical and chemical properties

10 mL of emulsifier solutions and emulsion without *S*. Typhimurium were prepared for pH value and water activity measurement. pH value and water activity were tested by pH meter (AB15; Fisher Scientific, Hampton, NH) and HygroPalm (HP23-A; Rotronic, Hauppauge, NY), respectively. Particle size was determined by dynamic light scattering (DLS) (BI-200SM, Brookhaven Instruments Corp., Holtsville, NY). The method was modified from J. Zhang et al., 2020 (J. Zhang, Mei, Chen, Yuan, Zeng, & Wang, 2020). Before detecting the size of emulsion samples, samples were diluted 1000-fold by distilled water. A 35 mW HeNe laser beam with 637 nm wavelength was applied to detect the diameters of emulsion samples. All measurements by DLS were measured at 25°C and in triplicate.

2.2.4 Bacterial growth and enumeration

S. Typhimurium was recovered from frozen culture, inoculated on TSA plates overnight (~20 hr) at 37°C, and stored at 4°C for up to a month. Before experiments, *S*. Typhimurium isolated from the plate was inoculated into 15 mL sterile tube with TSB overnight (~20 hr) at 37°C to reach early stationary phase (~9 log CFU/mL). With appropriate dilution, ~5 log CFU/mL of bacteria were inoculated into TSB (control), emulsifier solutions, and emulsions. Inoculated solutions were incubated at 37°C. Samples collected at specific time (0, 2, 4, 6, 8, 12, 18, 24 hr) were then inoculated on TSA plates via Spiral Plater (Eddy Jet; Neutec Group Inc., Farmingdale, NY). Inoculated plates were incubated overnight (~20 hr) at 37°C and counted by IUL Flash & Go automated colony counter (6010 colony counter; Neutec Group Inc., Farmingdale, NY).

2.2.5 Thermal treatment

S. Typhimurium was inoculated in TSB solution, emulsifier solutions, and emulsions overnight (~20 hr) at 37°C. 200 μL of aliquots were then placed into 55°C water bath (Model: 28L-M; Fisher Scientific, Hampton, NH), and collected at specific time point (0, 15, 30, 45, 60, 75, 90 min). Treated aliquots were plated on TSA plates, incubated overnight (~20 hr) at 37°C, counted by Plate Counter.

2.2.6 Statistical analysis

All experiments were performed in triplicate. Gompertz (1) and Logistic (2) models were used to describe the growth curve:

$$logN_t = logN_0 + Ce^{(-e^{(-\mu(t-d)})} \text{ ...... (1)}$$

$$logN_t = logN_0 + \frac{C}{1+e^{-\mu(t-d)}} \text{ ...... (2)}$$

At time t, the the number of surviving bacteria, N_t can be predicted using these models, where N_0 is the initial bacterial number, C is log bacterial number increase from t_0 to t_{MAX}, μ is the maximum growth rate, d is the inflection point (Dalgaard & Koutsoumanis, 2001; Tjørve et al., 2017). According to Buchanan and Cygnarowicz (Robert L. Buchanan & Cygnarowicz, 1990), the duration of lag phase can be determined by the following formula:

$$\lambda = d - (\frac{1}{\mu}) \text{ ...... (3)}$$

Where λ is the duration of lag phase.

In addition, Weibull model (3) was applied to describe the inactivation at 55°C heat treatment:

$$log\ N_t = \left(\frac{-1}{2.303}\right)\left(\frac{t}{\alpha}\right)^{\beta} \ \ldots\ldots (4)$$

Surviving bacterial number N_t can be predicted using this formula, where α is the shape parameter, and β is the scale parameter (van Boekel, 2002). According to van Boekel (van Boekel, 2002), 5D time, or the time required to achieve a 5-log reduction in bacterial population, can be calculated by the equation (5):

$$5\mathrm{D} = \alpha(-\ln(10^{-5}))^{\frac{1}{\beta}} \ \ldots\ldots (5)$$

Models were built by JMP Pro 15.2.0. The significant difference between each group was evaluated by Student's *t* test at a significance level of α = 0.05.

2.3 Results and Discussions

2.3.1 Physical and Chemical Properties

The pH value and water activity of 0, 20, 40, 60% (v/v) o/w emulsion with 2.0% (v/v) Tween 20, Tween 80, Triton X-100 were tested separately (Table 2-1). The pH value for all samples was approximately 7.0 with no significant difference among the samples ($P > 0.05$). Likewise, water activity values were approximately 0.95 with no significant difference between them ($P > 0.05$). Also, the size of the emulsion particles detected by the particle size analyzer varied from 200 nm to 700 nm (Table 2-1). The mean droplet size generally increased with an increase in the volumetric fraction of the oil phase inside the emulsion, irrespective of the emulsifier selected. Thus, regardless of their composition, all emulsion samples had similar pH, water activity and droplet size.

Table 2-1. Summary of emulsion characteristics. Each data point is an average of triplicate measurements ± standard deviation (SD).

Emulsifier	**Oil fraction (v/v)**	**pH**	**Aw**	**Particle size (nm)**
TSB	0%	6.96 ± 0.01	1.00 ± 0.01	-
Tween 20	0%	7.04 ± 0.02	0.96 ± 0.01	-
	20%	7.07 ± 0.03	0.98 ± 0.01	323.38 ± 68.95
	40%	7.03 ± 0.03	0.97 ± 0.01	320.25 ± 29.01
	60%	6.99 ± 0.05	0.97 ± 0.01	644.79 ± 24.88
Tween 80	0%	7.03 ± 0.00	0.95 ± 0.01	-
	20%	7.02 ± 0.01	0.98 ± 0.01	213.72 ± 78.84
	40%	7.00 ± 0.01	0.97 ± 0.01	261.20 ± 83.32
	60%	6.99 ± 0.02	0.978 ± 0.01	746.39 ± 110.34
Triton X-100	0%	7.06 ± 0.01	0.95 ± 0.01	-
	20%	7.01 ± 0.02	0.99 ± 0.01	267.70 ± 29.72
	40%	6.96 ± 0.00	0.98 ± 0.01	410.23 ± 86.86
	60%	6.91 ± 0.02	0.98 ± 0.01	486.72 ± 54.67

2.3.2 Effect of oil fraction and emulsifier type on the growth rate of S. Typhimurium

The effect of the emulsifier type and oil fraction in an emulsion on the growth behavior of *S*. Typhimurium was assessed. *S*. Typhimurium was inoculated in emulsions and emulsifier solutions (~5 log CFU/ml) and incubated at 37°C. Figure 2-1 shows the bacterial population measured at specific time (0, 2, 4, 6, 8, 12, 18, 24 hours) in TSB, emulsifier solutions and emulsions prepared from individual emulsifiers. Gompertz (supplemental information) and Logistic models were applied to this primary data to describe and predict bacterial growth with a specific growth rate (log CFU/hr), inflection point (hr), and the duration of lag phase (hr). We found that Logistic model fit the data slightly better than Gompertz model ($R^2 > 0.97$, compared to $R^2 > 0.96$). As a result, we performed the statistical comparison only on the parameters generated by Logistic model. The growth curve for *S*. Typhimurium in Tween 20 emulsifier had similar trend compared with that in TSB (Figure 2-1a). Presence of oil phase in Tween 20 stabilized emulsion had little impact on bacterial growth, except for 60% emulsion where visibly extended lag phase was observed. Figure 2-1b and 2-1c show qualitatively similar trends in emulsions stabilized by Tween 80 and Triton X-100 respectively.

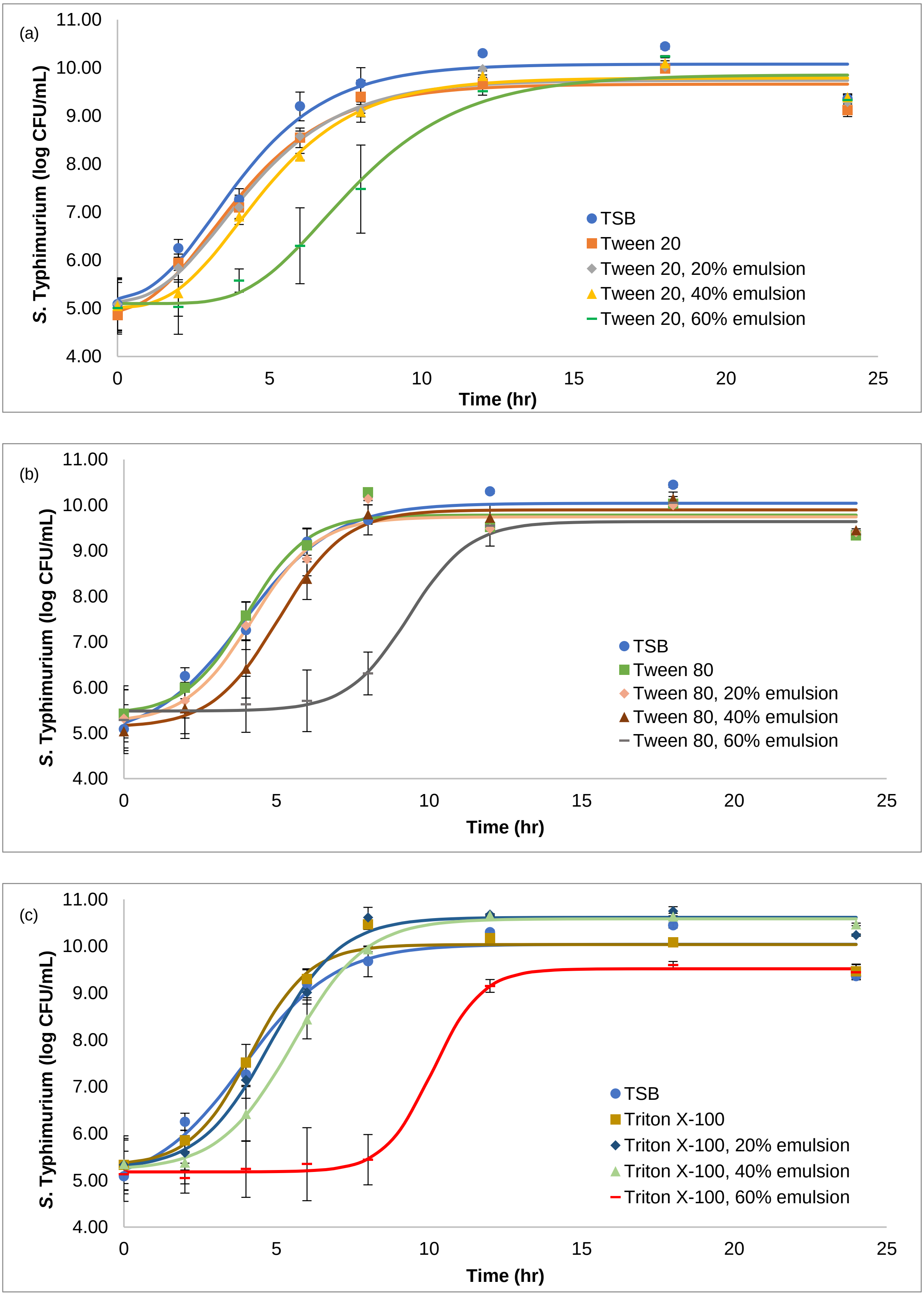
(a)
S. Typhimurium (log CFU/mL)
Time (hr)
TSB
Tween 20
Tween 20, 20% emulsion
Tween 20, 40% emulsion
Tween 20, 60% emulsion
(b)
S. Typhimurium (log CFU/mL)
Time (hr)
TSB
Tween 80
Tween 80, 20% emulsion
Tween 80, 40% emulsion
Tween 80, 60% emulsion
(c)
S. Typhimurium (log CFU/mL)
Time (hr)
TSB
Triton X-100
Triton X-100, 20% emulsion
Triton X-100, 40% emulsion
Triton X-100, 60% emulsion

Figure 2-1. The growth curve of *S*. Typhimurium at 37°C in (a) TSB, TSB supplemented with Tween 20 and 20, 40 and 60% (v/v) emulsions prepared using Tween 20; (b) TSB, TSB supplemented with Tween 80, and 20, 40 and 60% (v/v) emulsions prepared using Tween 80; (c) TSB, TSB supplemented with Triton X-100, and 20, 40 and 60% (v/v) emulsions prepared using Triton X-100. Experimental data was fitted into Logistics model and average model fit for each growth curve is depicted using the solid line. Each data point is an average of triplicate measurements ± standard deviation (SD).

Lag phase was considered as the reference to determine the effects of the factors in this study (Table 2-2). Among all cultured environments, there was a significant extension in the lag phase of bacteria cultured in every 60% emulsions, compared with *S.* Typhimurium cultured in TSB ($P < 0.05$). Specifically, the lag phase was 2.5-fold higher with Tween 20 stabilized 60% emulsion, 3.5-fold higher with Tween 80 stabilized 60% emulsion, and 4-fold higher with Triton X-100 stabilized 60% emulsion compared to that in TSB. However, there was no significant difference in the lag-phase between bacteria inoculated in the TSB and the three emulsifier solutions ($P > 0.05$) indicating that the addition of Tween 20, Tween 80 and Triton X-100 to TSB did not have any significant effect on bacteria growth ($P > 0.05$). Interestingly, the specific growth rate was not significantly different for all the samples ($P > 0.05$) except Tween 80 stabilized 60% emulsion which had marginally, but significantly higher growth rate than that in TSB ($P < 0.05$).

Table 2-2. *S*. Typhimurium growth model parameters in TSB, emulsifier solutions, and emulsions at 37°C. Each data point is an average of triplicate measurements ± standard deviation (SD). The unit of bacterial growth rate was log CFU/ hr.

	TSB	**Tween 20**	**Tween 20, 20% emulsion**	**Tween 20, 40% emulsion**	**Tween 20, 60% emulsion**
R-square	0.97	0.98	0.98	0.99	0.99
Growth rate	0.67 ± 0.28	0.60 ± 0.19	0.59 ± 0.19	0.59 ± 0.15	0.57 ± 0.17
Inflection point	3.92 ± 0.80	3.78 ± 0.71	4.07 ± 0.72	4.62 ± 0.54	7.73 ± 0.50
Lag phase (hr)	2.44 ± 0.85	2.13 ± 0.74	2.38 ± 0.74	2.93 ± 0.56	5.98 ± 0.53**
		Tween 80	**Tween 80, 20% emulsion**	**Tween 80, 40% emulsion**	**Tween 80, 60% emulsion**
R-square		0.97	0.98	0.99	0.99
Growth rate		1.00 ± 0.43	0.94 ± 0.33	0.92 ± 0.23	1.00 ± 0.33*
Inflection point		4.01 ± 0.46	4.20 ± 0.43	5.07 ± 0.33	9.34 ± 0.54
Lag phase (hr)		3.00 ± 0.63	3.13 ± 0.54	3.99 ± 0.40	8.34 ± 0.63***,††
		Triton X-100	**Triton X-100, 20% emulsion**	**Triton X-100, 40% emulsion**	**Triton X-100, 60% emulsion**
R-square		0.98	0.99	1.00	1.00
Growth rate		1.03 ± 0.38	0.87 ± 0.18	0.83 ± 0.07	1.26 ± 0.16
Inflection point		4.12 ± 0.39	4.80 ± 0.28	5.52 ± 0.11	10.12 ± 0.26

Lag phase (hr)		3.15 ± 0.55	3.65 ± 0.33	4.32 ± 0.13[†]	9.33 ± 0.30[**,††]

*: significant difference compared with inoculation in TSB (P < 0.05)

†: significant difference compared with inoculation in Tween 20 at the same amount of oil fraction (P < 0.05)

When comparing three types of emulsifiers, there were no significant difference among emulsions at 0 and 20% emulsion system. At 40% emulsion, *Salmonella* cultured in Triton X-100 had significantly longer lag phase than in Tween 20 or Tween 80 ($P < 0.05$); at 60% emulsion, *Salmonella* inoculated in Tween 80 and Triton X-100 had significantly longer lag phase than in Tween 20 ($P < 0.05$). Thus, in addition to the oil fraction, the type of emulsifier also affects bacterial lag phase in emulsions containing 60% oil fraction.

Previous studies have shown that Tween 20, Tween 80, or Triton X-100 has no antimicrobial effects on *S*. Typhimurium growth within the range of 0.5-10.0% (w/v) concentration (D'Aoust, Maishment, Stotland, & Boville, 1982; Dikici, Arslan, Yalcin, Ozdemir, Aydin, & Calicioglu, 2013; Zivanovic, Basurto, Chi, Davidson, & Weiss, 2004). Consistent with these findings, 2.0% v/v emulsifiers used in this study did not significantly affect bacterial growth. However, Prachaiyo & Mclandsborough found an extended lag phase (higher inflection point) in *E*. coli O157:H7 growth when Tween 20 was used as the emulsifier and the volume fraction of oil phase of the emulsion was over 40% (Prachaiyo & McLandsborough, 2003), which is also consistent with our findings. The result indicated that the type of emulsifier and the oil fraction in the emulsion influence bacterial growth, although the emulsifiers themselves may not affect bacterial growth. Several hypotheses may be proposed to explain this apparent inconsistency: According to previous research, bacterial growth is known to primarily occur in the aqueous phase of an emulsion (Cápiro, Wang, Hatt, Lebrón, Pennell,

& Löffler, 2014). As the oil fraction increases in the emulsion, the total aqueous volume available for bacterial growth decreases. Consequently, the lag phase becomes longer than that under normal conditions (inoculated at the TSB). Similarly, the bacterial metabolites are also excreted within the aqueous phase and due to lower available aqueous volume, their concentration would be higher in emulsions while the high oil fraction. It is known that metabolite accumulation in the extracellular environment may affect bacterial growth. This may explain the observed outcomes (Babynin, 2006; Shayanfar, Broumand, & Pillai, 2018).

2.3.3 Effect of emulsifier type on thermal inactivation rate of S. Typhimurium in high oil fraction emulsion

It is known that the environment in which bacteria grow influences their survival and inactivation in subsequent treatments (Birk, Henriksen, Müller, Hansen, & Aabo, 2016). As a result, we hypothesized that just as emulsions affect bacterial growth rate, they also affect bacterial inactivation rate. Because only oil fraction emulsions at 60% (v/v) had a significant impact on bacterial growth, inactivation experiments were conducted using only those emulsions, in addition to emulsifier and TSB solutions. Bacterial numbers reached 9-10 log CFU/mL after an overnight (20-hour) incubation in various medium (TSB, emulsifier solutions, and 60% emulsions). Figure 2-2 shows the bacterial inactivation data in TSB, individual emulsifiers and 60% oil-in-water emulsions prepared using those emulsifiers. It was discovered that bacteria inactivation in a 55°C water bath did not follow the log-linear inactivation kinetics, which was consistent with a previous study conducted by S. Warren (Warren, 1998). The tailing effect of inactivation curve was found during thermal treatment, and the effect became more significant as the increase of oil fraction in emulsion (Warren, 1998). Therefore, inactivation data was fitted into the Weibull model (Table 2-3). Based on the Weibull model parameters, $\beta < 1$ indicated that the remaining bacteria had a proclivity to adapt to heat. Furthermore, α was an analogue to the traditional D value (van Boekel, 2002). As a result, the 5D concept was chosen as the standard for evaluating effects on bacterial inactivation. Bacteria grown in emulsifier solutions had a significantly lower ($P < 0.05$) reduction in bacterial number after 90 minutes of thermal treatment than bacteria grown in TSB (Fig. 2-2). *S*. Typhimurium inoculated in Tween 20 had a comparable 5D value (1.3-fold lower) to bacteria inoculated in TSB, a 9-fold higher 5D in Tween 80, and a 4-fold higher 5D in Triton X-100, indicating that bacterial resistance significantly increased particularly when incubated with Tween 80 and Triton X-100 emulsifiers ($P < 0.05$).

However, 5D value was only 1.4-fold higher in bacteria cultured in Tween 20 emulsion than in bacteria cultured in TSB ($P > 0.05$). Furthermore, when *Salmonella* was inoculated in Tween 80 and Triton X-100 emulsions, 5D decreased 1.5-fold and increased 1.01-fold, respectively ($P > 0.05$).

Thus, a protective effect of emulsifiers against bacterial inactivation was found in 2% (v/v) emulsifier solution. According to Ren Yang et al. (2020), thermal treatment had a protective effect on bacteria when oil was present (Ren Yang, 2020). On the contrary, our findings revealed that the protective effect was found in emulsifier solutions rather than in emulsion systems.

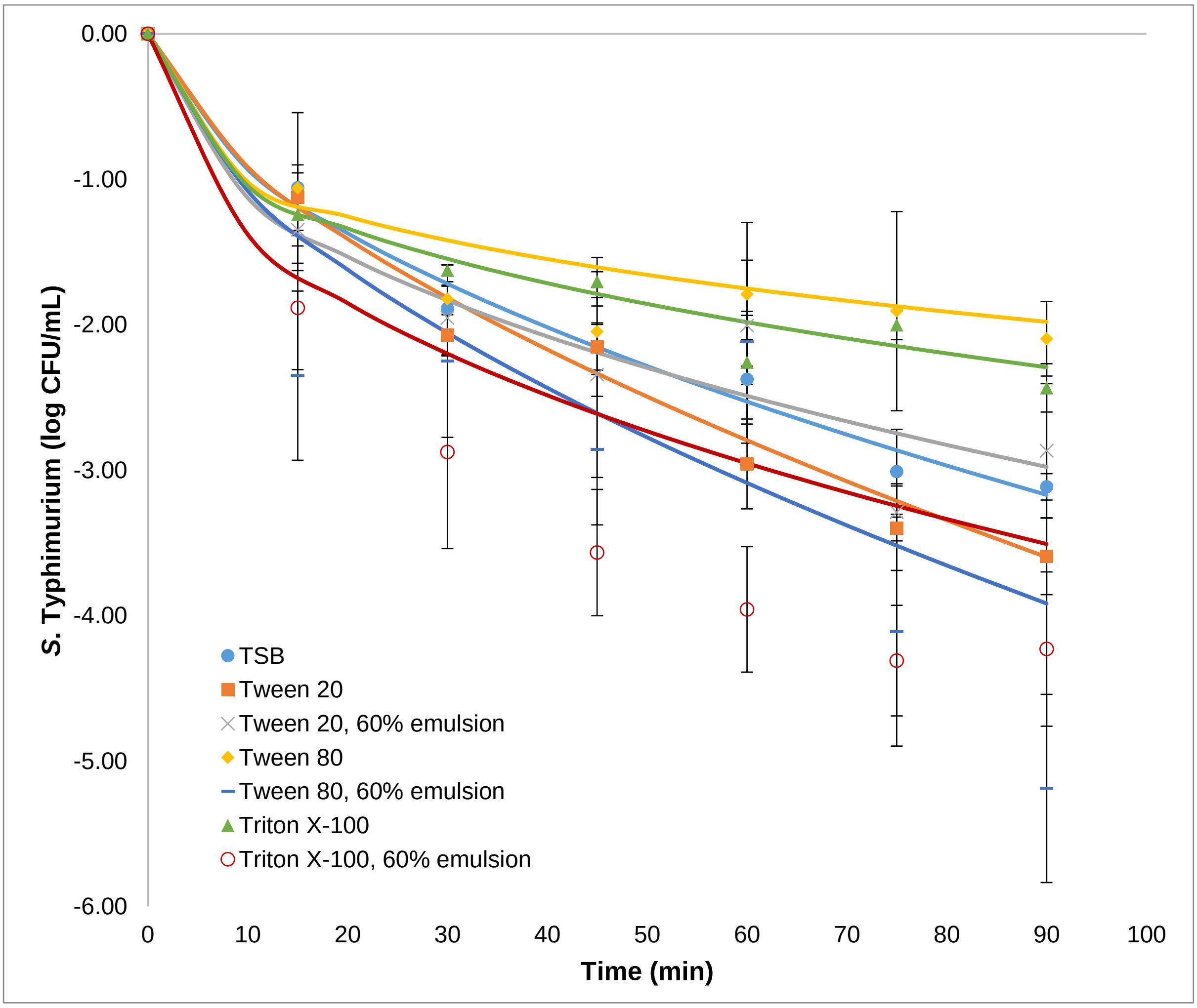

Figure 2-2. Thermal inactivation of *S*. Typhimurium at 55°C inoculated in TSB, Tween 20, Tween 80, and Triton X-100 emulsifiers and 60% (v/v) oil-in-water emulsions prepared using those emulsifiers. Experimental data was fitted into Weibull model and average model fit for each growth curve is depicted using the solid line. Each data point is an average of triplicate measurements ± standard deviation (SD).

Table 2-3. Thermal inactivation model parameters (Weibull model) for of S. Typhimurium at 55°C in TSB, Tween 20, Tween 80 and Triton X-100 emulsifiers and 60% (v/v) emulsions prepared thereof. Each data point is an average of triplicate measurements ± standard deviation (SD).

	TSB	Tween 20	Tween 20, 60% emulsion	Tween 80	Tween 80, 60% emulsion	Triton X-100	Triton X-100, 60% emulsion
α (min)	2.54 ± 0.23	3.03 ± 1.42	1.16 ± 0.57	0.60 ± 0.75	2.12 ± 1.71	0.86 ± 0.49	0.66 ± 1.01
β	0.56 ± 0.01	0.62 ± 0.07	0.44 ± 0.06	0.30 ± 0.07	0.59 ± 0.18	0.36 ± 0.06	0.43 ± 0.18
Avg. 5D concept predicted by Weibull model (min)	203.89 ± 10.18	152.56 ± 14.42	290.79 ± 73.66	1914.70 ± 706.35****	136.45 ± 30.56	795.34 ± 420.09***	207.23 ± 60.03

*: significant difference compared with inoculation in TSB (P < 0.05)

Since β was less than 1 in all the cases, we can conclude that *Salmonella* was able to adapt to thermal stress at 55°C. Furthermore, tailing effect was also observed across all groups as the 90-minute thermal treatment progressed. Thus, it is apparent that a subset of *Salmonella* Typhimurium was transferred to heat-resistant bacteria. Furthermore, the obvious protective effects were demonstrated when bacteria were cultured in Tween 80 and Triton X-100 emulsifier solutions using the 5D concept, which has not been noticed in previous studies. However, previous research has shown that oleic acid from Tween 80 can be released when heat energy is involved (Reitermayer, Kafka, Lenz, & Vogel, 2018). This released oleic acid may be able to stabilize bacterial membrane. Another study concerned with HLB value has also discovered that cells cultured in Triton X-100 (lower HLB value) had lower membrane permeability compared to cells grown in Tween 20 (higher HLB value) at the same concentration (BadrulHaswan, Hassan, Ali, Redhwan, & Nasir, 2022). This finding indicates that Triton X-100 can help to protect cells from external stress due to its lower HLB value. It is also possible that presence of different emulsifiers and mild heat trigger different bacterial stress response genes that are able to protect the bacteria against inactivation. Further research is needed to explore these possibilities.

2.4 Conclusion

Current research shows that *Salmonella* Typhimurium can thrive in high water activity oil-in-water emulsions. However, the impact of emulsifiers and oil content on bacterial growth and inactivation is unclear. The aim of this research was to gain a better understanding of these variables. Bacterial growth and thermal inactivation rates were measured in bacteria inoculated in TSB, Tween 20, Tween 80 and Triton X-100 emulsifiers and 20, 40 and 60% (v/v) oil-in-water emulsions prepared using those emulsions. The results showed that the growth of *S*. Typhimurium can be described and predicted using both the Gompertz and Logistic models, with the Logistic model being more accurate. The lag phase duration and specific growth rate were used to evaluate the effect on bacterial growth. While the presence of individual emulsifiers did not affect the duration of lag phase or the growth rate of the bacteria, presence of high amount of oil phase (60%) significantly extended the lag phase but not the growth rate and ultimate bacterial population. Among high oil fraction (60% (v/v)) emulsions, Tween 80 and Triton X-100 stabilized emulsions had significantly longer lag phase than Tween 20 emulsion. Thermal inactivation of bacteria was fitted into the Weibull model, and it was found that Tween 80 and Triton X-100 solutions provided significant protection against inactivation at 55°C, while presence of oil (emulsion) had no protective effect. We hypothesize that the oleic acid in Tween 80 and the reduced HLB value of Triton X-100 can help maintain membrane integrity and thus improve resistance of bacteria to thermal inactivation. The Weibull model also indicated that after 30 minutes of thermal treatment at 55°C, a sub-population of *S*. Typhimurium had become heat resistant. The mechanism behind this will be discussed in Chapter 5. Moreover, the results of this chapter demonstrate the importance of considering both emulsifier type and oil content when studying bacterial growth and thermal inactivation in heterogeneous environments. These findings will be used in the subsequent chapter to investigate the effect of temperature on *S*. Typhimurium behavior in oil-in-water emulsions, building on our understanding of the factors that influence bacterial survival in these complex systems.

Supplementary Information

Table S1. *S*. Typhimurium growth model parameters in TSB, emulsifier solutions, and emulsions at 37°C fitted in Gompertz model. Each data point is an average of triplicate measurements ± standard deviation (SD). The unit of bacterial growth rate was log CFU/ hr.

	TSB	**Tween 20**	**Tween 20, 20% emulsion**	**Tween 20, 40% emulsion**	**Tween 20, 60% emulsion**
R-square	0.96	0.98	0.98	0.99	0.98
Growth rate	0.49 ± 0.17	0.46 ± 0.12	0.46 ± 0.12	0.47 ± 0.09	0.40 ± 0.12
Inflection point	3.20 ± 0.74	3.13 ± 0.61	3.47 ± 0.56	3.97 ± 0.40	6.79 ± 0.54
Lag phase (hr)	1.14 ± 0.76	0.96 ± 0.62	1.30 ± 0.57	1.82 ± 0.41	4.27 ± 0.56
		Tween 80	**Tween 80, 20% emulsion**	**Tween 80, 40% emulsion**	**Tween 80, 60% emulsion**

R-square		0.97	0.98	0.98	0.99
Growth rate		0.74 ± 0.32	0.72 ± 0.27	0.71 ± 0.19	0.85 ± 0.42
Inflection point		3.50 ± 0.54	3.68 ± 0.46	4.52 ± 0.33	8.59 ± 0.40
Lag phase (hr)		2.15 ± 0.63	2.29 ± 0.53	3.11 ± 0.38†	7.42 ± 0.57**,††
		Triton X-100	**Triton X-100, 20% emulsion**	**Triton X-100, 40% emulsion**	**Triton X-100, 60% emulsion**
R-square		0.97	0.99	1.00	1.00
Growth rate		0.85 ± 0.40	0.66 ± 0.15	0.59 ± 0.04	0.87 ± 0.11
Inflection point		3.74 ± 0.42	4.20 ± 0.28	4.85 ± 0.11	9.21 ± 0.22
Lag phase (hr)		2.56 ± 0.58†	2.68 ± 0.32†	3.15 ± 0.11*,†	8.06 ± 0.24**,††

*: significant difference compared with inoculation in TSB ($P < 0.05$)

†: significant difference compared with inoculation in Tween 20 at the same amount of oil fraction ($P < 0.05$)

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3 Chapter 3 The effect of temperature on *S. enterica* Serovar Typhimurium growth and thermal inactivation in oil-in-water emulsion

Publication status

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Abstract

Salmonella Typhimurium is a significant foodborne pathogen that poses a severe threat to public health, particularly for immunocompromised individuals. Emulsions contaminated with *S*. Typhimurium have been identified as a common source of infection. Understanding the impact of temperature on the growth and inactivation of *S*. Typhimurium in emulsions is critical to developing effective control measures for this pathogen. In this study, the effect of temperature on the growth and inactivation kinetics of *S*. Typhimurium in various emulsion systems was investigated. Specifically, we inoculated *S*. Typhimurium in Tryptic Soy Broth (TSB), emulsifier solutions (Tween 20, Tween 80, Triton X-100), and emulsions with oil fractions of 20%, 40%, and 60%. The samples were incubated at different temperatures (7°C, 22°C, 37°C) for varying time intervals. Thermal inactivation was achieved using a submerged coil at 55°C, 58°C, and 60°C. Logistic model and the Weibull model were applied to fit and describe the data for growth and inactivation, respectively. The results revealed that temperature had a significant impact on the growth and inactivation kinetics of *S*. Typhimurium in emulsions. Temperature, rather than the type of emulsifier or oil fraction, was the primary factor influencing the bacterial kinetics. Specifically, increasing temperature led to a faster growth rate and inactivation rate of *S*. Typhimurium. The objective of this study is to understand the effect of temperature on S. Typhimurium growth and inactivation in emulsion systems.

3.1 Introduction

Salmonella Typhimurium is a pathogen that poses a serious threat to public health, particularly to individuals with compromised immune systems and elders (Lund, 2015; Monack, Mueller, & Falkow, 2004). Contaminated food products, such as emulsion, are a major source of *S.* Typhimurium infections (Chambers & Gong, 2011; Omotani et al., 2021; Vandeplas, Dubois Dauphin, Beckers, Thonart, & Théwis, 2010; Wu, Ricke, Schneider, & Ahn, 2017). The growth and survival of bacteria in emulsions are influenced by multiple parameters, including temperature, pH, and water activity (Karina, Julio, Leda, & Noemi, 2011; Kosegarten, Ramírez-Corona, Mani-López, Palou, & López-Malo, 2017; Valero, Pérez-Rodríguez, Carrasco, Fuentes-Alventosa, García-Gimeno, & Zurera, 2009). Among these variables, temperature is an essential environmental factor that directly affects the rate of chemical reaction, enzymatic activity, and microbial growth (József Baranyi et al., 1994; Pietikäinen, Pettersson, & Bååth, 2005). Therefore, understanding the impact of temperature on growth and inactivation of bacteria is important for developing effective strategies to control its proliferation and spread in food products. Previous study has reported the optimal temperature for the growth of *S*. Typhimurium is between 35°C and 43°C (Yates, 2011). Nevertheless, the influence of temperature on survival and inactivation of this pathogen in emulsions remains unexplored.

Thus, the primary objective of this study is to investigate the effect of temperature on the growth and inactivation of *S*. Typhimurium in emulsions. To achieve the goal, the growth kinetics of *S*.

Typhimurium at different temperatures (7°C, 22°C, and 37°C) in TSB, emulsifier solutions (2% (v/v) Tween 20, Tween 80, and Triton X-100), and emulsions (20%, 40%, and 60% (v/v) oil fraction) were evaluated. Furthermore, the inactivation kinetics of this pathogen were assessed at temperatures of 55°C, 58°C, and 60°C. The outcomes of this study are expected to provide novel insights into the behavior of Salmonella Typhimurium in emulsifier-containing systems and can inform the development of effective control strategies for this pathogen in the food industry.

3.2 Materials and Methods

3.2.1 Materials

Salmonella enterica serotype Typhimurium (CVM98) was obtained from the stock collection of the Department of Nutrition and Food Science at the University of Maryland, College Park. Tryptic Soy Agar (TSA) and Tryptic Soy Broth (TSB) were purchased from BD Biosciences (Franklin Lakes, NJ, USA). Buffered Peptone Water (BPW) was obtained from Thermo Scientific Remel Agar (Waltham, MA, USA). Emulsifiers, including Tween 20 and Tween 80 from Fisher Scientific (Hampton, NH, USA) and Triton X-100 from Acros Organics (Pittsburgh, PA, USA), were used in the study. The vegetable oil was purchased from a local grocery store and stored in a dark place at room temperature.

3.2.2 Sample preparation

Emulsifiers, including Tween 20, Tween 80, or Triton X-100, were added to varying volumes of TSB (10, 8, 6, 4 mL) at a concentration of 2.0% (v/v) and uniformly dispersed. To prepare oil-in-water emulsions with oil fractions of 0, 20, 40, and 60% (v/v), specific amounts of vegetable oil were added to TSB containing emulsifiers (0, 2, 4, 6 mL, respectively) to obtain a total volume of 10 mL. The solutions were then homogenized using an ultrasonic processor (FB505; Fisher Scientific, Hampton, NH; 200 W, 3 min) to prepare stable emulsions.

3.2.3 Bacterial growth and enumeration

S. Typhimurium was obtained from a frozen culture and inoculated onto TSA plates, followed by overnight incubation (~20 hr) at 37°C and storage at 4°C (< 1 month). To obtain bacteria in early stationary phase (~9 log CFU/mL), isolated *S*. Typhimurium was inoculated into a sterile tube containing 15 mL of TSB and incubated overnight (~20 hr) at 37°C. Using appropriate dilution, approximately 5 log CFU/mL of bacteria was cultured into TSB (as a control), emulsifier solutions, and emulsions. These inoculated solutions were then incubated at different temperatures of 7°C, 22°C, and 37°C, and samples were collected at specific time points (0, 1, 2, 3, 4, 5, 6, 7 days for 7°C; 0, 3, 6, 9, 12, 18, 24 hr for 22°C; and 0, 2, 4, 6, 8, 12, 18, 24 hr for 37°C). To quantify bacterial growth and inactivation, samples were inoculated on TSA plates using a Spiral Plater (Eddy Jet; Neutec Group Inc., Farmingdale, NY) and incubated overnight (~20 hr) at 37°C. Colony counts were determined using an IUL Flash & Go automated colony counter (6010 colony counter; Neutec Group Inc., Farmingdale, NY).

3.2.4 Thermal treatment

S. Typhimurium was cultured overnight (~20 hr) at 37°C in TSB, emulsifier solutions, and emulsions. Aliquots of 200 μL were then subjected to thermal treatment using a submerged coil apparatus (Coil-100; Sherwood Technologies, Lynnfield, MA) at temperatures of 55, 58, and 60°C for specific time intervals (0, 100, 200, 300, 400, 500 sec for 55°C, 0, 25, 50, 75, 100, 125 sec for 58°C, and 0, 10, 20, 30, 40, 50 sec for 60°C). Treated samples were subsequently plated on TSA, incubated at 37°C overnight (~20 hr), and the resulting colonies were counted using a Plate Counter. The protocol for thermal treatment using the submerged coil apparatus was adapted from Gao et al. (2021). Briefly, the device was placed in a class IIA biosafety cabinet, and 4-mL screw-cap vials, each containing 3.6 mL of sterile 0.1% peptone water, were arranged on the sample collection carousel. Following program setup, samples were delivered to the coil and subjected to the desired temperature. Upon completion of treatment, the sample vials, which served as a 10^{-1} dilution, were immediately capped and chilled on ice to stop further thermal inactivation. Between trials, the coil was cleaned with 70% ethanol and rinsed with sterile 0.1% peptone water. Temperature selection was based on achieving no more than a 6-log reduction, and the processing temperatures used in this study were determined via preliminary experiments and previous studies (Monfort, Saldaña, Condón, Raso, & Álvarez, 2012; Park & Kang, 2013; Topalcengiz & Danyluk, 2017).

3.2.5 Statistical analysis

All experiments were conducted in triplicate or more. The logistic model (1) was used to describe the growth curve as follows:

$$logN_t = logN_0 + \frac{C}{1+e^{-\mu(t-d)}} \ \ldots\ldots (1)$$

Here, N_t represents the number of surviving bacteria at time t, and N_0 is the initial bacterial count. C is the increase in log bacterial count from t_0 to t_{MAX}, μ is the maximum growth rate, and d is the inflection point (Dalgaard et al., 2001; Tjørve et al., 2017). The duration of the lag phase was determined using the formula proposed by Buchanan and Cygnarowicz (1990):

$$\lambda = d - (\frac{1}{\mu}) \ \ldots\ldots (2)$$

Here, λ represents the duration of the lag phase.

Furthermore, the Weibull model (3) was applied to describe the inactivation of thermal treatment as follows:

$$log\ N_t = \left(\frac{-1}{2.303}\right)\left(\frac{t}{\alpha}\right)^{\beta} \ \ldots\ldots (3)$$

Here, N_t represents the surviving bacterial count, α is the shape parameter, and β is the scale parameter (van Boekel, 2002). The time required to achieve a 5-log reduction in bacterial population, or the *5D* time, can be calculated using the following equation (4) proposed by van Boekel (2002):

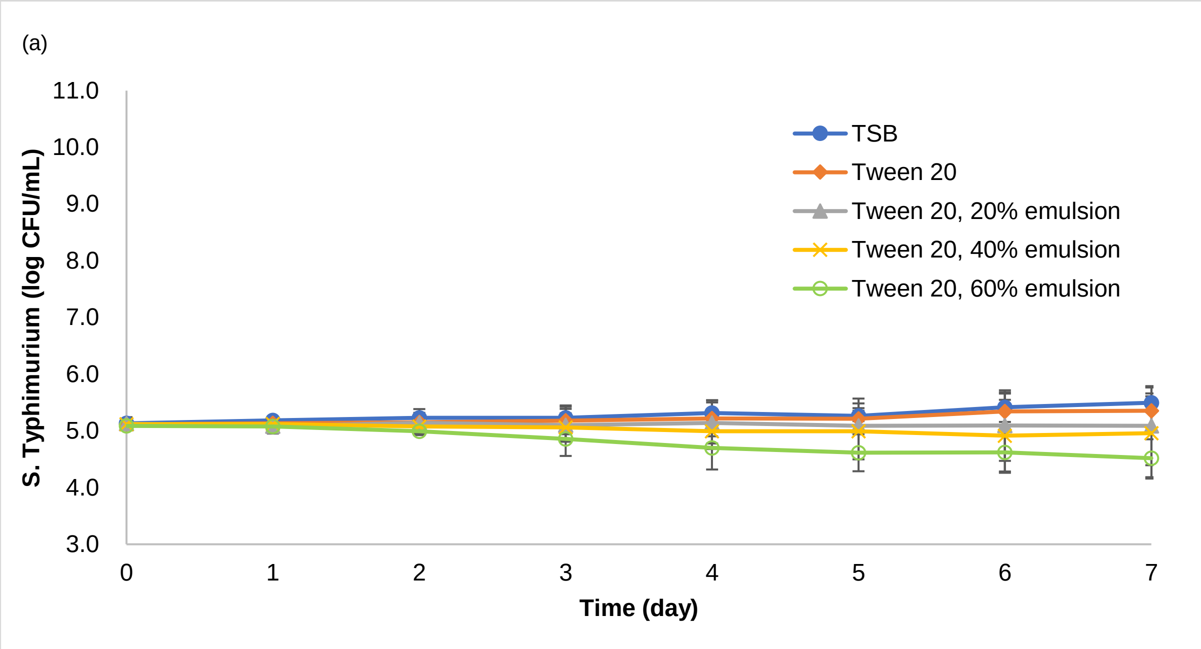
$$5D = \alpha(-\ln(10^{-5}))^{\frac{1}{\beta}} \ \ldots\ldots (4)$$

All models were developed using JMP Pro 15.2.0. The significance of the difference between groups was determined by Student's t-test at a significance level of α = 0.05.

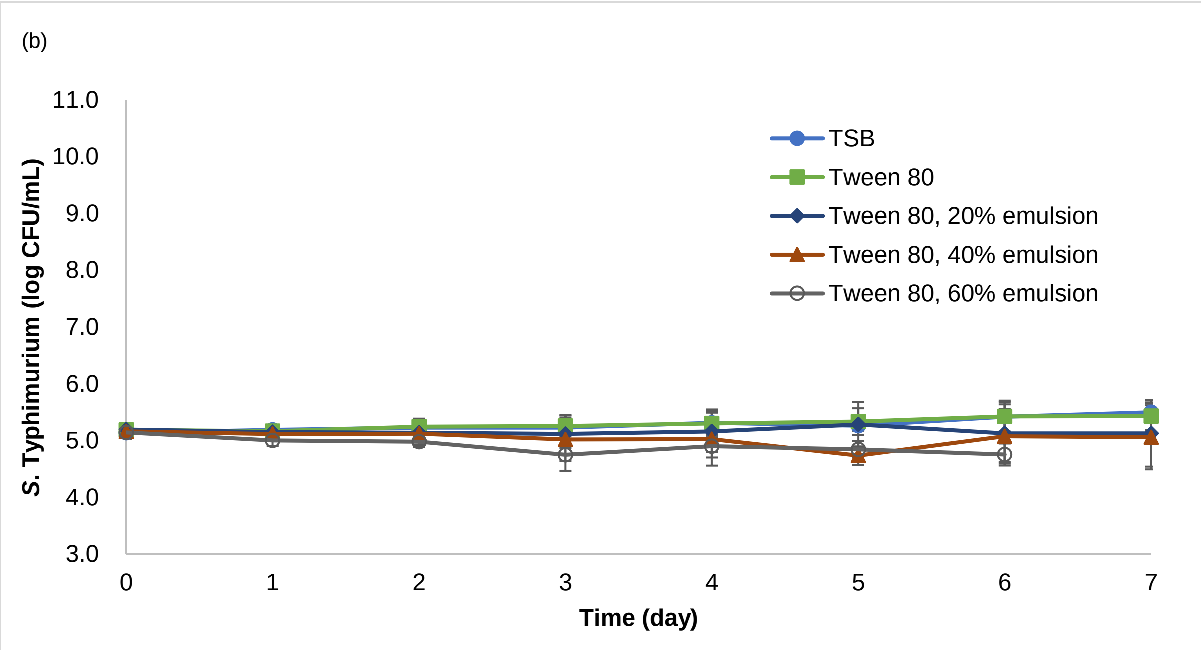
3.3 Results and Discussions

3.3.1 Effect of temperature on the growth rate of S. Typhimurium

The effect of temperature on the growth behavior of *S*. Typhimurium was investigated by inoculating it in TSB, emulsions and emulsifier solutions at an initial level of approximately 5 log CFU/mL, followed by incubation at 7, 22, and 37°C. The number of bacteria was determined at specific time intervals (0, 1, 2, 3, 4, 5, 6, 7 days for 7°C; 0, 3, 6, 9, 12, 18, 24 hr for 22°C; and 0, 2, 4, 6, 8, 12, 18, 24 hr for 37°C), and the results were presented in Figure 3-1 to 3-3, respectively. The logistic model was utilized to perform a statistical analysis on the parameters such as the maximum growth rate (log CFU/hr), inflection point (hr), and duration of lag phase (hr) analyzed, as it exhibited a good fit ($R^2 > 0.97$), except for the growth curve at 7°C.



(a)
S. Typhimurium (log CFU/mL)
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
0
1
2
3
4
5
6
7
Time (day)
TSB
Tween 20
Tween 20, 20% emulsion
Tween 20, 40% emulsion
Tween 20, 60% emulsion



(b)
S. Typhimurium (log CFU/mL)
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
0
1
2
3
4
5
6
7
Time (day)
TSB
Tween 80
Tween 80, 20% emulsion
Tween 80, 40% emulsion
Tween 80, 60% emulsion

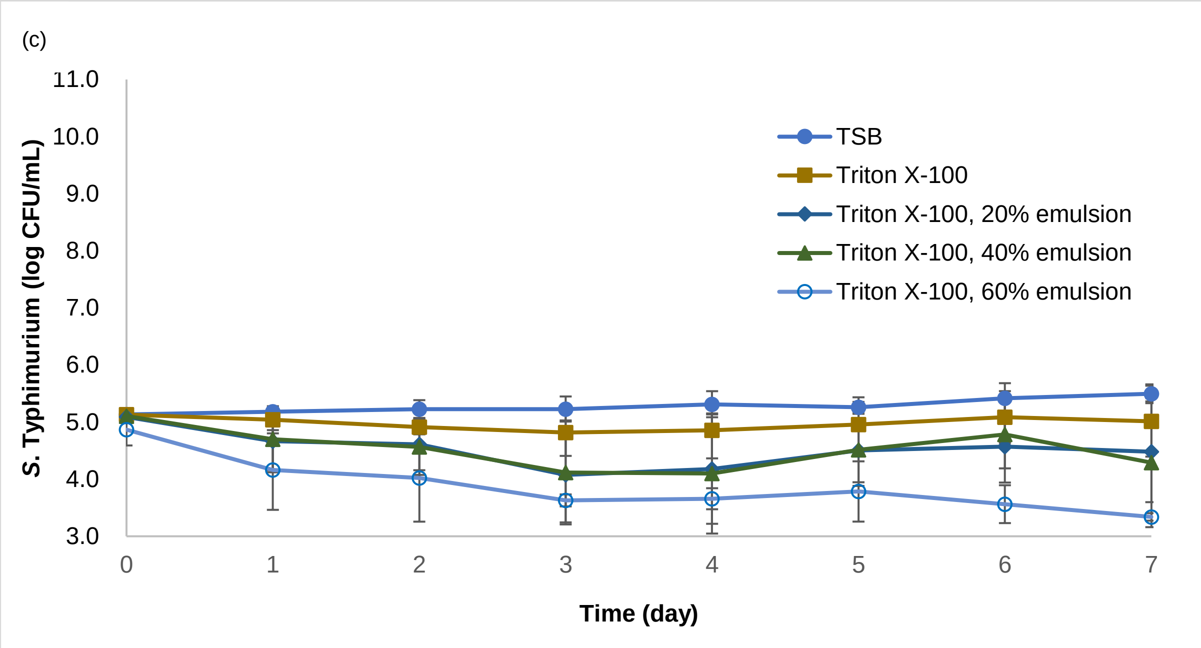
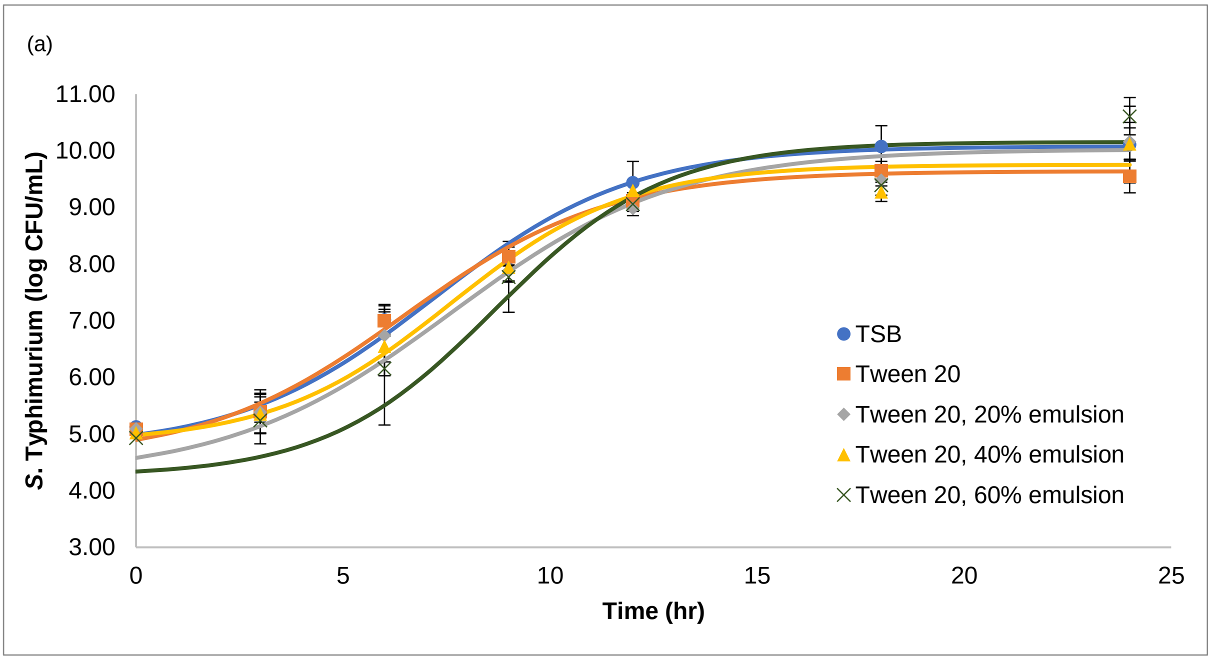
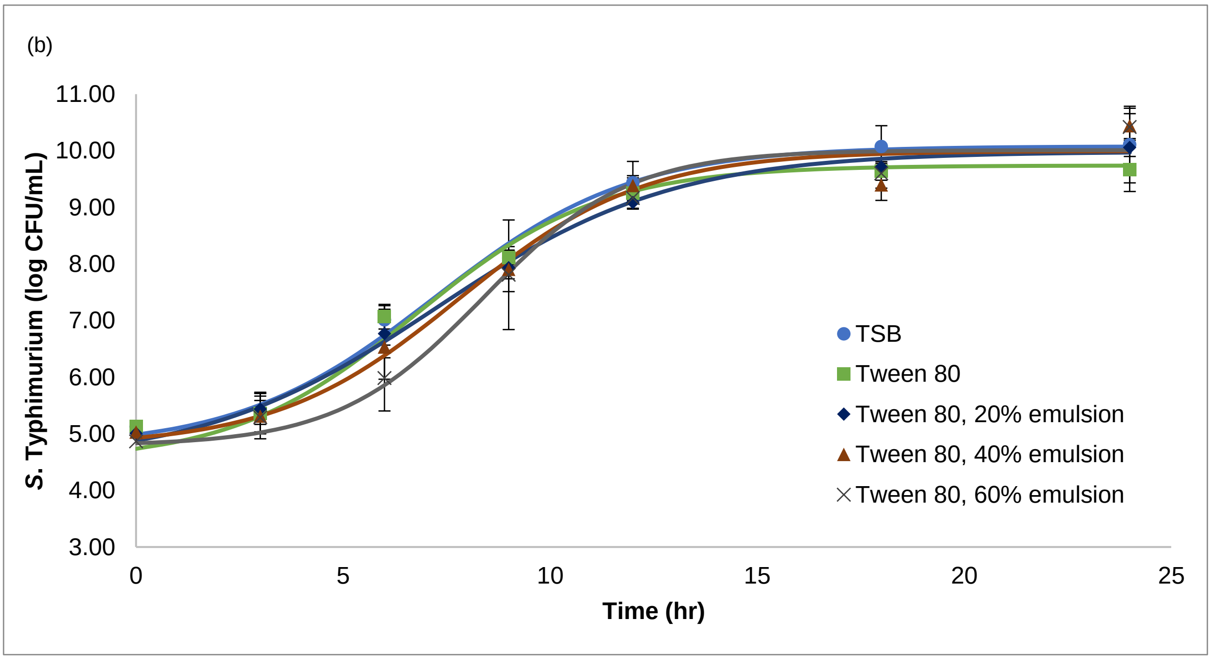


Figure 3-1. The growth curve of S. Typhimurium at 7°C (corresponding to refrigerating temperature) in (a) TSB, 2% (v/v) Tween 20 solution and 20, 40 and 60% (v/v) emulsions prepared using Tween 20; (b) TSB, 2% (v/v) Tween 80 solution and 20, 40 and 60% (v/v) emulsions prepared using Tween 80; (c) TSB, 2% (v/v) Triton X-100 solution and 20, 40 and 60% (v/v) emulsions prepared using Triton X-100. Each data point is an average of triplicate measurements ± standard deviation (SD).


(a)
S. Typhimurium (log CFU/mL)
11.00
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
0
5
10
15
20
25
Time (hr)
TSB
Tween 20
Tween 20, 20% emulsion
Tween 20, 40% emulsion
Tween 20, 60% emulsion


(b)
S. Typhimurium (log CFU/mL)
11.00
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
0
5
10
15
20
25
Time (hr)
TSB
Tween 80
Tween 80, 20% emulsion
Tween 80, 40% emulsion
Tween 80, 60% emulsion

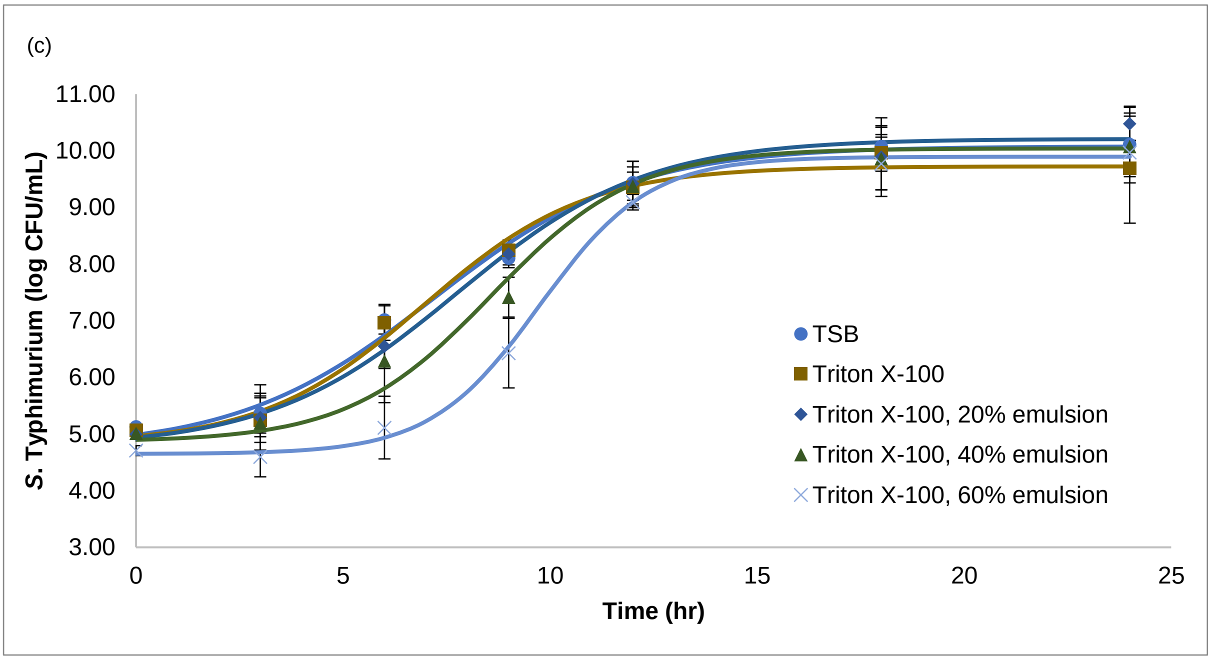
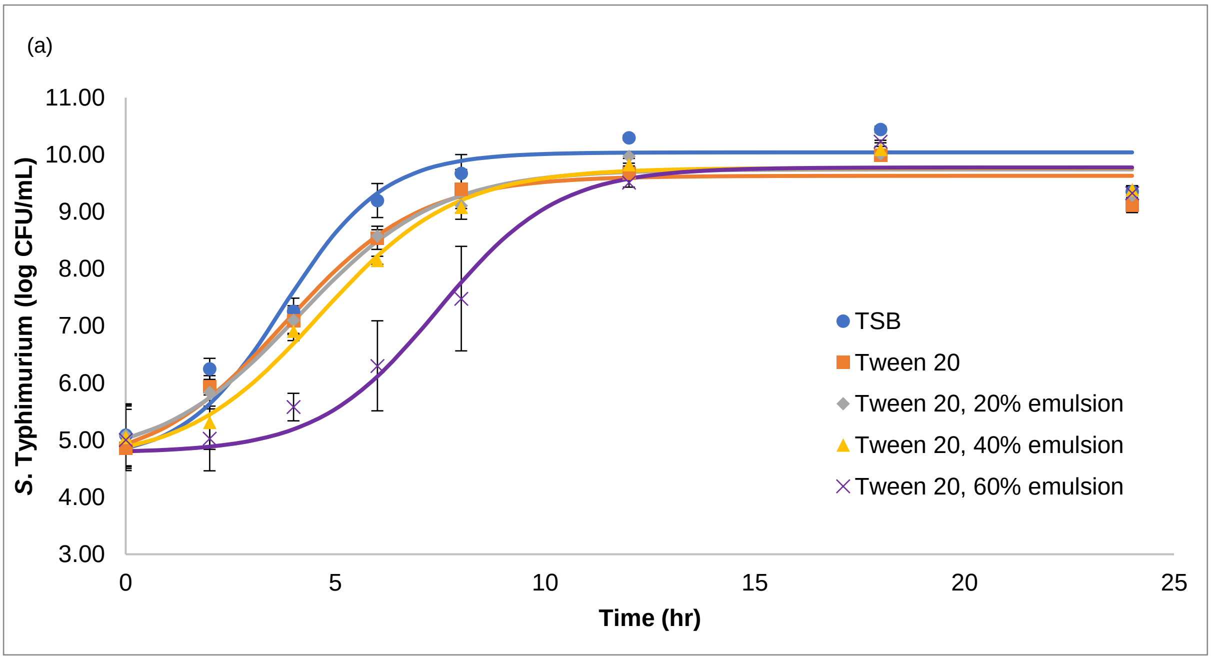
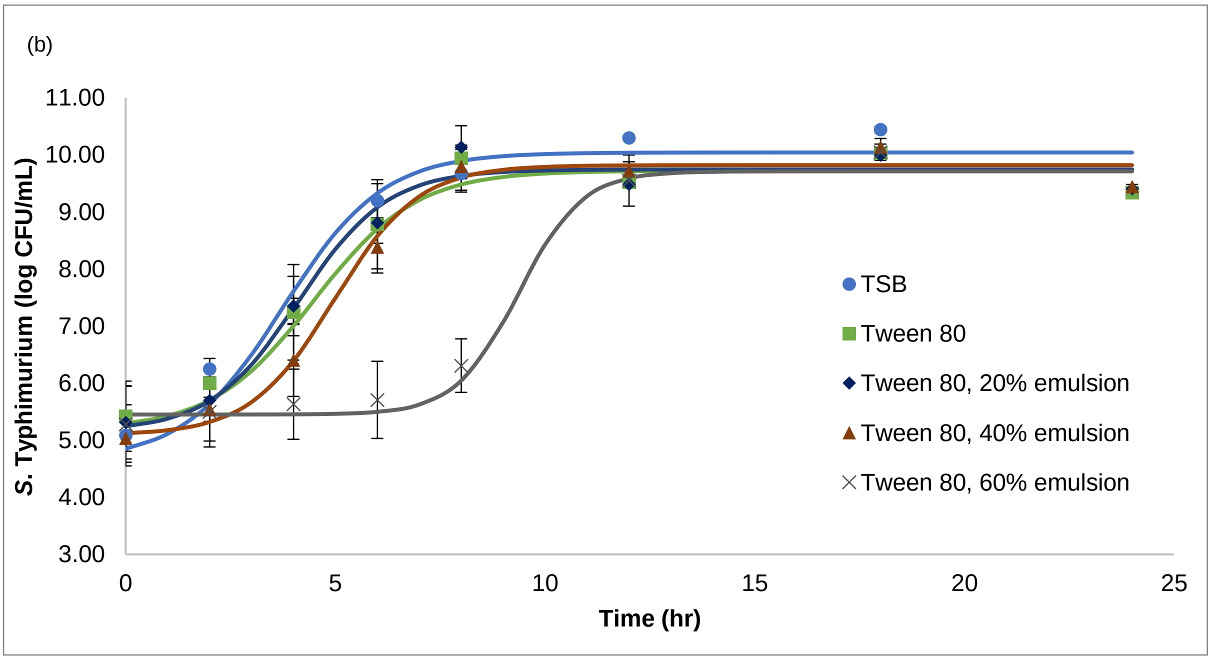


Figure 3-2. The growth curve of S. Typhimurium at room temperature (22°C) in (a) TSB, TSB supplemented with Tween 20 and 20, 40 and 60% (v/v) emulsions prepared using Tween 20; (b) TSB, TSB supplemented with Tween 80, and 20, 40 and 60% (v/v) emulsions prepared using Tween 80; (c) TSB, TSB supplemented with Triton X-100, and 20, 40 and 60% (v/v) emulsions prepared using Triton X-100. The experimental data was modeled using the Logistic model, and the average model fit for each growth curve is shown with a solid line. Each data point presented is the average of three measurements with standard deviation (SD).



(a)
11.00
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
S. Typhimurium (log CFU/mL)
0
5
10
15
20
25
Time (hr)
TSB
Tween 20
Tween 20, 20% emulsion
Tween 20, 40% emulsion
Tween 20, 60% emulsion



(b)
11.00
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
S. Typhimurium (log CFU/mL)
0
5
10
15
20
25
Time (hr)
TSB
Tween 80
Tween 80, 20% emulsion
Tween 80, 40% emulsion
Tween 80, 60% emulsion

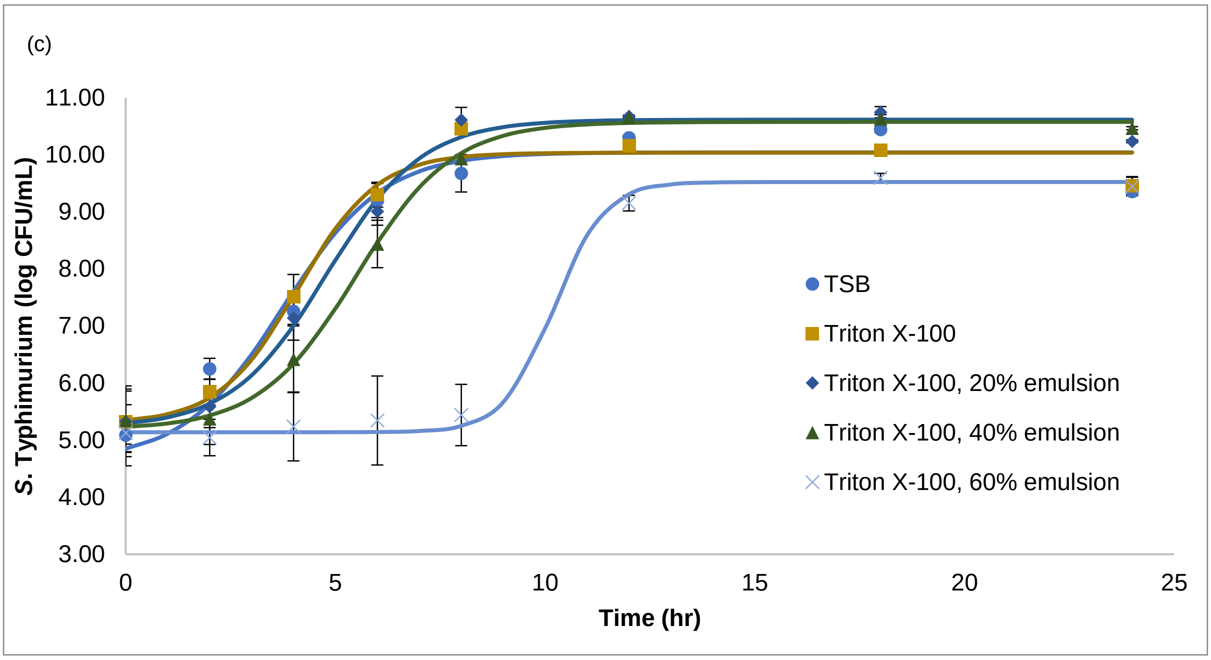


Figure 3-3. The growth curve of S. Typhimurium at optimal growth temperature (37°C) in (a) TSB, TSB supplemented with Tween 20 and 20, 40 and 60% (v/v) emulsions prepared using Tween 20; (b) TSB, TSB supplemented with Tween 80, and 20, 40 and 60% (v/v) emulsions prepared using Tween 80; (c) TSB, TSB supplemented with Triton X-100, and 20, 40 and 60% (v/v) emulsions prepared using Triton X-100. The experimental data was modeled using the Logistic model, and the average model fit for each growth curve is shown with a solid line. Each data point presented is the average of three measurements with standard deviation (SD).

The growth behavior of S. Typhimurium at 7°C was analyzed over a 7-day incubation period and the results were summarized in Table 3-1. No significant changes were observed in bacterial cultures except for those in Triton X-100 stabilized emulsions ($P > 0.05$). However, a decline in bacterial numbers was detected in Triton X-100 stabilized 20% and 40% emulsions on the third and fourth day of incubation at 7°C ($P < 0.05$). Bacterial numbers subsequently recovered to the same level as on the first day of incubation ($P > 0.05$). Furthermore, a notable decrease in bacterial numbers was observed in Triton X-100 stabilized 60% emulsion after 3 days of incubation at 7°C as compared to the first day ($P < 0.05$). The growing temperature for S. Typhimurium has been recoded as 5-46°C, and the optimistic range has been reported as 35-43°C (Yates, 2011). Thus, at 7°C, which was the temperature associated with the temperature in fridge, S. Typhimurium was merely able to survive instead of growing in TSB and emulsifier solutions. In contrast, the combination of oil and Triton X-100 had a negative impact on bacteria survive, which was consistent with previous research. Triton X-100 and oil may have the disruptive effects on cell membrane, leading to increased permeability and reduced viability (Hemaiswarya & Doble, 2009; Zdarta, Smułek, Pacholak, Dudzińska-Bajorek, & Kaczorek, 2020).

Table 3-1. *S*. Typhimurium numeration (log CFU/mL) in TSB, emulsifier solutions, and emulsions at 7°C. Each data point is an average of triplicate measurements ± standard deviation (SD). Lower case letter labels (a, b, c, etc.) were used to indicate significant differences of bacteria number with different time, based on two-way ANOVA. Upper case letter labels (A, B, C, etc.) were used to indicate significant differences of bacteria number with the same day but different environment, based on two-way ANOVA. Groups with the same letter were not significantly different (P > 0.05), while those with different letters were significantly different (P < 0.05).

Time (day)	TSB	Tween 20	Tween 20 20% emulsion	Tween 20 40% emulsion	Tween 20 60% emulsion	Tween 80	Tween 80 20% emulsion	Tween 80 40% emulsion	Tween 80 60% emulsion
0	$5.13 \pm 0.11^{a, A}$	$5.12 \pm 0.07^{a, A}$	$5.13 \pm 0.05^{a, A}$	$5.12 \pm 0.04^{a, A}$	$5.09 \pm 0.06^{a, A}$	$5.18 \pm 0.01^{a, A}$	$5.19 \pm 0.01^{a, A}$	$5.16 \pm 0.11^{a, A}$	$5.14 \pm 0.08^{a, A}$
1	$5.18 \pm 0.03^{a, A}$	$5.14 \pm 0.10^{a, A}$	$5.08 \pm 0.10^{a, A}$	$5.12 \pm 0.12^{a, A}$	$5.08 \pm 0.12^{a, A}$	$5.16 \pm 0.09^{a, A}$	$5.14 \pm 0.06^{a, A}$	$5.11 \pm 0.10^{a, A}$	$5.00 \pm 0.10^{a, A}$
2	$5.23 \pm 0.16^{a, A}$	$5.15 \pm 0.17^{a, A}$	$5.14 \pm 0.09^{a, AB}$	$5.08 \pm 0.12^{a, AB}$	$4.99 \pm 0.07^{a, AB}$	$5.24 \pm 0.10^{a, A}$	$5.14 \pm 0.13^{a, AB}$	$5.12 \pm 0.20^{a, AB}$	$4.98 \pm 0.09^{a, AB}$
3	$5.23 \pm 0.22^{a, AB}$	$5.18 \pm 0.24^{a, ABC}$	$5.10 \pm 0.28^{a, ABC}$	$5.06 \pm 0.25^{a, ABC}$	$4.86 \pm 0.30^{a, ABC}$	$5.25 \pm 0.18^{a, A}$	$5.12 \pm 0.24^{a, ABC}$	$5.02 \pm 0.38^{a, ABC}$	$4.75 \pm 0.28^{a, ABCD}$
4	$5.31 \pm 0.23^{a, A}$	$5.22 \pm 0.32^{a, ABC}$	$5.14 \pm 0.36^{a, ABC}$	$4.99 \pm 0.32^{a, ABC}$	$4.70 \pm 0.38^{a, ABCD}$	$5.30 \pm 0.25^{a, AB}$	$5.16 \pm 0.36^{a, ABC}$	$5.02 \pm 0.47^{a, ABC}$	$4.90 \pm 0.20^{a, ABC}$
5	$5.26 \pm 0.09^{a, A}$	$5.21 \pm 0.19^{a, A}$	$5.09 \pm 0.48^{a, A}$	$4.99 \pm 0.49^{a, A}$	$4.61 \pm 0.33^{a, A}$	$5.33 \pm 0.23^{a, A}$	$5.28 \pm 0.40^{a, A}$	$4.73 \pm 0.16^{a, AB}$	$4.84 \pm 0.14^{a, AB}$
6	$5.42 \pm 0.26^{a, A}$	$5.35 \pm 0.31^{a, A}$	$5.09 \pm 0.62^{a, A}$	$4.92 \pm 0.63^{a, A}$	$4.62 \pm 0.36^{a, A}$	$5.43 \pm 0.28^{a, A}$	$5.13 \pm 0.51^{a, A}$	$5.07 \pm 0.48^{a, A}$	$4.75 \pm 0.19^{a, A}$
7	$5.50 \pm 0.16^{a, A}$	$5.35 \pm 0.26^{a, AB}$	$5.09 \pm 0.70^{a, AB}$	$4.96 \pm 0.80^{a, AB}$	$4.52 \pm 0.33^{a, AB}$	$5.43 \pm 0.24^{a, A}$	$5.12 \pm 0.59^{a, AB}$	$5.06 \pm 0.56^{a, AB}$	$4.77 \pm 0.23^{a, AB}$

Table 3-1. (cont.)

Time (day)	Triton x-100	Triton x-100 20% emulsion	Triton x-100 40% emulsion	Triton x-100 60% emulsion
0	$5.13 \pm 0.08^{a, A}$	$5.09 \pm 0.02^{a, A}$	$5.11 \pm 0.12^{a, A}$	$4.87 \pm 0.28^{a, A}$
1	$5.04 \pm 0.24^{a, A}$	$4.67 \pm 0.55^{a, A}$	$4.70 \pm 0.55^{a, A}$	$4.16 \pm 0.70^{a, A}$
2	$4.91 \pm 0.10^{a, AB}$	$4.61 \pm 0.45^{a, AB}$	$4.56 \pm 0.49^{a, AB}$	$4.02 \pm 0.76^{a, B}$
3	$4.82 \pm 0.41^{a, ABC}$	$4.07 \pm 0.83^{b, CD}$	$4.12 \pm 0.91^{b, BCD}$	$3.63 \pm 0.10^{b, D}$
4	$4.86 \pm 0.49^{a, ABC}$	$4.18 \pm 0.96^{b, BCD}$	$4.10 \pm 1.05^{b, CD}$	$3.66 \pm 0.18^{b, D}$
5	$4.95 \pm 0.48^{a, A}$	$4.50 \pm 0.56^{a, AB}$	$4.51 \pm 0.63^{a, AB}$	$3.78 \pm 0.53^{b, B}$
6	$5.09 \pm 0.45^{a, A}$	$4.57 \pm 0.63^{a, AB}$	$4.78 \pm 0.59^{a, A}$	$3.56 \pm 0.33^{c, B}$
7	$5.02 \pm 0.62^{a, AB}$	$4.48 \pm 0.88^{a, AB}$	$4.29 \pm 1.13^{a, BC}$	$3.34 \pm 0.07^{d, C}$

Table 3-2 summarized the growth rate and lag phase of *S*. Typhimurium in TSB, emulsifier solutions, and emulsions at 22°C, which is a common model of room temperature in microbiological studies (Alshammari et al., 2021; Cho, Kim, Kim, Song, & Rhee, 2016). The lack of significant differences in the growth rate between emulsifier solutions, emulsions, and TSB ($P > 0.05$) suggests that temperature plays a critical role in suppressing *Salmonella* growth. However, a significantly higher lag phase was observed in Triton X-100 stabilized 60% emulsion compared to TSB ($P < 0.05$), indicating that the high oil content in Triton X-100 may pose a more severe environmental challenge, requiring the bacteria to take more time to adapt and initiate growth. Moreover, no significant difference in lag phase was observed among Tween 20, Tween 80, and Triton X-100 at the same oil fraction ($P > 0.05$), indicating that the type of emulsifier may not be an essential factor in influencing the growth of *S*. Typhimurium at 22°C. However, significantly higher lag phase was observed in Tween 20 stabilized 60% emulsion compared to Tween 20 solution ($P < 0.05$), and in Triton X-100 stabilized 60% emulsion compared to Triton X-100 solution and Triton X-100 stabilized 20% emulsion ($P < 0.05$) when comparing different oil fractions with the same type of emulsifier. Interestingly, Tween 80 solution showed no significant difference in comparison to any oil fraction of Tween 80 stabilized emulsions ($P > 0.05$). As Triton X-100 has a lower Hydrophilic-Lipophilic Balance (HLB) value and higher hydrophobicity than the other two emulsifiers, it has a greater affinity for oil, leading to increased interaction with cell membranes and oil droplets (Fang, Zhao, Li, Qiu, Wang, & Geng, 2022; Hollmann et al., 2016; Ullah, Baloch, & Durrani, 2011). Therefore, the impact of high oil fraction conditions on bacterial growth is more pronounced in Triton X-100 environments. On the other hand, since the oleic acid released from Tween 80 had a protective effect on bacteria membrane has been reported by Mohammad & Taha Daod (2021b), it is possible that the presence of oleic acid in Tween 80 emulsions may have contributed to the lack of significant difference in lag phase and growth rate observed between Tween 80 solution and different oil fractions of Tween 80 stabilized emulsions in our study.

Table 3-2. Summary of *S*. Typhimurium growth model in TSB, emulsifier solutions, and emulsions at 22°C. Each data point is an average of triplicate measurements ± standard deviation (SD). Lower case letter labels (a, b, c, etc.) were used to indicate significant differences of growth rate in different growth environment, based on two-way ANOVA. Upper case letter labels (A, B, C, etc.) were used to indicate significant differences of lag phase in different growth environment, based on two-way ANOVA. Groups with the same letter were not significantly different (P > 0.05), while those with different letters were significantly different (P < 0.05). The unit of bacterial growth rate was log CFU/ hr.

	TSB	**Tween 20**	**Tween 20, 20% emulsion**	**Tween 20, 40% emulsion**	**Tween 20, 60% emulsion**
R-square	0.98 ± 0.01	0.99 ± 0.01	0.99 ± 0.01	0.98 ± 0.01	0.97 ± 0.01
Growth rate	0.42 ± 0.04^{a}	0.41 ± 0.12^{a}	0.37 ± 0.06^{a}	0.47 ± 0.07^{a}	0.49 ± 0.19^{a}
Inflection point	7.21 ± 0.22	6.49 ± 0.45	7.61 ± 0.66	7.59 ± 0.95	8.68 ± 0.87
Lag phase (hr)	4.79 ± 0.33^{CD}	3.88 ± 1.18^{D}	4.85 ± 1.03^{CD}	5.44 ± 1.26^{CD}	6.44 ± 1.56^{BC}
		Tween 80	**Tween 80, 20% emulsion**	**Tween 80, 40% emulsion**	**Tween 80, 60% emulsion**
R-square		0.99 ± 0.01	0.99 ± 0.01	0.97 ± 0.02	0.98 ± 0.01
Growth rate		0.45 ± 0.05^{a}	0.35 ± 0.07^{a}	0.45 ± 0.10^{a}	0.57 ± 0.20^{a}
Inflection point		6.77 ± 0.30	7.26 ± 0.97	7.80 ± 1.16	8.38 ± 1.45
Lag phase (hr)		4.54 ± 0.52^{CD}	4.30 ± 1.52^{CD}	5.52 ± 1.63^{CD}	6.47 ± 2.03^{BC}

		Triton X-100	Triton X-100, 20% emulsion	Triton X-100, 40% emulsion	Triton X-100, 60% emulsion
R-square		0.97 ± 0.02	0.98 ± 0.01	0.98 ± 0.01	0.99 ± 0.01
Growth rate		0.51 ± 0.07[a]	0.44 ± 0.03[a]	0.58 ± 0.33[a]	0.76 ± 0.23[a]
Inflection point		7.75 ± 0.33	7.75 ± 1.17	8.59 ± 0.77	9.75 ± 0.45
Lag phase (hr)		4.97 ± 0.53[CD]	5.49 ± 1.30[CD]	6.52 ± 1.63[BC]	8.36 ± 0.79[AB]

The findings of this study, as presented in Table 3-3, suggested that the growth rate of *S*. Typhimurium in TSB, emulsifier solutions, and emulsions at 37°C was not significantly different ($P > 0.05$) among all samples. However, a significantly higher lag phase was observed in 60% emulsions stabilized with Tween 20, Tween 80, and Triton X-100 compared to in TSB ($P < 0.05$). In order to investigate the effect of emulsifier type on bacterial growth, a comparison at the same level of oil fraction was conducted between emulsifier types. Lower lag phase was found only in Tween 20 stabilized 60% emulsion when compared with 60% emulsions stabilized with Tween 80 or Triton X-100 ($P < 0.05$). Furthermore, the impact of oil fraction was assessed by comparing the different oil contents of emulsions with the same type of emulsifier. The results indicated that a 60% (v/v) oil content in emulsions resulted in a significantly higher lag phase compared to emulsifier solutions, 20%, and 40% emulsions ($P < 0.05$). In conclusion, while the individual usage of emulsifiers had no significant effect on bacterial growth at 37°C, the combination of emulsifier and high content of oil showed a negative impact on bacterial growth, as evidenced by the prolonged lag phase in all 60% emulsion samples. The significantly higher lag phase in the 60% emulsions may be attributed to the limited space for bacteria to survive. At optimal growing temperature, bacterial metabolites were secreted more into the aqueous phase, and in emulsions with high oil fraction, their concentration is likely to be higher due to the limited availability of aqueous phase. The accumulation of bacterial metabolites in the extracellular environment can affect bacterial growth, which may explain the observed results (Babynin, 2006). This finding is also consistent with previous studies that have shown that high oil concentrations in the environment can reduce bacterial growth rates (Mehdi & Giti, 2008).

Table 3-3. Summary of *S*. Typhimurium growth model in TSB, emulsifier solutions, and emulsions at 37°C. Each data point is an average of triplicate measurements ± standard deviation (SD). Lower case letter labels (a, b, c, etc.) were used to indicate significant differences of growth rate in different growth environment, based on two-way ANOVA. Upper case letter labels (A, B, C, etc.) were used to indicate significant differences of lag phase in different growth environment, based on two-way ANOVA. Groups with the same letter were not significantly different (P > 0.05), while those with different letters were significantly different (P < 0.05). The unit of bacterial growth rate was log CFU/ hr.

	TSB	**Tween 20**	**Tween 20, 20% emulsion**	**Tween 20, 40% emulsion**	**Tween 20, 60% emulsion**
R-square	0.97 ± 0.01	0.98 ± 0.01	0.98 ± 0.01	0.99 ± 0.01	0.98 ± 0.01
Growth rate	0.84 ± 0.52^{a}	0.62 ± 0.06^{a}	0.60 ± 0.05^{a}	0.62 ± 0.12^{a}	0.70 ± 0.31^{a}
Inflection point	3.76 ± 0.85	3.80 ± 0.51	4.13 ± 0.83	4.62 ± 0.64	7.44 ± 1.64
Lag phase (hr)	2.28 ± 1.54^{C}	2.18 ± 0.64^{C}	2.47 ± 0.97^{C}	2.98 ± 0.93^{C}	5.83 ± 2.20^{B}
		Tween 80	**Tween 80, 20% emulsion**	**Tween 80, 40% emulsion**	**Tween 80, 60% emulsion**
R-square		0.98 ± 0.01	0.98 ± 0.01	0.98 ± 0.01	0.97 ± 0.03
Growth rate		0.82 ± 0.07^{a}	0.95 ± 0.20^{a}	1.00 ± 0.28^{a}	1.33 ± 0.87^{a}
Inflection point		4.49 ± 0.68	4.13 ± 0.52	4.97 ± 0.46	9.37 ± 0.65
Lag phase (hr)		3.26 ± 0.59^{C}	3.06 ± 0.66^{C}	3.91 ± 0.74^{BC}	8.42 ± 1.04^{A}

		Triton X-100	Triton X-100, 20% emulsion	Triton X-100, 40% emulsion	Triton X-100, 60% emulsion
R-square		0.98 ± 0.01	0.99 ± 0.01	0.99 ± 0.01	0.98 ± 0.02
Growth rate		1.06 ± 0.22^{a}	0.88 ± 0.05^{a}	0.87 ± 0.12^{a}	1.65 ± 1.09^{a}
Inflection point		4.11 ± 0.19	4.80 ± 0.42	5.50 ± 0.60	10.21 ± 0.67
Lag phase (hr)		3.14 ± 0.28^{C}	3.66 ± 0.49^{C}	4.34 ± 0.76^{BC}	9.43 ± 1.07^{A}

In summary, the results of this study showed that *S*. Typhimurium was unable to grow in TSB, emulsifier solutions, and emulsions at 7°C, indicating that the presence of emulsifiers and oil does not support bacterial growth at refrigeration temperatures. At room temperature (22°C), the 60% emulsion stabilized with Triton X-100 provided the most challenging environment for bacterial growth, as evidenced by the extended lag phase. The effect of oil content varied depending on the emulsifier type, with Tween 20 and Triton X-100 showing extended lag phase in 60% emulsion compared to their solutions, while Tween 80 showed no difference between emulsions and emulsifier solution. This suggests that the oleic acid released from Tween 80 provides protection to bacterial cells by maintaining the integrity of the cell membrane. However, at the optimal growing temperature of 37°C, the impact of emulsifier type and oil fraction became more pronounced. In 60% emulsions, a prolonged lag phase was observed with Tween 20, Tween 80, and Triton X-100, indicating a negative impact of high oil fraction on bacterial cells. The limited space for bacterial growth due to the higher oil fraction in the environment led to a higher concentration of metabolites, which was more evident at the optimal growing temperature.

3.3.2 Effect of temperature on thermal inactivation kinetics of S. Typhimurium in high oil fraction emulsion

The growth environment of bacteria has been reported to have an impact on their survival and subsequent inactivation in subsequent treatments (Birk et al., 2016). Based on this knowledge, it was hypothesized that the emulsions which affect bacterial growth would also impact their inactivation rate. Since only emulsions with 60% oil fraction had a significant effect on bacterial growth, only these emulsions, along with TSB and individual emulsifiers, were used for inactivation experiments. After an overnight incubation (~20 hr), bacteria reached 9-10 log CFU/mL in all media. Figure 3-4 to 3-6 presented the bacterial inactivation data in TSB, individual emulsifiers, and 60% oil-in-water emulsions at 55, 58, and 60°C. It was found that bacterial inactivation at 55°C or higher temperature did not follow the log-linear inactivation kinetics, consistent with a previous study by S. Warren (1998). The tailing effect of the inactivation curve became more significant with an increase in oil fraction in emulsion. Therefore, the Weibull model was used to fit the inactivation data (Table 3-4). The Weibull model parameters showed that $\beta < 1$, indicating that the remaining bacteria had a propensity to adapt to heat. Additionally, α was an analogue to the traditional D value, and the 5D concept, which indicated the time that reached 5-log reduction, was chosen as the standard for evaluating effects on bacterial inactivation (van Boekel, 2002).

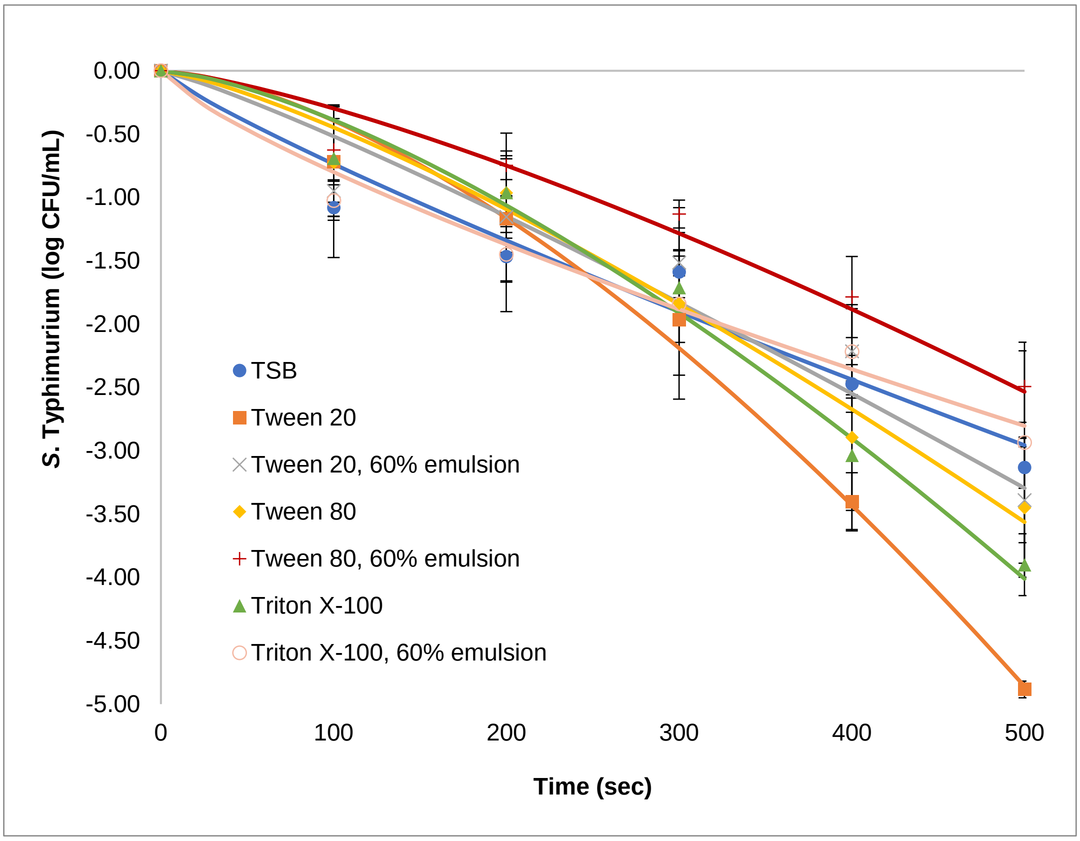


Figure 3-4. Thermal inactivation, conducted by submerged coil apparatus, of *S*. Typhimurium at 55°C inoculated in TSB, Tween 20, Tween 80, Triton X-100 emulsifier solutions and 60% (v/v) oil-in-water emulsions prepared using those emulsifiers. Experimental data was fitted into Weibull model and average model fit for each growth curve is depicted using the solid line. Each data point is an average of triplicate measurements ± standard deviation (SD).

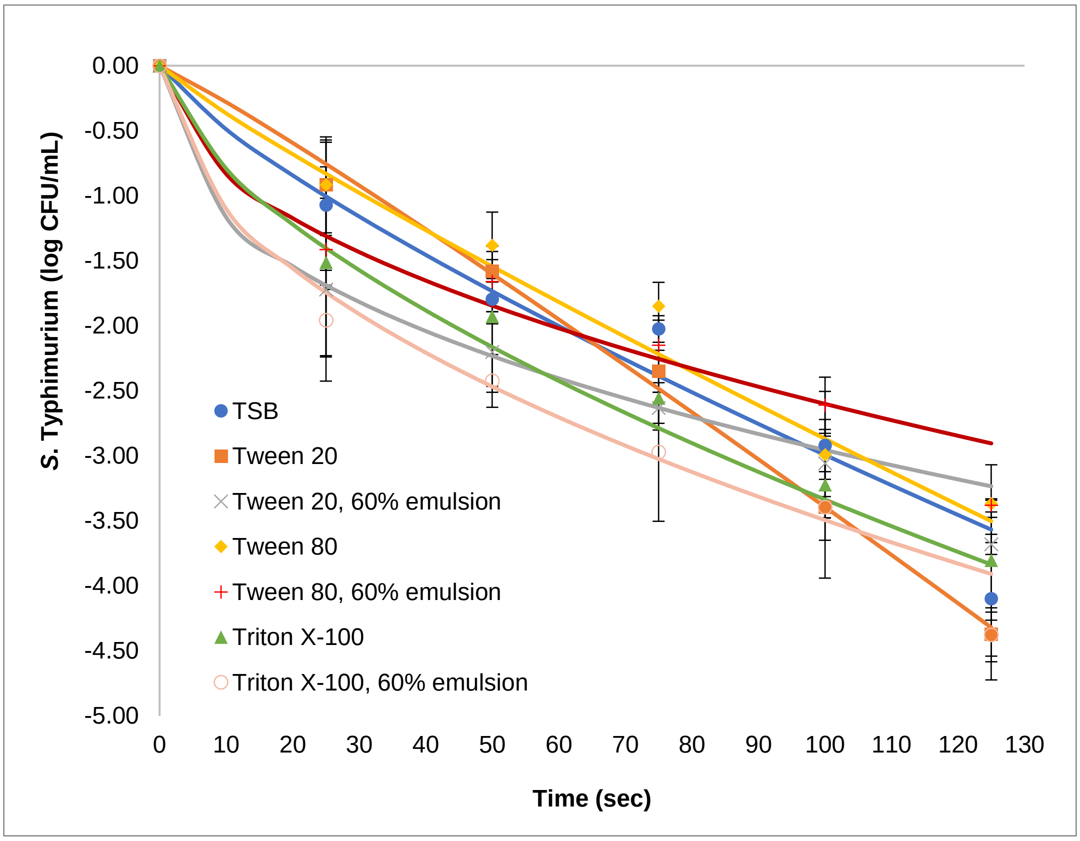


Figure 3-5. Thermal inactivation, conducted by submerged coil apparatus, of *S*. Typhimurium at 58°C inoculated in TSB, Tween 20, Tween 80, Triton X-100 emulsifier solutions and 60% (v/v) oil-in-water emulsions prepared using those emulsifiers. Experimental data was fitted into Weibull model and average model fit for each growth curve is depicted using the solid line. Each data point is an average of triplicate measurements ± standard deviation (SD).

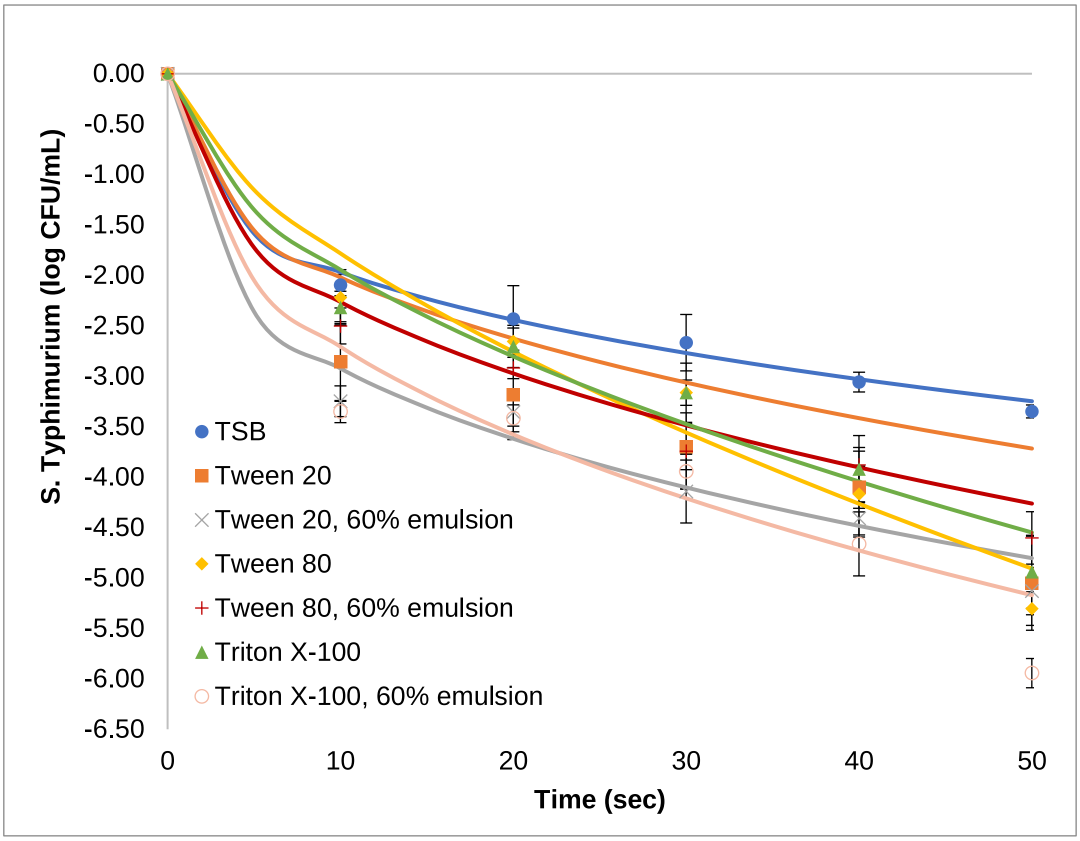


Figure 3-6. Thermal inactivation, conducted by submerged coil apparatus, of *S*. Typhimurium at 60°C inoculated in TSB, Tween 20, Tween 80, Triton X-100 emulsifier solutions and 60% (v/v) oil-in-water emulsions prepared using those emulsifiers. Experimental data was fitted into Weibull model and average model fit for each growth curve is depicted using the solid line. Each data point is an average of triplicate measurements ± standard deviation (SD).

Table 3-4. Thermal inactivation model parameters (Weibull model) for of *S*. Typhimurium at 55°C, 58°C, and 60°C in TSB, Tween 20, Tween 80, and Triton X-100 emulsifiers, as well as 60% (v/v) emulsions prepared from these emulsifiers. Each data point is an average of triplicate measurements ± standard deviation (SD). Lower case letter labels (a, b, c, etc.) were used to indicate significant differences between groups with the same temperature, based on two-way ANOVA. Upper case letter labels (A, B, C, etc.) were used to indicate significant differences within groups with different temperature, based on two-way ANOVA. Groups with the same letter were not significantly different ($P > 0.05$), while those with different letters were significantly different ($P < 0.05$).

		TSB	Tween 20	Tween 20, 60% emulsion	Tween 80	Tween 80, 60% emulsion	Triton X-100	Triton X-100, 60% emulsion
55°C	α (sec)	54.29 ± 15.35	106.39 ± 22.37	85.85 ± 7.16	97.69 ± 66.58	132.81 ± 25.44	107.88 ± 41.61	45.69 ± 19.80
	β	0.86 ± 0.09	1.56 ± 0.23	1.15 ± 0.10	1.29 ± 0.47	1.33 ± 0.17	1.45 ± 0.37	0.78 ± 0.08
	5D concept based on Weibull model (sec)	915.06 ± 48.58$^{a, A}$	513.17 ± 10.45$^{d, A}$	727.12 ± 71.15$^{bc, A}$	664.37 ± 67.23$^{cd, A}$	841.79 ± 65.56$^{ab, A}$	598.45 ± 46.80$^{cd, A}$	1031.79 ± 226.70$^{a, A}$
58°C	α (sec)	8.61 ± 3.30	14.99 ± 1.92	0.87 ± 0.80	12.10 ± 1.73	2.69 ± 2.20	3.83 ± 0.61	1.57 ± 0.28
	β	0.79 ± 0.14	1.08 ± 0.08	0.40 ± 0.08	0.89 ± 0.04	0.50 ± 0.11	0.63 ± 0.02	0.50 ± 0.03
	5D concept based on Weibull model (sec)	195.68 ± 26.75$^{bc, B}$	154.49 ± 5.52$^{c, B}$	324.68 ± 64.04$^{abc, B}$	185.82 ± 9.54$^{c, B}$	365.32 ± 92.61$^{a, B}$	190.49 ± 20.57$^{bc, B}$	207.09 ± 36.34$^{abc, B}$
60°C	α (sec)	0.08 ± 0.04	0.17 ± 0.22	0.02 ± 0.01	1.06 ± 0.38	0.15 ± 0.09	0.59 ± 0.16	0.11 ± 0.07
	β	0.31 ± 0.02	0.38 ± 0.13	0.31 ± 0.02	0.63 ± 0.07	0.39 ± 0.05	0.53 ± 0.03	0.40 ± 0.05
	5D concept based on Weibull model (sec)	187.63 ± 16.29$^{a, B}$	61.27 ± 12.40$^{a, B}$	54.17 ± 4.52$^{a, C}$	51.37 ± 5.16$^{a, C}$	68.58 ± 9.94$^{a, C}$	58.65 ± 3.88$^{a, C}$	41.54 ± 2.83$^{a, C}$

At a thermal treatment of 55°C, the bacterial inactivation rate of *S*. Typhimurium was found to be influenced by the emulsifier involved environment, with lower 5D values observed in all environments except for Tween 80 and Triton X-100 stabilized 60% emulsions ($P < 0.05$). Interestingly, no significant difference was observed in the 5D value of the latter two environments compared to TSB ($P > 0.05$). This suggests that the negative effects of Tween 80 or Triton X-100 were alleviated by the presence of oil. This observation is consistent with a previous study that described the lower heat conductivity in food matrix with increased fat content (Murphy, Osaili, Duncan, & Marcy, 2004). However, the result was opposite to that of Ch2, which showed a protective effect in Tween 80 and Triton X-100 emulsifier solutions instead of their emulsions. The potential reason for this will be revealed in the next paragraph. Additionally, comparing the effects of oil inclusion, the 5D value was found to be higher in the emulsion compared to the corresponding emulsifier solution ($P < 0.05$), suggesting that emulsion may provide protection to bacteria due to the presence of oil (Ren, 2021). At a temperature of 58°C, *S*. Typhimurium inoculated in Tween 80 stabilized 60% emulsion showed a significantly higher 5D value than in TSB ($P < 0.05$). However, no significant difference in 5D values was observed when comparing different emulsifier types within their respective emulsions and solutions ($P > 0.05$). Moreover, when comparing emulsifier solutions and their corresponding emulsions, only *S*. Typhimurium cultured in Tween 80 stabilized 60% emulsion exhibited a higher 5D value compared to in its solution counterpart ($P < 0.05$), indicating that the emulsion environment can still have an impact on bacterial inactivation under specific conditions. However, the lack of significant differences in 5D values between different emulsifier types within their respective emulsions and solutions may suggest that temperature becomes the main factor influencing bacterial inactivation, rather than the specific properties of the emulsifiers. This hypothesis was further supported by the results of the inactivation experiment at 60°C, where the 5D value was more than 2 times less in emulsifier solutions and emulsions compared to TSB. However, statistical analysis showed no difference among all of the environments ($P > 0.05$), indicating that neither emulsifier type nor the presence of oil had an influence on the inactivation of bacteria at higher temperatures. Therefore, it can be concluded that temperature is the main factor that influences bacterial inactivation in these environments.

In Table 3-4, the comparison between different temperatures within the same environment was also conducted to investigate the effect of temperature on bacterial inactivation. As expected, the 5D value decreased with an increase in temperature, indicating that higher temperature led to faster inactivation rate of *S*. Typhimurium. However, it is interesting to note that S. Typhimurium cultured in TSB and Tween 20 solution showed no significant difference in bacterial inactivation between 58°C and 60°C ($P > 0.05$). This finding suggests that these two temperatures might be too close to have a significant impact on the inactivation rate of *S*. Typhimurium in TSB and Tween 20 solution. Specifically, it is possible that the difference in thermal resistance of the bacteria at these two temperatures is not large enough to result in a statistically significant difference in inactivation rate. Interestingly, this lack of significant difference between 58°C and 60°C is only observed in TSB and Tween 20 solution. Further investigation is required to determine if this phenomenon is able to be observed in other environments or with other bacteria. It is also noted that the 5D value is the only parameter to evaluate the effect of inactivation in this study and may not comprehensively uncover the influence of temperature on microbial viability. Hence, further study is needed to be done to fully understand the impact of temperature on bacteria inactivation in different environments.

An interesting finding from this chapter was that the emulsions displayed a protective effect in the inactivation model at 55°C, which was opposite to the result reported in chapter 2 for the emulsifier solutions. This discrepancy was due to the different devices used for thermal treatment. In chapter 2, a water bath was used, while a submerged coil apparatus was used in this chapter. The reason for the change was that the samples required more time to reach the target temperature when subjected to the water bath. It took about 5 minutes to reach 55°C with the water bath, but only a few seconds with the submerged coil apparatus. This can also result in the difference of inactivation curve between chapter 2 and this chapter. For instance, a tailing effect was evident after a 30-minute thermal treatment in chapter 2, whereas this effect was observed at the beginning of treatment in this study. This observation suggested that rapid thermal processing potentially augmented the influence of oil incorporation in food matrix, while slow heating provided a comparatively less effect of oil. This is in line with the findings reported by Juneja and Marks (2003), in their study on *Salmonella* in sous vide cooked beef, which demonstrated that slow heating rates resulted in greater heat resistance.

3.4 Conclusion

The objective of this study was to investigate the effect of temperature on the growth and inactivation of *S*. Typhimurium in emulsion system. It was found that *S*. Typhimurium was only survive but not grown in emulsions and emulsifier solutions at refrigeration temperatures (7°C). At room temperature (22°C), the 60% emulsion stabilized with Triton X-100 was the most challenging environment for bacterial growth. The impact of emulsifier type and oil fraction became more pronounced at the optimal growing temperature (37°C), with a prolonged lag phase observed in 60% emulsions with Tween 20, Tween 80, and Triton X-100. On the other hand, the impact of temperature on bacteria inactivation in emulsions was also investigated. Emulsions with 60% oil fraction had a significant effect on bacterial growth and therefore were used in inactivation experiments. It is important to note that the inactivation device in this chapter is submerged coil apparatus, which showed the opposite effect on the behavior of bacteria inactivation. At 55°C, the presence of emulsifiers had a negative impact on bacterial inactivation, but the presence of oil had a protective effect. At higher temperatures (58°C and 60°C), temperature was found to be the main factor influencing bacterial inactivation in emulsions and emulsifier solutions, rather than the type of emulsifier or oil fraction. The 5D value decreased with an increase in temperature, and there were no significant differences in 5D values between different emulsifier types within their respective emulsions and solutions, indicating that temperature was the main factor to affect bacterial inactivation. Oleic acid released from Tween 80, higher hydrophobicity of Triton X-100, and the inclusion of oil were potentially provided protection against bacteria inactivation to some extent. However, temperature remained the most important factor influencing bacterial growth and inactivation. Hence, further research may need to be conducted to fully understand the effect of temperature on bacteria growth and inactivation in emulsion systems.

Based on the findings of this chapter, it is clear that temperature plays a crucial role in the growth and inactivation of *S*. Typhimurium in emulsion systems. Moreover, the impact of emulsifier type and oil fraction on bacterial growth becomes more pronounced at higher temperatures. This highlights the importance of investigating the molecular properties of emulsifiers, such as their molecular weight and surface charge, as explored in next chapter. By examining the relationship between the physicochemical properties of emulsifiers and their effect on bacterial growth and inactivation, it will be possible to gain a deeper understanding of the mechanisms that underlie bacterial behavior in emulsion systems.

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4 Chapter 4 The effect of molecular weight and surface charge of emulsifier on *S. enterica* Serovar Typhimurium growth and thermal inactivation in oil-in-water emulsion

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Abstract

The comprehension of pathogenic bacterial growth and inactivation rates in emulsion systems is essential in preventing bacterial contamination and ensuring food safety. Optimal selection of emulsifiers, taking into account their molecular weight and surface charge, can assist in achieving the desired product attributes while minimizing the potential for bacterial growth and spoilage in the emulsion system. In this research, oil-in-water emulsions were prepared using three different emulsifiers (whey protein isolate (WPI), whey protein hydrolysate (WPH), and whey protein isolate at a pH lower than the isoelectric point (WPI$^+$)) and varying oil fractions (20%, 40%, and 60% (v/v)). The emulsions, along with emulsifier solutions and control (TSB), were inoculated with S. Typhimurium and subsequently incubated at 37°C for up to 24 hours, after which the growth and inactivation of S. Typhimurium were determined by plate counting. The findings indicated that the molecular weight of emulsifiers exerted a greater impact on bacterial growth than their surface charge when employed separately in the system, which showed no lag phase with WPH and no difference of lag phase with WPI and WPI$^+$ when compared to TSB. However, in the case of 60% emulsion stabilized with a cationic emulsifier (WPI$^+$), a higher growth rate was observed in comparison to the anionic emulsifier (WPI) stabilized 60% emulsion. After thermal treatment at 55°C, neither the molecular weight nor surface charge of emulsifiers had a significant effect on bacterial inactivation. Nonetheless, the emulsion system exhibited more protective effects on bacterial inactivation when stabilized with WPI$^+$. This may be attributed to the ability of positively charged emulsifiers to interact with the bacterial membrane, thereby

providing protective effects during thermal treatment. This study aimed to investigate how the molecular weight and surface charge of emulsifiers affect microbial growth and inactivation in oil-in-water emulsions, with the ultimate goal of developing more effective preservation strategies to ensure food safety.

4.1 Introduction

Understanding of pathogenic bacterial growth and inactivation rates in emulsion systems is critical as improper treatment can lead the emulsion to be contaminated or spoiled by bacterial contamination (Thilini P. Keerthirathne, Ross, Fallowfield, & Whiley, 2016; T. P. Keerthirathne, Ross, Fallowfield, & Whiley, 2019; Leistner & Gorris, 1995). To ensure the safety of food processing, it is important to understand factors that affect microbial growth and inactivation rates in an emulsion system (Podolak, Whitman, & Black, 2020). Emulsifiers are widely used in the food industry to improve the physical properties and stability of emulsions (Tan & McClements, 2021). The use of different types of emulsifiers results in different final properties for emulsion products. For example, low molecular weight compounds such as monoglycerides and diglycerides in food products generally lead to higher clarity, and better flavor retention of products (Moonen & Bas, 2014). However, at the same concentration, these products also tend to be less stable than traditional products made using high molecular weight emulsifiers such as soybean lecithin (Hasenhuettl, 2019).

Based on their net charge, emulsifiers are classified as neutral, negatively, and positively charged (Marhamati, Ranjbar, & Rezaie, 2021). The electrical charge of the emulsifier affects the electrostatic interactions between bacteria and emulsion droplets (Ly et al., 2008). Theoretically, the negatively charged emulsifiers in the emulsion create an electric field that repels the negative charges on the surface of bacteria and can inhibit the growth of harmful bacteria on the surface of food particles (Li, McClements, & McLandsborough, 2001). In contrast, a neutrally charged emulsifier does not interact with bacterial cells electrostatically and therefore may not affect bacteria growth in emulsion (Li et al., 2001). However, a positively charged emulsifier is attracted to the surface of the bacteria and may clump together to block the entry of nutrients or the heat energy from thermal treatment into bacterial cells (Satpute, Banpurkar, Dhakephalkar, Banat, & Chopade, 2010). Thus, the food manufacturer must select the emulsifier that has the best combination of properties to achieve the desired product characteristics while minimizing the risk of bacterial growth and spoilage in the emulsion system. Recent studies have explored the potential of emulsifiers in inhibiting bacterial growth, both in fresh produce and in biofilms, and the populations of various pathogens were effectively reduced (Kang & Song, 2015; Kolomaznik, Nova, & Calkovska, 2017; Simões, Pereira, & Vieira, 2005). However, most of these studies have not considered the effect of molecular weight and surface charge of emulsifiers on bacteria growth and inactivation rates.

The issue of microbial growth and inactivation in oil-in-water emulsions by comparing the factors that influence these rates was addressed in this study. To better understand this, we used vegetable oil as a model ingredient and employed three different emulsifiers with the same chemical composition but different molecular weights and surface charges: whey protein isolate (WPI), whey protein hydrolysate (WPH), and whey protein isolate at a pH lower than the isoelectric point (5.5) (WPI^{+}) (Durham & Hourigan, 2007). This approach was able to control and systematically vary emulsifier properties and evaluate their impact on microbial growth and inactivation rates in emulsion systems. By utilizing this novel approach, a deeper understanding of the role of emulsifier properties in modulating microbial growth and inactivation in oil-in-water emulsions was revealed, and insights that can be useful for developing more effective preservation strategies was provided.

4.2 Materials and Methods

4.2.1 Materials

Salmonella enterica serotype Typhimurium (CVM98) strain was obtained from the stock collection of the Department of Nutrition and Food Science at the University of Maryland, College Park. Tryptic Soy Agar (TSA) and Tryptic Soy Broth (TSB) were procured from BD Biosciences (Franklin Lakes, NJ) under product codes 236920 and 211825, respectively. Buffered Peptone Water (BPW) was acquired from Thermo Scientific Remel Agar (Waltham, MA), product code R452672. Emulsifiers, Whey Protein Isolate (molecular weight approximately 18,000 Daltons) and Whey Protein Hydrolysate (molecular weight ranging from 450-500 Daltons), were obtained from Hilmar Ingredients (Hilmar, CA) with product codes Hilmar 9400 and Hilmar 8390, respectively. The vegetable oil used in the experiment was purchased from a local grocery store and stored in dark at room temperature.

4.2.2 Sample preparation

A 2.0% (v/v) concentration of either WPI or WPH was added to TSB in varying volumes of 10, 8, 6, and 4 mL, and the resulting mixture was homogenized to ensure a uniform dispersion. To prepare oil-in-water emulsions with concentrations of 0%, 20%, 40%, and 60% (v/v), a precise volume of vegetable oil was added to TSB containing the emulsifiers to yield a final volume of 10 mL of solution. These solutions were subjected to homogenization using an ultrasonic processor (FB505; Fisher Scientific, Hampton, NH) with a power output of 200 W for a duration of 3 minutes to produce stable emulsions. In order to transfer the surface charge of emulsifier to positive, the pH of the solution was adjusted to 5.0, which was below the isoelectric point (pH = 5.5) of WPI (Durham et al., 2007).

4.2.3 Bacterial growth and enumeration

S. Typhimurium was retrieved from a frozen culture, and then inoculated onto TSA plates and incubated overnight at 37°C for approximately 20 hours. The plates were stored at 4°C for a maximum of one month until needed. Prior to the experiments, S. Typhimurium was isolated from the plates and inoculated into sterile 15 mL tubes with TSB and incubated overnight at 37°C to reach early stationary phase of approximately 9 log CFU/mL. With appropriate dilution, approximately 5 log CFU/mL of bacteria were introduced into TSB (control), emulsifier solutions, and emulsions. The inoculated solutions were then incubated at 37°C and samples were collected at specific intervals (0, 2, 4, 6, 8, 12, 24 hours) and plated on TSA plates via Spiral Plater (Eddy Jet; Neutec Group Inc., Farmingdale, NY). The inoculated plates were then incubated overnight (~20 hr) at 37°C and colony counts were obtained using an IUL Flash & Go automated colony counter (6010 colony counter; Neutec Group Inc., Farmingdale, NY).

4.2.4 Physical and chemical properties

To evaluate the physical and chemical properties of the emulsifier solutions and emulsions without S. Typhimurium, 10 mL samples were prepared. The pH value and water activity were measured using a pH meter (AB15; Fisher Scientific, Hampton, NH) and HygroPalm (HP23-A; Rotronic, Hauppauge, NY), respectively. The particle size and ζ-potential were determined using a Particle Sizer and ζ-Potential Analyzer (NanoBrook Omni; Brookhaven Instruments, Holtsville, NY). Prior to particle size detection of the emulsion samples, they were diluted 10,000-fold with distilled water. Dynamic Light Scattering (DLS) was employed for particle sizing and distribution, while Doppler velocimetry (electrophoretic lightscattering, ELS) was used to detect the ζ-potential. A 10 mW HeNe laser beam with a wavelength of 633 nm was used to detect the diameter of the emulsion samples. All measurements using DLS and ELS were performed in triplicate at 25°C.

4.2.5 Thermal treatment

To evaluate the effect of temperature on *S*. Typhimurium, bacteria were introduced into TSB solution, emulsifier solutions, and emulsions and allowed to incubate for approximately 20 hours at 37°C. Aliquots of 200 μL were collected and placed in a water bath (Model: 28L-M; Fisher Scientific, Hampton, NH) set to 55°C, a temperature above the appropriate growth temperature of *Salmonella* that has been extensively utilized for *Salmonella* inactivation in scientific research (Jin, Zhang, Hermawan, & Dantzer, 2009; Park & Kang, 2013; Sung, Song, Kim, Ryu, & Kang, 2014). Samples were collected at specific intervals (0, 15, 30, 45, 60, 75, 90 minutes). Treated aliquots were then plated onto TSA plates, which were incubated overnight (~20 hr) at 37°C. Colony counts were subsequently obtained using a Plate Counter, allowing for the evaluation of the bacteria’s ability to withstand exposure to elevated temperatures.

4.2.6 Statistical analysis

All experiments were conducted in triplicate. The logistic model (1) was employed to describe the growth curve as follows:

$$logN_t = logN_0 + \frac{C}{1+e^{-\mu(t-d)}} \text{ ...... (1)}$$

where N_t represents the bacterial count at time t, N_0 is the initial bacterial count, C is the logarithmic increase in bacterial count from t_0 to t_{MAX}, μ is the maximum growth rate, and d is the inflection point. Moreover, the duration of the lag phase was determined using the formula proposed by Buchanan and Cygnarowicz (1990):

$$\lambda = d - (\frac{1}{\mu}) \text{ ...... (2)}$$

where λ denotes the duration of the lag phase. The Weibull model (3) was applied to describe the inactivation of bacteria during 55°C heat treatment:

$$log\ N_t = \left(\frac{-1}{2.303}\right)\left(\frac{t}{\alpha}\right)^{\beta} \text{ ...... (3)}$$

where N_t is the bacterial count at time t, α is the shape parameter, and β is the scale parameter (van Boekel, 2002). The 5D value was calculated according to van Boekel (2002) using the following formula (4):

$$5\mathrm{D} = \alpha(-\ln(10^{-5}))^{\frac{1}{\beta}} \text{ ...... (4)}$$

All models were developed using JMP Pro 15.2.0. To assess the significance of differences between groups, the student's t-test was performed at a significance level of $\alpha = 0.05$.

4.3 Results and Discussions

4.3.1 Physical and Chemical Properties

The pH value and water activity of 0%, 20%, 40%, and 60% (v/v) o/w emulsions containing 2.0% (v/v) WPI and WPH were tested, and the results are shown in Table 4-1. The pH values of WPI and WPH were adjusted to approximately 7.0, with no significant difference observed between the samples ($P > 0.05$). The pH value of the WPI solution was reduced to approximately 5.0 to alter the charge of whey protein (WPI^+), which was less than its isoelectric point (PI). The water activity values were approximately 0.99, with no significant difference among the samples ($P > 0.05$) (Table 4-1). The particle size of emulsion droplets varied with the emulsifier used, with droplet sizes of about 500 nm, 10 μm, and 50 μm observed with WPI, WPI^+, and WPH, respectively (Table 4-1). WPH emulsifier was found to significantly increase the mean droplet size, even when the oil content of the emulsion was varied. Despite differences in droplet size based on the molecular weight and surface charge of the emulsifiers, stability of the emulsions was observed within a 24-hour period (Data not shown). Based on prior research, the size of droplets, whether on a nanoscale or a microscale, was found to have no significant impact on the growth or inactivation of bacteria (Brocklehurst, Parker, Gunning, Coleman, & Robins, 1995; Buranasuksombat, Kwon, Turner, & Bhandari, 2011). In addition, the droplet size is consistent with previous finding that demonstrated WPH exhibited weaker emulsifying properties with a bimodal droplet size distribution and droplet sizes of approximately 30 μm (van der Ven, Gruppen, de

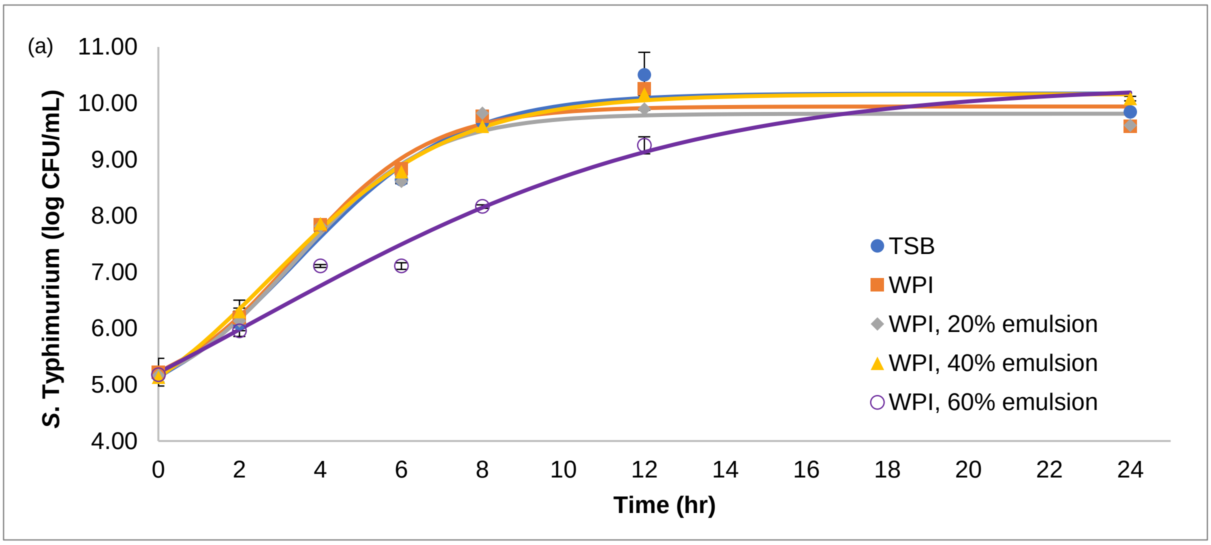
Bont, & Voragen, 2001). The ζ-potential of WPI and WPI^+ was approximately -10 mV and +4 mV, respectively, in 20%, 40%, and 60% (v/v) o/w emulsions (Table 4-1). The charge was negative when the pH value was higher than PI and positive when the pH value was lower than PI.

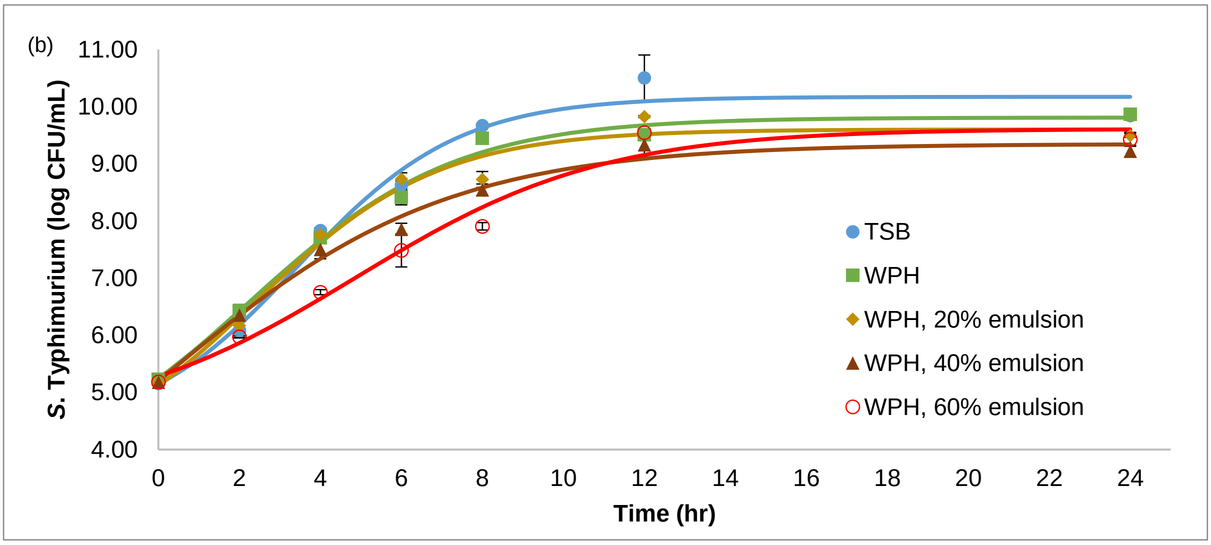
Table 4-1. Summary of emulsion characteristics. Each data point is an average of triplicate measurements ± standard deviation (SD).

Emulsifier	**Oil fraction (v/v)**	**pH**	**Aw**	**Particle size (nm)**	**Zeta potential (mV)**
TSB	0%	6.99 ± 0.01	1.00 ± 0.01	-	-
WPI	0%	6.91 ± 0.00	0.99 ± 0.01	-	-
	20%	6.91 ± 0.01	0.99 ± 0.01	448.64 ± 29.51	-8.66 ± 0.41
	40%	6.90 ± 0.04	0.99 ± 0.01	788.76 ± 403.06	-9.83 ± 2.22
	60%	6.85 ± 0.03	0.99 ± 0.01	695.54 ± 26.31	-12.32 ± 0.98
WPH	0%	6.91 ± 0.00	0.99 ± 0.01	-	-
	20%	6.91 ± 0.00	0.99 ± 0.01	9647.47 ± 4484.73	-
	40%	6.90 ± 0.03	0.99 ± 0.01	50477.26 ± 31369.80	-
	60%	6.89 ± 0.02	0.99 ± 0.01	47328.51 ± 15248.08	-
WPI^+	0%	5.11 ± 0.02	0.99 ± 0.01	-	-
	20%	5.11 ± 0.01	0.99 ± 0.01	11293.33 ± 6654.34	+5.63 ± 0.36
	40%	5.15 ± 0.02	0.98 ± 0.01	4475.36 ± 4806.54	+4.10 ± 0.78
	60%	5.12 ± 0.01	0.98 ± 0.01	13709.53 ± 9049.58	+2.77 ± 1.02

4.3.2 <u>Effect of molecular weight and surface charge of emulsifier on the growth rate of S. Typhimurium</u>

To investigate the impact of molecular weight and electric charge of emulsifiers, as well as oil fraction in the emulsion on the growth behavior of *S*. Typhimurium, it was inoculated in emulsions and emulsifier solutions at an initial level of approximately 5 log CFU/mL, followed by incubation at 37°C. Bacterial enumeration was conducted at specific time intervals (0, 2, 4, 6, 8, 12, and 24 hours), and the results are illustrated in Figure 4-1. The logistic model was chosen to conduct a statistical analysis on the parameters as it generally had a good fit (R^2>0.98) with parameters such as the maximum growth rate (log CFU/hr), inflection point (hr), and duration of lag phase (hr) analyzed (Table 4-2).


(a)
S. Typhimurium (log CFU/mL)
Time (hr)
TSB
WPI
WPI, 20% emulsion
WPI, 40% emulsion
WPI, 60% emulsion


(b)
S. Typhimurium (log CFU/mL)
Time (hr)
TSB
WPH
WPH, 20% emulsion
WPH, 40% emulsion
WPH, 60% emulsion

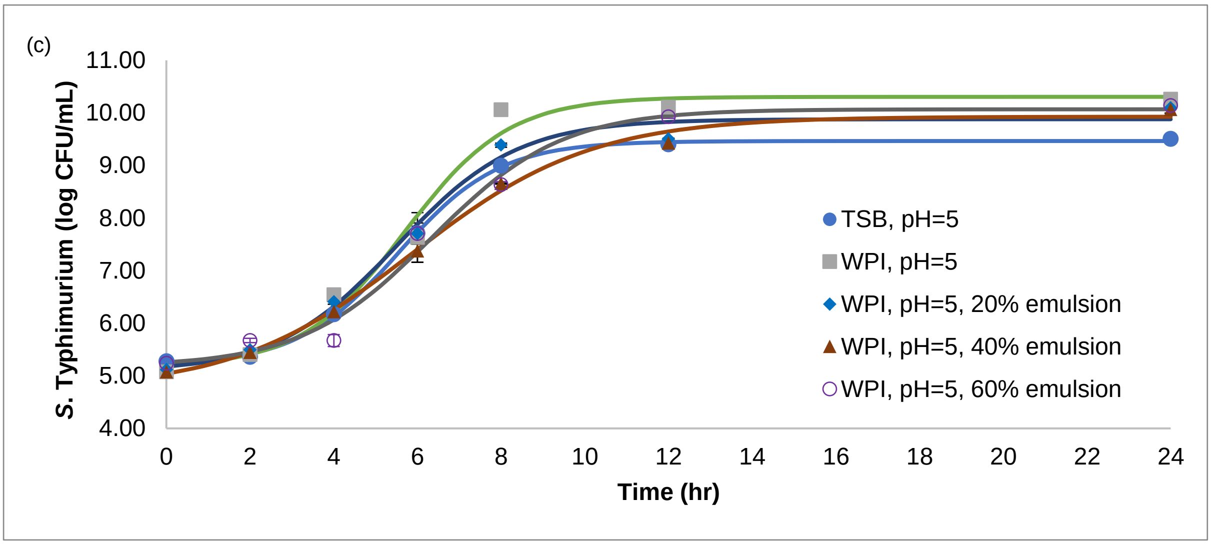


Figure 4-1. The growth curve of *S*. Typhimurium at 37°C in (a) TSB, TSB supplemented with WPI and 20, 40 and 60% (v/v) emulsions prepared using WPI; (b) TSB, TSB supplemented with WPH, and 20, 40 and 60% (v/v) emulsions prepared using WPH; (c) TSB (pH = 5.0), TSB (pH = 5.0) supplemented with WPI$^+$, and 20, 40 and 60% (v/v) emulsions prepared using WPI$^+$. Experimental data was fitted into Logistics model and average model fit for each growth curve is depicted using the solid line. Each data point is an average of triplicate measurements ± standard deviation (SD).

Table 4-2. Summary of *S*. Typhimurium growth model in TSB, emulsifier solutions, and emulsions at 37°C. Each data point is an average of triplicate measurements ± standard deviation (SD). Letter labels (a, b, c, etc.) were used to indicate significant differences between groups, based on a multiple t-test. Groups with the same letter were not significantly different (P > 0.05), while those with different letters were significantly different (P < 0.05). NA indicated not applicable for the lag phase calculation. The unit of bacterial growth rate was log CFU/ hr.

	TSB	WPI	WPI, 20% emulsion	WPI, 40% emulsion	WPI, 60% emulsion
R-square	0.98	0.99	0.99	0.99	0.99
Growth rate	0.51 ± 0.02^{c}	0.61 ± 0.04^{c}	0.60 ± 0.05^{c}	0.44 ± 0.04^{c}	0.19 ± 0.04^{e}
Inflection point	3.46 ± 0.20^{c}	3.41 ± 0.23^{c}	3.32 ± 0.28^{cd}	2.73 ± 0.51^{c}	1.83 ± 2.86^{acde}
Lag phase (hr)	1.48 ± 0.15^{c}	1.75 ± 0.32^{c}	1.64 ± 0.43^{c}	0.59 ± 0.51^{c}	NA
		WPH	**WPH, 20% emulsion**	**WPH, 40% emulsion**	**WPH, 60% emulsion**
R-square		0.99	0.98	0.99	0.98
Growth rate		0.39 ± 0.05^{d}	0.44 ± 0.03^{d}	0.31 ± 0.02^{d}	0.33 ± 0.04^{d}
Inflection point		2.17 ± 0.31^{d}	2.31 ± 0.65^{d}	0.79 ± 0.77^{e}	4.81 ± 0.76^{c}
Lag phase (hr)		NA	NA	NA	1.75 ± 1.10^{c}

	TSB, pH=5	WPI, pH=5	WPI, pH=5, 20% emulsion	WPI, pH=5, 40% emulsion	WPI, pH=5, 60% emulsion
R-square	0.99	0.98	0.99	0.99	0.99
Growth rate	0.83 ± 0.04[a]	0.83 ± 0.01[a]	0.70 ± 0.03[b]	0.47 ± 0.03[c]	0.65 ± 0.03[b]
Inflection point	5.54 ± 0.14[b]	5.72 ± 0.33[ab]	5.51 ± 0.17[b]	5.88 ± 0.28[ab]	6.33 ± 0.12[a]
Lag phase (hr)	4.34 ± 0.19[b]	4.52 ± 0.31[ab]	4.08 ± 0.14[b]	3.74 ± 0.31[b]	4.77 ± 0.06[a]

According to the data presented in Table 4-2, the duration of the lag phase for *S*. Typhimurium was comparable between WPI solution and TSB (P > 0.05), indicating that there was no significant difference in the initial adaptation period of the bacteria in these two solutions. In contrast, no lag phase was observed when *S*. Typhimurium was inoculated in WPH solution. Moreover, the growth rate of *S*. Typhimurium in WPI solution was found to be similar to that in TSB (P > 0.05). However, the growth rate of *S*. Typhimurium in WPH solution was significantly lower than that in TSB as well as in WPI solution (P < 0.05), indicating that the emulsifier with a lower molecular weight was able to inhibit bacterial growth to some extent. Therefore, the use of emulsifiers with a lower molecular weight could potentially decrease the growth rate and lag phase of *S*. Typhimurium at 37°C. Furthermore, the effect of the emulsifier on bacterial growth was also assessed in WPI^+ solution, which had a different surface charge compared to WPI solution. The results showed that *S*. Typhimurium inoculated in WPI^+ solution had a similar lag phase and growth rate to that in TSB (pH = 5.0) (P > 0.05). A similar effect was observed in WPI solution, which had opposite surface charge to the WPI^+ solution, as no differences were found in the lag phase and growth rate compared to TSB (P > 0.05). Overall, in emulsifier solutions, the molecular weight but not the surface charge affected the rate of bacterial growth.

The effect of different oil fractions on the growth of *S*. Typhimurium was also conducted within emulsions. The results showed that an increase in oil fraction can affect the growth of *S*. Typhimurium differently depending on the type of emulsifier used. Within WPI-stabilized emulsions, an increase in oil fraction from 20 to 60% led to a decrease in both lag phase and growth rate ($P < 0.05$). One possible explanation for the observed effect is that the increase in oil fraction may have led to a greater stabilization of the emulsion by the 2% WPI used as an emulsifier, as reported by Sun & Gunasekaran (2009). This suggests that at higher oil fractions, WPI may function primarily as an emulsifier rather than as a nutrient source for bacterial growth. This shift in function could lead to a decrease in the growth rate of bacteria as the oil fraction increases. In contrast, within WPH-stabilized emulsions, an increase in oil fraction did not affect the growth rate. This observation may be attributed to the presence of small protein peptides (<5 kDa) in WPH, which may exhibit weaker interaction with the oil droplets (Schröder, Berton-Carabin, Venema, & Cornacchia, 2017). The small size of these peptides may limit their ability to form a stable interfacial layer at the oil-water interface, resulting in limited influence on bacterial growth. The observed decrease in growth rate within WPI^{+}-stabilized emulsions as the oil fraction increased from 20 to 40% may be attributed to the interaction of cationic emulsifiers with bacterial cells. It has been reported that cationic emulsifiers can adsorb onto the surface of bacterial cells due to their positive charge, leading to alterations in cell membrane properties and impaired bacterial growth (Yegin, Oh, Akbulut, & Taylor, 2019). Interestingly, the WPI^{+}-stabilized 60% emulsion showed a significantly higher growth rate and longer lag phase compared to the WPI^{+}-stabilized 40% emulsion ($P < 0.05$), suggesting a potential dose effect of oil inclusion. However, the effect has not been reported in previous research.

Previous studies have demonstrated that *S*. Typhimurium is capable of growing at a pH of 4.3 at 37°C, although the growth rate and lag phase are known to be affected by the level of acidity, with higher acidity leading to lower growth rates and longer lag phases (Chung & Goepfert, 1970; Theys et al., 2008). In our study, WPI was selected as the model emulsifier to investigate the impact of surface charge on bacterial growth, as it has a well-defined composition and structure. To mitigate the effects of pH, the ratio between the emulsifier-containing environment and TSB at the same pH was employed as a reliable method. The average growth rate ratio for WPI solution, WPI-stabilized 20%, and 40% emulsions compared to TSB was 1.20 ± 0.09, 1.18 ± 0.10, and 0.87 ± 0.08, respectively. Similarly, the growth rate ratio for WPI^+, WPI^+-stabilized 20%, and 40% emulsions compared to TSB (pH = 5.0) was 1.01 ± 0.02, 0.84 ± 0.04, 0.57 ± 0.03, respectively (Table 4-3). Statistically significant higher growth rates were observed in negatively charged emulsifiers at the same level of oil fraction ($P < 0.05$). However, for 60% emulsion, the cationic emulsifier had a higher growth rate ratio than the anionic emulsifier, which were 0.78 ± 0.04 and 0.37 ± 0.07, respectively ($P < 0.05$). Moreover, the lag phase was only observed in WPI^+-stabilized 60% emulsion, but not in WPI-stabilized 60% emulsion. The results suggested that the cationic emulsifier had a greater effect on bacterial growth, leading to a decreased growth rate, except in the case of the 60% emulsion. As noted by Falk (2019), cationic compounds have been found to have higher antimicrobial activity than anionic compounds due to their high affinity for attaching to bacterial membranes. However, under high oil fraction environment (60% (v/v)), when cationic compound was used as an emulsifier, the same high affinity may function as a protective mechanism for bacterial cells, potentially reducing the effect of oil on bacterial growth. Thus, the observed higher growth rate ratio for the cationic WPI^+-stabilized 60% emulsion in this study may be attributed by the protective effect of the cationic emulsifier on bacterial cells, allowing them to overcome the stress from oil and grow at a higher rate compared to the anionic WPI-stabilized 60% emulsion.

Table 4-3. Growth rate ratio for WPI solution, WPI-stabilized 20%, 40%, and 60% emulsions compared to TSB and growth rate ratio for WPI^+ solution, WPI^+-stabilized 20%, 40%, and 60% emulsions compared to TSB (pH = 5). Each data point is an average of triplicate measurements ± standard deviation (SD). Lower case letter labels (a, b, c, etc.) were used to indicate significant differences of growth rate between groups, based on a multiple t-test. Upper case letter labels (A, B) were used to indicate significant differences of lag phase between groups, based on a multiple t-test. Groups with the same letter were not significantly different (P > 0.05), while those with different letters were significantly different (P < 0.05).

	Growth rate		Lag phase	
	TSB	TSB, pH = 5	TSB	TSB, pH = 5
WPI / WPI^+	1.19 ± 0.09^{a}	1.01 ± 0.02^{b}	1.18 ± 0.22^{AB}	1.04 ± 0.07^{AB}
WPI / WPI^+, 20% emulsion	1.18 ± 0.10^{ab}	0.84 ± 0.04^{c}	1.11 ± 0.29^{AB}	0.94 ± 0.03^{B}
WPI / WPI^+, 40% emulsion	0.87 ± 0.08^{bc}	0.57 ± 0.03^{d}	0.40 ± 0.35^{B}	0.86 ± 0.07^{B}
WPI / WPI^+, 60% emulsion	0.37 ± 0.07^{e}	0.78 ± 0.04^{c}	NA	1.10 ± 0.01^{A}

4.3.3 *Effect of molecular weight and surface charge of emulsifier on thermal inactivation rate of S. Typhimurium in high oil fraction emulsion*

The survival and response of microorganisms to treatments are influenced by various environmental factors (Birk, Henriksen, Müller, Hansen, & Aabo, 2016). It was hypothesized that emulsions, which have been shown to modulate bacterial growth rates, may also affect bacterial inactivation rates. Building upon previous research that has demonstrated the effect of 60% (v/v) oil fraction emulsions on bacterial inactivation, a series of experiments were performed using emulsions, emulsifier solutions, and TSB media (CH 2). Following a 20-hour incubation period, the bacterial population in the different media reached 9-10 log CFU/mL. The bacterial inactivation kinetics during thermal treatment at 55°C exhibited a non-linear pattern, which is consistent with earlier observations made by S. Warren (1998) (Fig. 4-2). Specifically, a tailing effect was observed during thermal treatment, and the effect was more pronounced with increasing oil fraction in the emulsion (Warren, 1998).

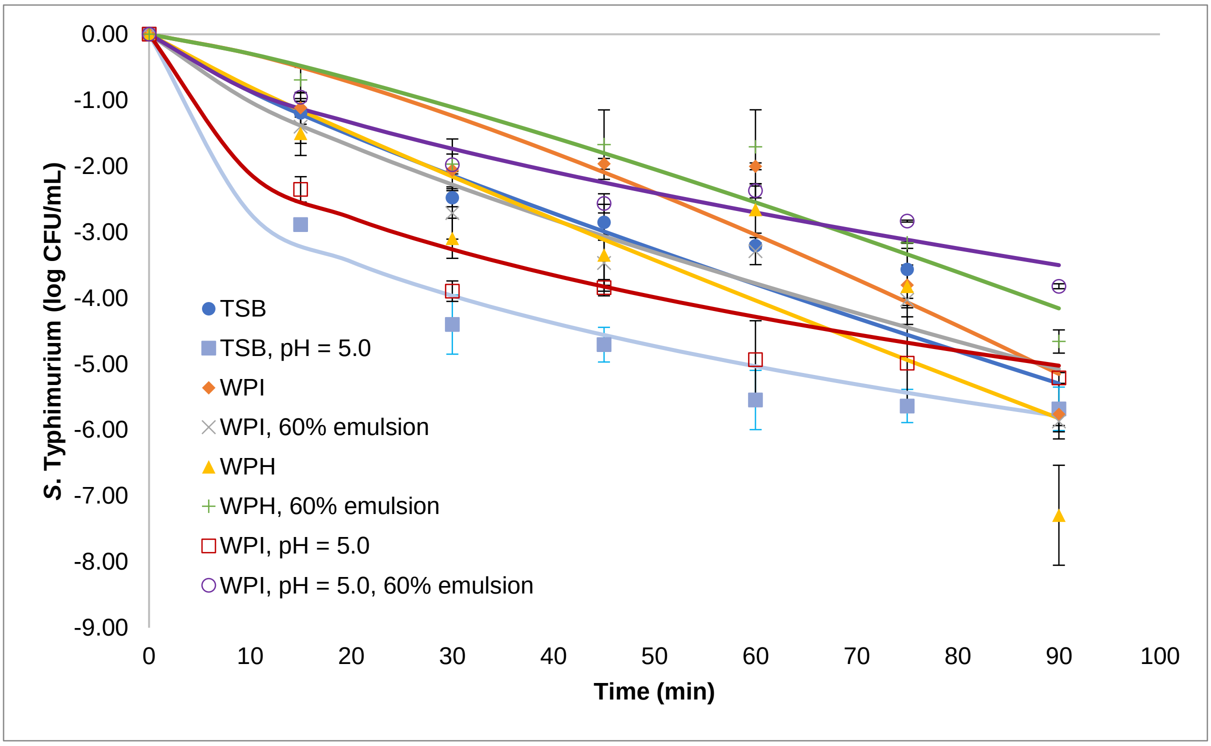


Figure 4-2. Thermal inactivation of *S*. Typhimurium at 55°C inoculated in TSB, TSB (pH = 5.0), WPI, WPH, WPI+ emulsifiers and 60% (v/v) oil-in-water emulsions prepared using those emulsifiers. Experimental data was fitted into Weibull model and average model fit for each growth curve is depicted using the solid line. Each data point is an average of triplicate measurements ± standard deviation (SD).

In this study, the inactivation data were analyzed using the Weibull model, as presented in Table 4-4. The value of $\beta < 1$ indicated that the surviving bacteria tended to adapt to the heat, while $\beta > 1$ suggested that the bacteria were more susceptible to thermal treatment. Furthermore, the parameter α in the Weibull model was found to be analogous to the traditional D value (van Boekel, 2002). The 5D concept, which was selected as a standard measure for evaluating bacterial inactivation efficacy, was utilized to compare the inactivation kinetics of *S*. Typhimurium in diverse systems, including WPI, WPI-stabilized 60% emulsion, WPH, WPH-stabilized 60% emulsion, WPI^+, WPI^+-stabilized 60% emulsion and TSB. The obtained results revealed that the 5D values of *S*. Typhimurium in WPI, WPI-stabilized 60% emulsion, and WPH solution were not statistically different from those in TSB ($P > 0.05$), indicating similar inactivation kinetics in these food systems. However, the 5D value of *S*. Typhimurium in WPH-stabilized 60% emulsion was significantly higher than that in TSB ($P < 0.05$), indicated that the bacteria had a higher resistance to thermal inactivation in the WPH-stabilized emulsion system. Furthermore, the obtained results demonstrated that the presence of oil in the emulsion system may have a protective effect on the bacteria with lower molecular weight emulsifier. On the other hand, the 5D value of S. Typhimurium in WPI^+ solution was found to be similar to that of TSB (pH = 5.0) ($P > 0.05$). However, in WPI^+-stabilized 60% emulsion, the 5D value was observed to be three times higher than that in TSB (pH = 5.0) ($P < 0.05$). The results of the study indicated that cationic emulsifiers presented a protective effect to some extent on bacteria during thermal treatment at 55°C, particularly when oil is present in the system. This finding is consistent with the observation made by Yang et al. (2020), who reported that oil has a protective effect on bacteria under thermal treatment. Furthermore, Yegin et al. (2019) mentioned that cationic emulsifiers have the ability to attach to and repair the cell membrane. Therefore, it is possible that the synergistic effects of oil and cationic emulsifiers enhance the protective effects on bacteria.

Table 4-4. Thermal inactivation model parameters (Weibull model) for of *S*. Typhimurium at 55°C in TSB, TSB (pH = 5.0), WPI, WPH and WPI^{+} emulsifiers and 60% (v/v) emulsions prepared thereof. Each data point is an average of triplicate measurements ± standard deviation (SD). Letter labels (a, b, c, etc.) were used to indicate significant differences between groups, based on a multiple t-test. Groups with the same letter were not significantly different (P > 0.05), while those with different letters were significantly different (P < 0.05).

	TSB	**TSB, pH = 5.0**	**WPI**	**WPI, 60% emulsion**	**WPH**	**WPH, 60% emulsion**	**WPI, pH = 5.0**	**WPI, pH = 5.0 60% emulsion**
α (min)	4.33 ± 0.25^{b}	0.05 ± 0.03^{d}	13.41 ± 1.65^{a}	3.09 ± 1.81^{bc}	5.08 ± 0.67^{b}	13.81 ± 2.80^{a}	0.18 ± 0.17^{cd}	3.42 ± 0.99^{b}
β	0.82 ± 0.02^{c}	0.34 ± 0.03^{e}	1.30 ± 0.09^{a}	0.73 ± 0.13^{bcd}	0.90 ± 0.03^{b}	1.21 ± 0.11^{a}	0.39 ± 0.07^{e}	0.64 ± 0.06^{d}
Avg. 5D value predicted by Weibull model (min)	83.92 ± 2.33^{c}	53.35 ± 7.83^{d}	88.02 ± 1.88^{c}	84.38 ± 2.28^{c}	75.96 ± 8.12^{c}	104.62 ± 3.60^{b}	76.48 ± 11.96^{cd}	156.70 ± 13.28^{a}

To investigate the effect of surface charge on bacterial inactivation at 55°C, comparison between WPI and WPI^+ as well as WPI-stabilized 60% emulsion and WPI^+-stabilized 60% emulsion was conducted. The ratio of WPI/TSB and WPI+/TSB at pH 5.0 was used to eliminate the potential pH effect (Table 4-5). The statistical analysis revealed that there were no significant differences in the relative α, β, and 5D between negatively and positively charged emulsifiers ($P > 0.05$). However, it was observed that *S*. Typhimurium inoculated in 60% WPI^+ emulsion exhibited significantly ($P < 0.05$) higher α, β, and 5D values when compared to those in WPI-stabilized 60% emulsion. The observed differences in the inactivation kinetics of *S*. Typhimurium between WPI-stabilized and WPI^+-stabilized emulsions can be attributed to the surface charge of the emulsifiers. WPI had a negative surface charge, while WPI^+ had a positive charge. The effect of surface charge on bacterial inactivation has been previously reported in literature, which suggested that positively charged particles may interact more strongly with bacterial cells, leading to a reduction in bacterial inactivation during thermal treatment (Abbaszadegan et al., 2015). It is worth noting that the observed differences in inactivation kinetics were only significant in the 60% emulsion systems. This can be attributed to the presence of oil in the emulsions, which may have acted as a protective barrier for the bacteria during thermal treatment (Senhaji & Loncin, 1977).

Table 4-5. The ratio of α, β, and 5D value for WPI solution, WPI-stabilized 60% emulsion compared to TSB and the ratio of α, β, and 5D value for WPI^+ solution, WPI^+-stabilized 60% emulsion compared to TSB (pH = 5). Each data point is an average of triplicate measurements ± standard deviation (SD). Letter labels (a, b, c, etc.) were used to indicate significant differences between groups, based on a multiple t-test. Groups with the same letter were not significantly different (P > 0.05), while those with different letters were significantly different (P < 0.05).

	α	β	5D value
WPI	3.10 ± 0.38^{b}	1.57 ± 0.11^{ab}	1.05 ± 0.02^{b}
WPI, 60% emulsion	$0.71 \pm 0.42_{c}$	0.88 ± 0.15^{c}	1.01 ± 0.03^{b}
WPI^+	3.66 ± 3.47^{bc}	1.14 ± 0.21^{bc}	1.43 ± 0.22^{b}
WPI^+, 60% emulsion	70.28 ± 20.38^{a}	1.85 ± 0.18^{a}	2.94 ± 0.25^{a}

4.4 Conclusion

Emulsifiers play a crucial role in the food industry for the preparation of emulsions. Previous studies have examined the effects of emulsifiers on bacterial growth and inactivation, but the impact of emulsifier molecular weight and electrical charge on these processes remains unclear. Therefore, the objective of this study was to investigate the impact of these variables on bacterial growth and inactivation. Our findings revealed that *S*. Typhimurium growth could be predicted using the Logistic model, with the duration of lag phase and maximum growth rate considered as references to assess the impact of emulsifiers on bacterial growth. The results showed that the molecular weight of emulsifiers played a more significant role in bacterial growth than their surface charge when used individually in the system. On the other hand, 60% emulsion stabilized with cationic emulsifier (WPI^+) showed a higher growth rate compared to anionic emulsifier (WPI) stabilized 60% emulsion, likely due to the high affinity that functions as a protective mechanism for bacterial cells. The thermal inactivation kinetics were modeled using the Weibull model, and our results indicated that the molecular weight and surface charge of the emulsifier had no significant effect on bacterial inactivation, as evidenced by the comparable 5D values obtained in the individual use of emulsifiers in the system ($P > 0.05$). However, we found that the emulsion environment had more protective effects on bacterial inactivation with WPI^+. Emulsifiers with a positive charge can interact with the bacterial membrane, thus leading to protective effects during thermal treatment. In sum, our research provides valuable insights into the parameters affecting bacterial growth in emulsions, enabling food manufacturers to improve food safety.

The previous chapters have explored the effect of various factors on bacterial growth and thermal inactivation in oil-in-water emulsions, including the impact of emulsifier type, oil fraction, temperature, and molecular weight and surface charge of the emulsifier. Building on these findings, next chapter will investigate how stress-related RNA expression of *S*. Typhimurium is affected by the presence of emulsifiers and oil in the heterogeneous environment. Specifically, chapter 5 focuses on investigating how the expression of stress response-related genes is affected by the presence of different emulsifiers and oil in emulsions, aiming to shed light on the potential mechanisms responsible for bacterial survival and adaptation in such heterogeneous environments.

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5 Chapter 5 The effect of emulsifier type and oil presence on stress-related RNA expression of *Salmonella enterica* Serovar Typhimurium in oil-in-water emulsion

<u>Publication status</u>

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Abstract

Salmonella, a Gram-negative pathogen, has been associated with numerous outbreaks resulting from the consumption of contaminated food products, including emulsions. Emulsions, which are mixtures of immiscible liquids stabilized by emulsifiers, are influenced by emulsifier type and oil presence, which can have varying degrees of stress or protection on bacteria under optimal temperature and thermal treatment conditions. While previous research has shown that emulsifier solutions, rather than emulsions, provide a protective effect on *S*. Typhimurium after thermal treatment, the underlying mechanism remains unclear. To investigate this, this study selected *S*. Typhimurium as the model microorganism, and utilized the same emulsifiers (Tween 20, Tween 80, Triton X-100) to create emulsifier solutions and emulsions with the same oil fraction (60% (v/v)) to examine their effect on nine selected gene expressions (*rpoE, rpoH, otsB, proV, fadA, fabA, dnaK, ibpA, ompC*) associated with stress response. Specifically, the study analyzed stress-related gene expression after 20 hours of incubation at 37°C and after thermal treatment at 55°C. By identifying how *Salmonella* responds to various environmental stresses, it may be possible to develop effective strategies to mitigate its growth and spread in food products.

5.1 Introduction

Salmonella is a Gram-negative, rod-shaped bacterium that can cause a range of illnesses in humans, including diarrhea, fever, and abdominal cramps (Coburn et al., 2007). It is commonly transmitted through the consumption of contaminated food products, including meat, poultry, eggs, and dairy products (O'Bryan et al., 2022; Sher et al., 2021). One transmission way of *Salmonella* is through the consumption of emulsions, which are mixtures of two immiscible liquids that are stabilized by the addition of emulsifiers (Akbari et al., 2018; Prachaiyo et al., 2003; Warren, 1998). Additionally, emulsifiers are commonly used in food processing to improve the texture, stability, and sensory qualities of products (Marhamati, Ranjbar, & Rezaie, 2021). By reducing the surface tension between the two liquids, emulsifiers are able to mix and form a stable emulsion (Cambiella et al., 2006). However, emulsifiers may also affect the survival and virulence of pathogens like *Salmonella* (R. Zhang et al., 2015). For example, some emulsifiers can disrupt the cell membranes of bacteria, making them more susceptible to environmental stresses and less able to cause infection (Christina K. Nielsen et al., 2016). The impact of oil on bacteria has been documented in previous research (Senhaji & Loncin, 1977). Studies indicate that oil may offer protective benefits for certain types of bacteria, shielding them from potential harm caused by environmental stressors like high temperatures (Ren, 2021; Ren Yang, 2020). However, the degree of protection provided by oil can vary based on the specific type of bacteria and environmental factors present. It is worth noting that while oil may provide a level of protection, it does not confer complete immunity to harmful agents or environmental conditions. On the other hand, the stress-related RNA expression of *Salmonella* is an important indicator of the pathogen's ability to survive and thrive in different environments (Roncarati & Scarlato, 2017). RNA expression refers to the process by which genes are transcribed into RNA molecules, which then serve as templates for the synthesis of proteins (Cooper, Wan, & Dreyfuss, 2009). Specifically, the expression of certain genes, such as *rpoE* and *ompC*, can be used to monitor the survival and virulence of *Salmonella* in different environments (Du et al., 2011; Q. Li et al., 2021).

The type of emulsifier and the presence of oil in emulsions are important factors that can significantly influence the stress-related RNA expression of *Salmonella*. Given the importance of controlling the growth and spread of this pathogen in food products, it is crucial to understand the effects of emulsifier type and oil presence on the stress-related RNA expression of *Salmonella* in oil-in-water emulsions. Previous research has shown that a high oil fraction in emulsion system can extend the lag phase of *S*. Typhimurium growth; while emulsifier solutions alone (Tween 80 and Triton X-100), rather than emulsions, have been found to be protective against thermal inactivation (CH 2). To build on this knowledge, this study aims to investigate the effects of different emulsion conditions on stress-related gene expression in *Salmonella*. Specifically, we will analyze stress-related gene expression after 20 hours of incubation at 37°C and after thermal treatment at 55°C. By characterizing how *Salmonella* responds to various environmental stresses, effective strategies to mitigate its growth and spread in food products can be developed.

5.2 Materials and Methods

5.2.1 Materials

Salmonella enterica serotype Typhimurium (CVM98) was obtained from the stock collection of the Department of Nutrition and Food Science at the University of Maryland, College Park. *Salmonella enterica* serotype Typhimurium with green fluorescent protein gene (GFP) labeled was purchased from ATCC (14028GFP). Tryptic soy agar (TSA) and Tryptic soy broth (TSB) were purchased from BD Biosciences (236920, 211825; Franklin Lakes, NJ). Phosphate buffer saline (PBS) tablets were obtained from Fisher Scientific (BP2944-100; Hampton, NH), while Buffered peptone water (BPW) was sourced from Thermo Scientific Remel Agar (R452672; Waltham, MA). Emulsifiers Tween 20, Tween 80, and Triton X-100 were procured from Fisher Scientific (BP337-500, BP338-500; Hampton, NH), and Acros Organics (21568-2500; Pittsburgh, PA), respectively. Vegetable oil was purchased from a local grocery store and stored in the dark at room temperature. For RNA extraction, RNAprotect Bacteria Reagent was obtained from Qiagen (76506; Hilden, Germany), while RNA isolation and purification were performed using the RNeasy Mini Kit (74104; Qiagen, Hilden, Germany). The concentration of extracted RNA was determined using the Qubit RNA BR Assay Kit (Q10210; Molecular Probes, Invitrogen, Waltham, MA), and RNase-free water from Fisher Scientific (7732185; Waltham, MA) was used to prevent potential RNase contamination. For the real-time quantitative PCR (qPCR) experiment, primers (standard desalted) were purchased from IDTDNA (Integrated DNA Technologies, Coralville, Iowa) (Table 5-1). PowerTrack SYBR Green Master Mix was procured from Invitrogen (A46109; Waltham, MA).

Table 5-1. Selected genes and primers used in this study. F: Forward, R: Reverse.

Gene	Direction	Primer sequence (5' → 3')	Reference
16s rRNA	F	CGATCCCTAGCTGGTCTGAG	(Castelijn, van der Veen, Zwietering, Moezelaar, & Abee, 2012)
	R	GTGCAATATTCCCCACTGCT	
rpoE	F	GTCTACAACATGACAAACAAAAACAAATGC	(Humphreys et al., 1999)
	R	CCTTTTCCACTATCCCGCTATCGTCAACGC	
rpoH	F	GTTTCCTTCGCCGTACACTG	(Kang, Kim, Jeong, Park, & Seo, 2018)
	R	CCACCATTTCAACCTCATCC	
otsB	F	ACCTTGATGGCACATTGGCAGA	(H. Li, Bhaskara, Megalis, & Tortorello, 2012)
	R	ACGCCCTGAAATCAATGCCA	
proV	F	CCACAATGGTACGCCTTCTCA	(Z. Chen & Jiang, 2017)
	R	GCATGAGCGCAAATGACTGGA	
fadA	F	ATCTCTCCGCCCACTTAATGCGTA	(Fong & Wang, 2016)
	R	AGCCTTGCTCCAGCGTTTGTTGTA	
fabA	F	ACTCCCTGCGCCGAACATGC	(W. Chen, Golden, & Critzer, 2014)
	R	CACTTCGCCCACGCCCAGAG	
dnaK	F	CGATTATGGATGGAACGCAGG	(Fong et al., 2016)
	R	GGCTGACCAACCAGAGTT	
ibpA	F	GCCGCCAACCGTTTCA	(Carroll, Bergholz, Hildebrandt, & Marks, 2016)
	R	TGCTTACCGTGAGCGTTCCT	
ompC	F	ACGCTGCTGCATAAAGTTGTCA	(Kollanoor Johny et al., 2017)
	R	CCGATGTTCTGCCGGAGTT	

5.2.2 Emulsion preparation

Different volumes of TSB (10, 8, 6, 4 mL) were mixed with 2% (v/v) of Tween 20, Tween 80, or Triton X-100 and thoroughly mixed. Specific amounts of vegetable oil were added to each emulsifier-containing TSB solution (0, 2, 4, 6 mL, respectively) to obtain a total volume of 10 mL, resulting in 0%, 20%, 40%, and 60% (v/v) oil-in-water emulsions. The solutions were homogenized using an ultrasonic processor (FB505; Fisher Scientific, Hampton, NH; 200 W, 3 min) to create stable emulsions.

5.2.3 Bacteria growth

S. Typhimurium was retrieved from a frozen culture and streaked on TSA plates, which were incubated overnight (~20 hr) at 37°C and stored at 4°C for up to a month. Before conducting experiments, a single colony of *S*. Typhimurium was inoculated into a 15 mL sterile tube containing TSB and incubated overnight (~20 hr) at 37°C until early stationary phase was reached (~9 log CFU/mL). With appropriate dilution, ~5 log CFU/mL of bacteria were inoculated into TSB (control), emulsifier solutions, and emulsions. Inoculated solutions were incubated at 37°C for 20 hr, and pellets were then collected by centrifugation at 5000 g for 10 min.

5.2.4 Thermal treatment

S. Typhimurium was cultured in TSB, emulsifier solutions, and emulsions overnight (~20 hr) at 37°C. After that, 200 μL of each sample was subjected to 45 min of heat treatment in a 55°C water bath

(Model: 28L-M; Fisher Scientific, Hampton, NH). The bacterial pellets were collected by centrifugation at 5000 g for 10 min.

5.2.5 <u>Confocal imaging</u>

The GFP-labeled *S*. Typhimurium was cultured in 60% emulsions overnight (~20 hr) at 37°C, and then collected for confocal imaging. Before imaging, the bacterial samples were diluted at least 10,000 times. The imaging was performed using an SP5X Laser Scanning Confocal with an inverted microscope (Leica Microsystems CMS GmbH, Germany), and a 63× oil immersion objective was used to increase the magnification of the images.

5.2.6 <u>RNA expression</u>

RNA was extracted from bacterial samples collected after 20 hours of growth and after 45 minutes of thermal treatment at 55°C. A control group consisting of stationary phase *S*. Typhimurium cells directly harvested from TSB was also included. To minimize RNA synthesis and degradation during extraction and further processing and to stabilize the gene expression levels, RNAprotect Bacteria Reagent was used (Rocha, Castro, Aguiar, & Pacheco, 2020). Specifically, the samples were mixed with RNAprotect Bacteria Reagent at a ratio of 1:2 (samples:reagent) and incubated at room temperature for 5 minutes after vortexing. The mixture was then centrifuged at 5000 g for 10 minutes, and the supernatant was discarded. The resulting cell pellets were stored at -80°C for further processing for up to one week.

RNA isolation and purification from the stabilized samples were conducted using the RNeasy Mini Kit with an automated QIAcube instrument (Qiagen, Hilden, Germany) following the manufacturer's protocol. The concentration of the extracted RNA was determined using the Qubit RNA BR Assay Kit on a Qubit 2.0 Fluorometer (Invitrogen, Waltham, MA), following the instructions from the manufacturer. Reverse transcription to complementary DNA (cDNA) was performed using the QuantiTect Reverse Transcription Kit, which includes a gDNA Wipeout Buffer to eliminate any contaminating genomic DNA (gDNA) present in the RNA extract. The resulting cDNA was stored at -20°C for up to one month for future processing.

5.2.7 Real-time quantitative PCR (qPCR)

To prepare for qPCR, primer preparation and RNA quality control were performed. The designed primers were dissolved in RNase-free water to create individual working stock solutions at a concentration of 8 μM. RNA quality was assessed by measuring the $OD_{260/280}$, as impurities in the RNA sample can result in inaccurate quantification (Fleige & Pfaffl, 2006). An $OD_{260/280}$ value between 1.8 and 2.0 indicated high-quality RNA that was free from protein and phenol contamination.

The qPCR experiment was conducted using a C1000 Touch Thermal Cycler coupled with a CFX96 Optical Reaction Module (Bio-Rad, Hercules, CA), with some modifications to the manufacturer's recommended protocol, based on previous research (Nolan, Hands, & Bustin, 2006). In brief, each 10 μL reaction mixture contained 5 μL of Master Mix, 0.5 μL of cDNA samples (~150 ng), 7.15 µL of forward and reverse primers (400 nM), 0.25 µL of yellow sample buffer, and 3.75 µL of molecular-grade water. The PCR reaction consisted of 40 cycles after an initial enzyme activation step (95 °C for 2 min), with 5 s of denaturation (95 °C) and 30 s of annealing (60 °C). A subsequent dissociation step was performed by increasing the incubation temperature from 65 °C to 90 °C with a 0.1 °C/s ramp. The resulting melt curve was analyzed to detect potential contamination or primer binding. The relative expression of each target gene in the samples was calculated using the $2^{-\Delta\Delta C_T}$ method, as described by Livak & Schmittgen (2001) in Eq. 1.

$$\text{Fold change} = 2^{-\Delta\Delta C_T} \ldots\ldots (1)$$

The purpose of Eq. 1 was to compare the gene expression levels of the target gene with those of a reference gene. This equation can be expanded as shown in the following equation (Schmittgen & Livak, 2008):

$$\Delta\Delta C_T = [(C_T \text{ of target gene} - C_T \text{ of reference gene})_{\text{sample}} -$$

$$(C_T \text{ of target gene} - C_T \text{ of reference gene})_{\text{control}}] \ldots\ldots (2)$$

Where 16S ribosomal RNA (16s rRNA) was used as the reference gene and stationary phase S. Typhimurium cultured in TSB as untreated control sample.

5.2.8 Statistical analysis

cDNA for qPCR analysis was generated from three independently treated samples, each with two on-plate replicates. Statistical significance was determined using 2-way ANOVA analysis in GraphPad Prism 6 software (Boston, MA), with a significance level of α = 0.05. Multi-dimensional scaling analysis and hierarchical cluster analysis were conducted in JMP Pro 15.2.0. Furthermore, the threshold for significant difference in this study was set at one $\log_2$ fold change in gene expression.

5.3 Results and Discussions

5.3.1 Confocal imaging

To determine whether bacteria had entered oil droplets, confocal microscopy images were obtained after all samples were cultured at 37°C for 20 hours. Fluorescence-labeled *Salmonella* Typhimurium was used to visualize the bacteria, while small pores in the image were indicative of oil droplets. As seen in Figure 5-1, the length of the labeled bacteria was approximately 3 μm, while the diameters of the small pores were less than 1 μm. Therefore, it is unlikely that the observed protective effect on bacteria in emulsion samples after thermal treatment resulted from bacterial movement to oil droplets due to the larger size of the bacteria. It is possible that other mechanisms, such as emulsifier stress, oil stress, and high temperature, may have contributed to the observed protective effect following thermal treatment, as well as the extended lag phase observed when *S*. Typhimurium was inoculated in emulsions with a high oil fraction (Ch. 2).

The potential mechanism of protective effect of emulsifiers after thermal treatment observed in previous study remains to be fully elucidated (Ch. 2). While it is clear bacterial movement to oil droplets is unlikely to be the cause of the effect, other mechanisms may be at play. One possible explanation is the emulsifier stress induced by thermal treatment, which can cause changes in the surface properties of the emulsion droplets, such as surface charge, hydrophobicity, or released compounds, and lead to the formation of a protective barrier around bacterial cells (Ellouze, Vial, Attia, & Ayadi, 2021;

Mohammad & Taha Daod, 2021a). As an example, a previous study found that emulsifiers with positive charge, such as whey protein isolate with positive charge (WPI^+), had a higher 5D value compared to negatively charged whey protein isolate (WPI). This result implies that the interaction between the positively charged emulsifier and cell membrane may be stronger, potentially providing greater protection against thermal treatment (Ch. 4). Another possible explanation is the stress caused by the high temperature itself, which can cause changes in bacterial membrane permeability and fluidity, and disrupt cellular functions, ultimately leading to the effect on bacterial inactivation (Cebrián, Condón, & Mañas, 2019; Guillén, Nadal, Álvarez, Mañas, & Cebrián, 2021). Thus, the protective effect in this study was be focused on various stress response mechanisms, including emulsifier stress and high temperature stress.

(a)

(b)

(c)

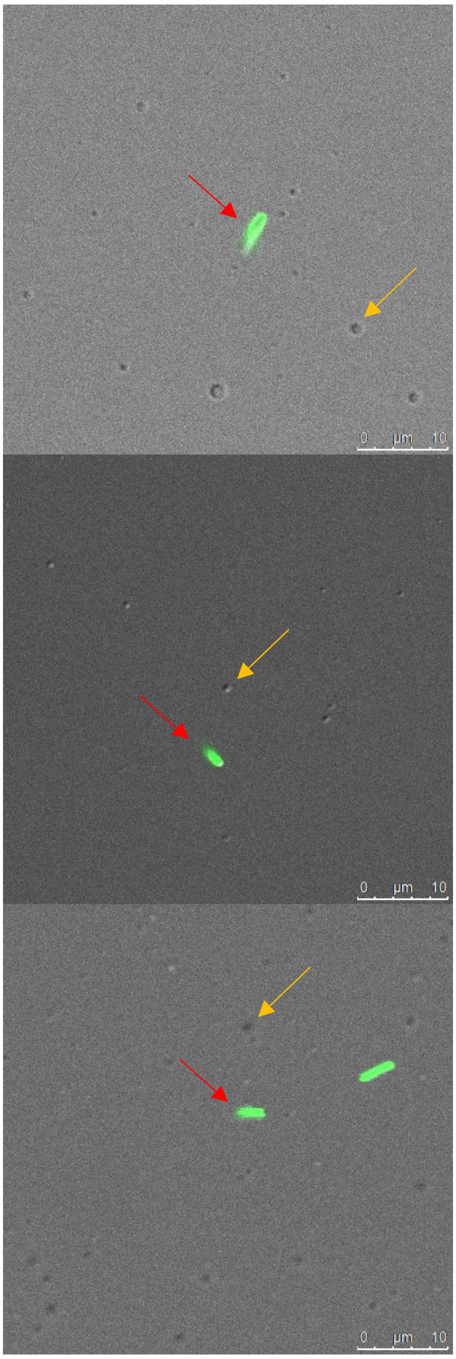

Figure 5-1. Fluorescent images of GFP-labeled *S*. Typhimurium inoculated in three different emulsions: (a) Tween 20 stabilized 60% emulsion, (b) Tween 80 stabilized 60% emulsion, and (c) Triton X-100 stabilized 60% emulsion, taken in the merge of bright field and GFP modes. In the images, oil droplets were indicated by yellow arrows, while GFP-labeled *S*. Typhimurium were indicated by red arrows. Magnification was 63x/1.4 NA (numerical aperture) oil lens.

5.3.2 RNA expression

5.3.2.1 <u>Expression of rpoE</u>

In various stress environments, including heat, oxidation, UV-light, and desiccation, the alternative σ factor, σE, is regulated by the transcriptional regulator *rpoE*, which controls its expression levels (Amar, Pezzoni, Pizarro, & Costa, 2018; Guillén et al., 2021). The expression of *rpoE* is suppressed by a specific anti-σ factor that binds to it on the inner cell membrane under unstressed conditions (Du et al., 2011). Dawoud et al. (2017). showed that suppressed cell viability in *E. coli* is a result of a lack of *rpoE* expression, particularly under cell membrane stress. Activated *rpoE* under stress conditions leads to upregulation of outer membrane proteins and the β-barrel assembly machinery complex, which works to alleviate stress on the outer membrane (Palmer & Slauch, 2020). In addition, studies have indicated that *rpoE* mutant *Salmonella* exhibited reduced sensitivity to heat shock temperature, deficiencies in intracellular survivability, and non-virulence in mouse infection models (Dawoud et al., 2017). However, recent studies have reported inconsistent results regarding *rpoE* expression under different stress conditions. As an example, the expression of *rpoE* has been observed to be downregulated when bacteria are exposed to low temperatures (4°C), while its expression is upregulated following exposure to high temperatures (Kumar, Datta, & Lalitha, 2015; Sirsat et al., 2011). Moreover, different desiccation methods were shown to modulate *rpoE* expression differently (Fong et al., 2016). These results suggested that different stress types may trigger different *rpoE* responses and indicated the complexity of its regulatory function under stress conditions.

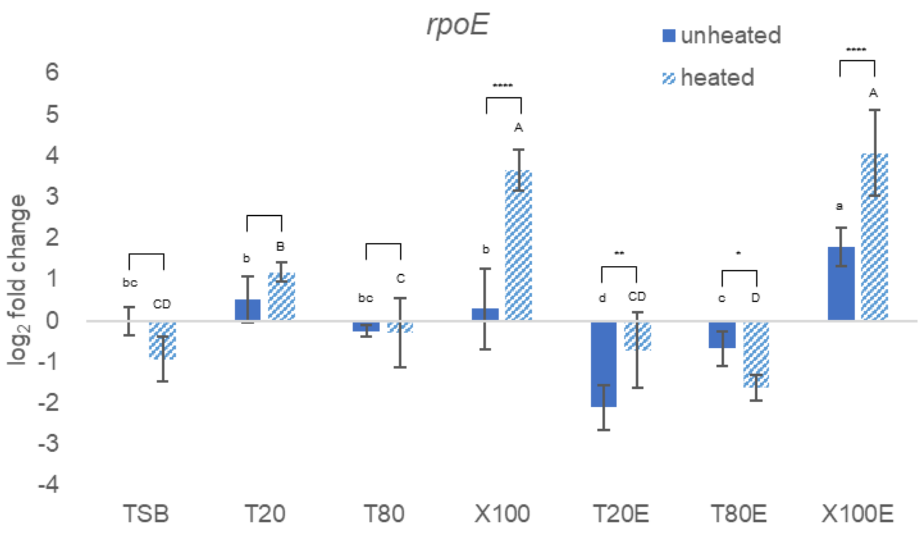

Figure 5-2A. Gene expression of *rpoE*. Log$_2$ fold change in the relative expression of genes in *S.* Typhimurium in TSB, Tween 20 solution (T20), Tween 80 solution (T80), Triton X-100 solution (X100), Tween 20 stabilized 60% emulsion (T20E), Tween 80 stabilized 60% emulsion (T80E), and Triton X-100 stabilized 60% emulsion (X100E) with (orange bar) or without (blue bar) 45-minute thermal treatment at 55°C. Lower case letter labels (a, b, c, etc.) were used to indicate significant differences between groups without thermal treatment, upper case letter labels (A, B, C, etc.) were used to indicate significant differences between groups with thermal treatment, and the asterisks indicate significant differences before and after thermal treatment. The determination of significant difference was based on a two-way ANOVA. Groups with the same letter were not significantly different ($P > 0.05$), while those with different letters were significantly different ($P < 0.05$).

The expression levels of *rpoE* were measured in bacterial cultures grown in emulsifier solutions and emulsions for 20 hours at 37°C, compared to those grown in TSB, as shown in Figure 5-2A. A significant downregulation of *rpoE* was observed in 60% emulsion stabilized with Tween 20 ($P < 0.05$), while 60% emulsion stabilized with Triton X-100 showed an upregulation of *rpoE* ($P < 0.05$). However, no significant differences in the expression of *rpoE* were observed in any other emulsifier solution-based cultures compared to the TSB ($P > 0.05$). The observed downregulation of *rpoE* in Tween 20 stabilized 60% emulsion reflect a decrease in bacterial heat sensitivity, which may have a negative impact following thermal treatment (Dawoud et al., 2017). Conversely, the upregulation of *rpoE* in the presence of Triton X-100 and oil may indicate an adaptive response to alleviate stress, which was supported by the comparable growth numbers of *S*. Typhimurium after 18 hours in Triton X-100 stabilized 60% emulsion and TSB (Ch. 2).

To investigate the influence of emulsifier type on *rpoE* expression, we also compared expression levels in *S.* Typhimurium grown in different emulsifier solutions and emulsions for 20 hours. No significant difference was found in *rpoE* expression among 2% Tween 20, Tween 80, and Triton X-100 solutions ($P > 0.05$). In contrast, *S.* Typhimurium cultured in Triton X-100 stabilized 60% emulsion showed a significant increase in *rpoE* expression compared to those in Tween 20 and Tween 80 stabilized 60% emulsions ($P < 0.05$). Conversely, *rpoE* expression was significantly lower in *Salmonella* inoculated in Tween 20 stabilized 60% emulsion compared to in Tween 80 stabilized 60% emulsion (P

< 0.05). These findings suggested that Tween 20 stabilized 60% emulsion was potentially a less heat sensitive environment than Tween 80 stabilized 60% emulsion, but there was no significant difference compared to the TSB (P > 0.05). Dawoud et al. (2017) reported that *Salmonella* mutants with disrupted *rpoE* expression showed decreased responsiveness to heat shock temperatures, suggesting decreased susceptibility to heat shock and less sensitivity to thermal stress. The presence of oil in emulsions had an impact on *rpoE* expression of *S*. Typhimurium. In Tween 20 stabilized 60% emulsion, *rpoE* expression was significantly downregulated compared to Tween 20 solution (P < 0.05). The presence of oil in Tween 20 environment was found to decrease susceptibility to thermal stress, which was associated with a lower expression of *rpoE*. On the other hand, in the Triton X-100 stabilized 60% emulsion, *rpoE* expression was significantly (P < 0.05) upregulated compared to the Triton X-100 solution. This indicated that the oil in emulsion with Triton X-100 increased the stress on the bacteria, leading to a higher expression of *rpoE*. Thus, the impact of oil on *rpoE* expression was found to vary depending on the type of emulsifier utilized. Prior studies have demonstrated that the expression of *rpoE* is stress-dependent, with increasing stress levels leading to upregulated expression of *rpoE* (Dawoud et al., 2017; Palmer et al., 2020). Specifically, the presence of oil in a Tween 20 environment in this study has been observed to induce intracellular defects and less sensitivity to thermal stress. On the other hand, the presence of oil in a Triton X-100 emulsion has been found to elevate stress levels in the environment, subsequently activating stress-related responses through *rpoE*.

Following a 45-minute thermal treatment at 55°C, *rpoE* expression in *S*. Typhimurium cultured in TSB did not differ significantly (P > 0.05), when comparing before and after thermal treatment within the group. A similar effect was found by Kim et al. (2020), the expression of *rpoE* was downregulated within 10 minutes after thermal treatment at 42°C, and subsequently returned to untreated levels. However, in bacteria cultured in Triton X-100 solution, Triton X-100 stabilized 60% emulsion, and Tween 20 stabilized 60% emulsion, *rpoE* expression was significantly increased (P < 0.05). This implied that the stress on the bacteria was not only due to heat, but also to the presence of Triton X-100 and oil. The results also indicated that the stress was severe enough that the expression of *rpoE* in the Triton X-100 system was not regulated by RseA within 45 minutes. Conversely, *rpoE* expression decreased in bacteria inoculated in Tween 80 stabilized 60% emulsion after thermal treatment (P < 0.05).

A comparison was also conducted between the emulsifier-including environment and TSB to determine the potential protective effect. Inoculation of *S*. Typhimurium in Tween 20 stabilized 60% emulsion, Tween 80 solution, and Tween 80 stabilized 60% emulsion did not show upregulation of *rpoE* expressions, whereas upregulation was observed in other samples (P < 0.05). These results suggested that the presence of oil in Tween 20 and Tween 80 including environment may decrease the heat sensitivity when thermal stress was included, while the presence of individual emulsifier may enhance the stress in the system after thermal treatment, except for Tween 80.

The comparison was conducted within emulsifier solutions and emulsions to investigate the impact of emulsifier type and presence of oil on *rpoE* expression following thermal treatment. The results showed that the expression level of *rpoE* of *S*. Typhimurium was the highest in the Triton X-100 solution, followed by Tween 20 and then Tween 80 ($P < 0.05$). A similar trend was observed in emulsions ($P < 0.05$). These findings suggested that Triton X-100 environment induced more severe stress on the bacteria compared to Tween 20 and Tween 80 environments, as evidenced by the significantly higher expression level of *rpoE*. Furthermore, the impact of oil on the expression of *rpoE* was observed to decrease heat sensitivity in both Tween 20 and Tween 80 environments, with a significant decrease of approximately 5-fold and 2-fold, respectively, in their emulsions ($P < 0.05$). However, in the presence of Triton X-100, the addition of oil did not have any effect on *rpoE* expression, suggesting that stress was not exacerbated or alleviated by the presence of oil. This was plausibly because Tritox-X alone caused a significantly high stress by itself. Therefore, the type of emulsifier used played a crucial role in evaluating the effect of oil on stress response.

5.3.2.2 Expression of rpoH

The *rpoH* gene is known to play a critical role in the induction of heat shock proteins through σ factor 32 (Ban, Kang, & Yoon, 2015; Roncarati et al., 2017). Under normal growth and survival conditions, *rpoH* expression is not typically upregulated; however, exposure to heat treatment has been observed to result in an increase in *rpoH* expression (Dawoud et al., 2017; Takashi Yura & Nakahigashi, 1999). Moreover, *rpoH* has been reported to act as an RNA thermometer at high temperatures, where the expression of *rpoH* is induced by ribosomes (Narberhaus, Waldminghaus, & Chowdhury, 2006). In a subset of Gram-negative bacteria, such as Pseudomonas, the *dnaK* chaperone system has been shown to negatively regulate *rpoH* expression (Kobayashi, Ohtsu, Fujimura, & Fukumori, 2011).

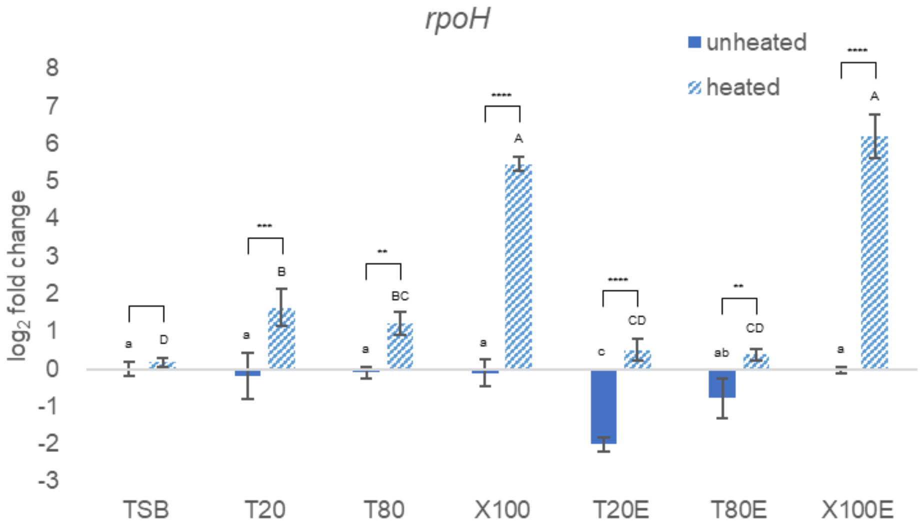

Figure 5-2B. Gene expression of *rpoH*. Log_2 fold change in the relative expression of genes in *S.* Typhimurium in TSB, Tween 20 solution (T20), Tween 80 solution (T80), Triton X-100 solution (X100), Tween 20 stabilized 60% emulsion (T20E), Tween 80 stabilized 60% emulsion (T80E), and Triton X-100 stabilized 60% emulsion (X100E) with (orange bar) or without (blue bar) 45-minute thermal treatment at 55°C. Lower case letter labels (a, b, c, etc.) were used to indicate significant differences between groups without thermal treatment, upper case letter labels (A, B, C, etc.) were used to indicate significant differences between groups with thermal treatment, and the asterisks indicate significant differences before and after thermal treatment. The determination of significant difference was based on a two-way ANOVA. Groups with the same letter were not significantly different ($P > 0.05$), while those with different letters were significantly different ($P < 0.05$).

Figure 5-2B displays the expression of *rpoH* in *S*. Typhimurium cultured in emulsifier solutions and emulsions, which were compared to bacteria cultured in TSB after 20 hours of incubation at 37°C. No significant difference was observed in *rpoH* expression between the emulsifier solutions and TSB (P > 0.05) or between the emulsions and TSB (P > 0.05), except for Tween 20 stabilized 60% emulsion, where *rpoH* expression was downregulated (P < 0.05). The results showed no upregulation of *rpoH* which was consistent with previous study, and indicated that the environments were suitable for growth without thermal treatment (Takashi Yura et al., 1999).

A comparative analysis of *S*. Typhimurium cultured in emulsifier solutions and emulsions for a duration of 20 hours was conducted to evaluate the impact of both emulsifier type and oil presence on *rpoH* expression. However, the results showed no significant difference in *rpoH* expression among the 2% Tween 20, Tween 80, and Triton X-100 solutions (P > 0.05). In contrast, a significant decrease in *rpoH* expression was observed in *S*. Typhimurium cultured in Tween 20 stabilized 60% emulsion compared to Tween 80 and Triton X-100 stabilized 60% emulsions, as well as compared to the Tween 20 emulsifier solution (P < 0.05). The observed results were consistent with previous study that suggests *rpoH* expression remains unchanged in appropriate growth conditions without thermal treatment , and implied that emulsifiers and oils may have no direct effects on *rpoH* expression. (Kang et al., 2018). However, the cause of the decrease in *rpoH* expression observed in this study remains unknown. While the data and previous studies did not allow for a definitive explanation of this phenomenon, and further investigations were needed to elucidate the mechanisms involved in this phenomenon.

Following a 45-minute thermal treatment at 55°C, no significant difference in *rpoH* expression was observed in *S*. Typhimurium cultured in TSB (P > 0.05) when comparing the blue and orange bars within the group. This may be due to the DnaK-DnaJ chaperone system, which has been reported to downregulate *rpoH* expression (to untreated level) as an adaptive reponse to thermal environments (Nakahigashi, Yanagi, & Yura, 2001; Y. Yang, Khoo, Zheng, Chung, & Yuk, 2014). In contrast, a significant increase in *rpoH* expression was observed in the remaining samples following the 45-minute thermal treatment (P < 0.05). This finding was consistent with previous studies indicating that *rpoH* upregulation was able to be induced by thermal treatment (Kang et al., 2018; Y. Yang et al., 2014).

A comparative analysis was also performed between the emulsifier including environment and TSB to investigate the potential protective effect after thermal treatment. Notably, inoculation of *S*. Typhimurium in Tween 20 stabilized 60% emulsion and Tween 80 stabilized 60% emulsion did not exhibit any upregulation of *rpoH* expressions (P > 0.05), while upregulation was observed in the remaining samples (P < 0.05). Drawing upon the concept of RNA thermometer, it can be postulated that the presence of oil in Tween 20 and Tween 80 containing environment may have a protective effect against increased temperature, whereas the presence of individual emulsifier may not provide significant protection against heat-induced stress.

The comparison was conducted within emulsifier solutions and emulsions to investigate the impact of emulsifier type and presence of oil on *rpoH* expression following thermal treatment. Results indicated that only Triton X-100 solution significantly upregulated *rpoH* expression in comparison to other emulsifier solutions ($P < 0.05$). Similarly, Triton X-100 stabilized 60% emulsion also demonstrated a significant effect in comparison to other emulsions ($P < 0.05$). These findings aligned with previous studies that suggested a correlation between higher *rpoH* expression and heat resistance, which has been observed in Triton X-100 solution and Triton X-100 stabilized 60% emulsion (H. J. Chung, Bang, & Drake, 2006; Y. Yang et al., 2014). The fact that only Triton X-100 solutions and Triton X-100 stabilized 60% emulsions exhibited a significant upregulation of *rpoH* expression implied that the specific physicochemical properties of Triton X-100 may play a role in the observed effect. However, our previous research has shown that the heat-resistant effect was specifically observed in Triton X-100 solution (5D = 795.34 ± 420.09 min) in comparison to TSB (5D = 203.89 ± 10.18 min) and not in Triton X-100 stabilized emulsion (5D= 207.23 ± 60.03 min) (Ch. 2). The contrasting results between Triton X-100 solution and Triton X-100 stabilized 60% emulsion indicated that the effect of emulsification on bacterial heat resistance is not necessarily predictable based on the properties of the emulsifier alone. It was possible that the observed effect was due to greater-than-anticipated damage to the cell membrane in Triton X-100 stabilized 60% emulsion.

5.3.2.3 <u>Expression of otsB</u>

The present study examined the expression of *otsB* as an indicator of metabolic response. OtsB is an essential enzyme in the trehalose biosynthesis pathway, and it encodes for trehalose-6-phosphate (T6P), a metabolite derived from glucose (Balaji, O'Connor, Lucas, Anderson, & Csonka, 2005; Fong et al., 2016; H. Li et al., 2012). Trehalose plays a vital role in maintaining the appropriate osmotic gradient potential in cells (Finn, Condell, McClure, Amézquita, & Fanning, 2013). Previous studies have shown that the upregulation of *otsB* occurs following desiccation and thermal treatment (Abdelhamid & Yousef, 2020). Additionally, the expression of *otsB* is impacted by storage duration, with a higher fold change in *otsB* expression observed after a 2-day storage period compared to a 14-day storage period (Abdelhamid et al., 2020). Specifically, upregulation of *otsB* implied an increased demand for metabolites to be transferred to trehalose-6-phosphate (T6PP) to maintain osmotic potential. On the other hand, according to a study carried out by Abdelhamid and Yousef in 2019, paenibacterin can lead to the destruction of the cell membrane of *Salmonella*, which is the cause of the reduction of *otsB* expression (Abdelhamid & Yousef, 2019).

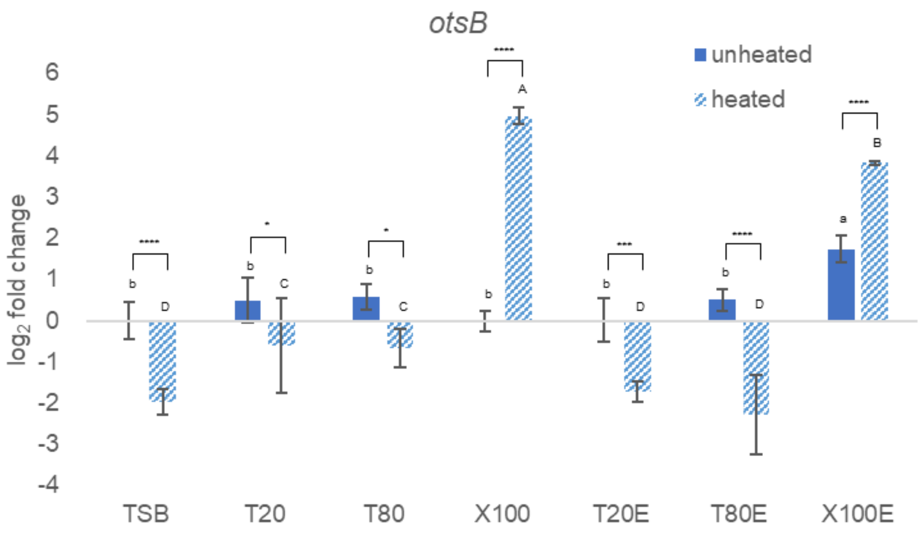

Figure 5-2C. Gene expression of *otsB*. Log_2 fold change in the relative expression of genes in *S.* Typhimurium in TSB, Tween 20 solution (T20), Tween 80 solution (T80), Triton X-100 solution (X100), Tween 20 stabilized 60% emulsion (T20E), Tween 80 stabilized 60% emulsion (T80E), and Triton X-100 stabilized 60% emulsion (X100E) with (orange bar) or without (blue bar) 45-minute thermal treatment at 55°C. Lower case letter labels (a, b, c, etc.) were used to indicate significant differences between groups without thermal treatment, upper case letter labels (A, B, C, etc.) were used to indicate significant differences between groups with thermal treatment, and the asterisks indicate significant differences before and after thermal treatment. The determination of significant difference was based on a two-way ANOVA. Groups with the same letter were not significantly different ($P > 0.05$), while those with different letters were significantly different ($P < 0.05$).

The expression of *otsB* was analyzed in this study, as shown in Figure 5-2C. After 20 hours of incubation at 37°C, the *otsB* expression of bacteria cultured in emulsifier solutions and emulsions was compared to that of bacteria cultured in TSB. Upregulation of *otsB* was only observed in Triton X-100 stabilized 60% emulsion ($P < 0.05$), while no significant differences in *otsB* expression were found for other samples ($P > 0.05$). The observed upregulation of *otsB* expression in Triton X-100 stabilized 60% emulsion may indicate that the presence of Triton X-100 and oil combination created a more challenging environment for bacterial growth when compared to the other samples. This result was in line with the pivotal role of *otsB* in the biosynthesis of trehalose, which maintained the osmotic gradient potential in cells. Additionally, the upregulated *otsB* in Triton X-100 stabilized 60% emulsion implied that more metabolites need to be transferred to T6P-phosphate (T6PP) to maintain the osmotic potential.

To investigate the effect of the emulsifier type and the presence of oil on *otsB* expression, *S*. Typhimurium cultured in emulsifier solutions and emulsions for 20 hours were also compared. Consistent with the previous results, upregulation of *otsB* was only found in Triton X-100 stabilized 60% emulsion ($P < 0.05$). Again, the combination of Triton X-100 and oil created the most severe growth environment for bacteria compared to other samples. These results reflect the tendency of *otsB* to activate T6P to T6PP with increasing metabolites in Triton X-100 stabilized 60% emulsion. This adaptation to the harsh environment may contribute to the observed growth inhibition, which was consistent with previous data (Lag phase = 9.326 ± 0.301 hr) in comparison to TSB (Lag phase = 2.435 ± 0.849 hr) (Ch. 2).

After subjecting *S*. Typhimurium to a 45-minute thermal treatment at 55°C, *otsB* was downregulated in those cultured in TSB, Tween 20 solution, Tween 80 solution, Tween 20 stabilized 60% emulsion, and Tween 80 stabilized 60% emulsion ($P < 0.05$). There are two possible reasons for the downregulation of *otsB*: (a) lower stress level is detected in the system compared to samples prior to thermal treatment, and (b) damage to cell membrane, which may have been caused by thermal treatment. However, the former reason is less likely since thermal treatment is known to increase stress levels in the system. Additionally, a previous study has suggested a possible correlation between membrane damage and *otsB* downregulation (Abdelhamid et al., 2019).

On the other hand, it was interesting to note that there were no significant differences in *otsB* expression between *S*. Typhimurium cultured in TSB and those cultured in Tween 20 stabilized 60% emulsion and Tween 80 stabilized 60% emulsion after thermal treatment ($P > 0.05$). We hypothesized that the combination of Tween 20 or Tween 80 with oil in the culture medium may confer some level of protection to bacterial cells under thermal stress, thereby promoting a more stable *otsB* expression. Additionally, *S*. Typhimurium cultured in Triton X-100-containing medium showed a significant upregulation of *otsB* expression compared to that in TSB ($P < 0.05$). The upregulation of *otsB* expression in the presence of Triton X-100 indicated that this emulsifier had a significant impact on bacterial metabolism and growth. It was consistent with previous studies that have shown the disruptive effects of Triton X-100 on bacterial membranes, which can lead to increased permeability and changes in cellular physiology (Sałek, Kaczorek, Guzik, & Zgoła-Grześkowiak, 2015). The observed increase in *otsB* expression was attributed to the need for increased production of metabolites in response to the altered cellular environment, which may be necessary for maintaining proper osmotic balance and other essential cellular processes.

In order to assess the impact of both emulsifier type and the inclusion of oil on *otsB* expression following thermal treatment, comparisons were conducted in emulsifier solutions and emulsions. It was observed that among all emulsifier solutions, *S*. Typhimurium cultured in Triton X-100 exhibited an increase in otsB expression, while among all emulsions, Triton X-100 stabilized 60% emulsion demonstrated a significantly higher level of *otsB* expression ($P < 0.05$). Again, these results confirmed the effects of metabolites on *otsB* expression. Conversely, the presence of oil resulted in the downregulation of *otsB* in Tween 20, Tween 80, and Triton X-100 environments, indicating that the inclusion of oil potentially increased cell membrane damage after thermal treatment.

5.3.2.4 <u>Expression of proV</u>

The expression of *proV* is a reliable indicator of proline accumulation and changes in osmotic pressure within the cell (Abdelhamid & Yousef, 2022; Gruzdev, McClelland, Porwollik, Ofaim, Pinto, & Saldinger-Sela, 2012). Proline is one of the most important solutes that helps bacteria maintain normal osmotic pressure (Takasu & Nagata, 2015). Thus, upregulation of *proV* indicates increased adaptation and survival of bacteria in stressful environments, particularly in salt-rich or metabolite-rich conditions (Z. Chen et al., 2017). Additionally, *proV* is responsible for transporting osmoprotectant molecules into the cell. Previous research has shown that reduced expression of *proV* results in lower importation of osmoprotectant compounds, leading to increased susceptibility to desiccation in *Salmonella* (Abdelhamid et al., 2022).

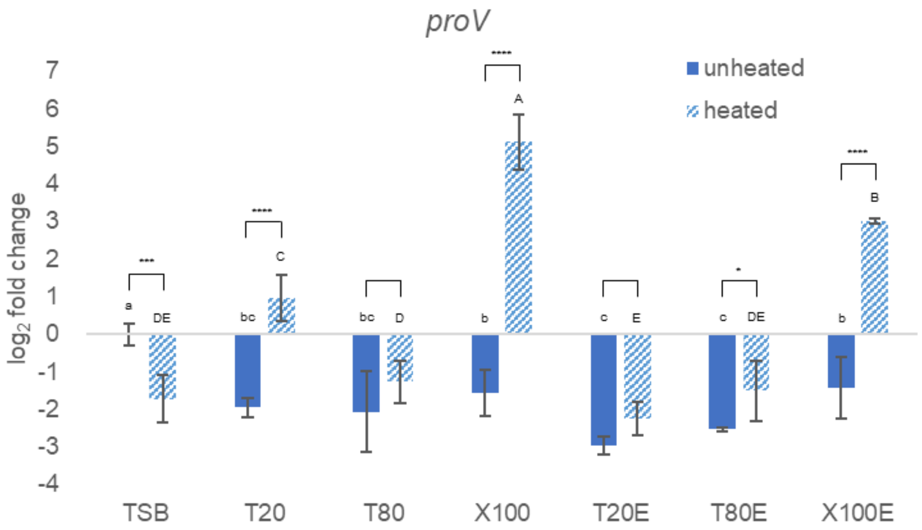

Figure 5-2D. Gene expression of *proV*. Log$_2$ fold change in the relative expression of genes in *S.* Typhimurium in TSB, Tween 20 solution (T20), Tween 80 solution (T80), Triton X-100 solution (X100), Tween 20 stabilized 60% emulsion (T20E), Tween 80 stabilized 60% emulsion (T80E), and Triton X-100 stabilized 60% emulsion (X100E) with (orange bar) or without (blue bar) 45-minute thermal treatment at 55°C. Lower case letter labels (a, b, c, etc.) were used to indicate significant differences between groups without thermal treatment, upper case letter labels (A, B, C, etc.) were used to indicate significant differences between groups with thermal treatment, and the asterisks indicate significant differences before and after thermal treatment. The determination of significant difference was based on a two-way ANOVA. Groups with the same letter were not significantly different ($P > 0.05$), while those with different letters were significantly different ($P < 0.05$).

The expression of *proV* was analyzed and presented in Figure 5-2D. After a 20-hour incubation period at 37°C, the *proV* expression of bacteria cultured in emulsifier solutions and emulsions was compared to those cultured in TSB. Downregulation of *proV* was observed in all samples ($P < 0.05$). The downregulation of *proV* expression indicates a decrease in the ability of bacteria to accumulate proline and maintain osmotic balance, which is a critical response for bacterial survival in stress-related environments (Z. Chen et al., 2017).

A comparison of *S*. Typhimurium cultured in emulsifier solutions and emulsions for 20 hours was conducted to examine the impact of emulsifier type and oil on *proV* expression. No significant differences in *proV* expression were observed among 2% (v/v) Tween 20, Tween 80, and Triton X-100 solutions ($P > 0.05$). In contrast, *S*. Typhimurium cultured in Triton X-100 stabilized 60% emulsion demonstrated significantly higher *proV* expression compared to those cultured in Tween 20 and Tween 80 stabilized 60% emulsion ($P < 0.05$). It is important to note that the difference in *proV* expression was minimal, therefore, marginal cases may be observed. Furthermore, no significant differences were observed in any corresponding emulsifier solution and emulsion groups ($P > 0.05$), which revealed that the presence of oil did not affect the expression of *proV*.

Following thermal treatment at 55°C for a duration of 45 minutes, a significant downregulation of *proV* was observed in *S*. Typhimurium cultured in both TSB and Tween 80 stabilized 60% emulsion ($P < 0.05$), as evidenced by the comparison of the blue and orange bars within the group. In contrast, upregulation of *proV* expression was observed in Tween 20 solution, Triton X-100 solution, and in Triton X-100 stabilized 60% emulsion following the thermal treatment ($P < 0.05$). Therefore, the effect of thermal stress on *proV* expression may vary depending on the environmental condition. It was reported by previous study that *proV* gene was involved in the regulation of osmotic pressure within the cell, which was critical for survival in high-stress environments (Z. Chen et al., 2017). The upregulation of *proV* expression in Tween 20 and Triton X-100 solution and Triton X-100 stabilized 60% emulsion with thermal treatment indicated that a protective effect was potentially possessed on the cell under heat stress in these environments. In addition, its downregulation in response to heat stress could also indicate a prioritization of other stress responses over the maintenance of osmotic pressure.

Compared to TSB, *S*. Typhimurium cultured in Tween 20 and Triton X-100 solution, as well as Triton X-100 stabilized 60% emulsion, exhibited significant upregulation of *proV* expression ($P < 0.05$). Interestingly, these emulsifiers may act as a trigger to *S*. Typhimurium, which led to an adaptive response to maintain cellular homeostasis. However, the specific mechanism remained unclear and needed further investigation.

In order to investigate the impact of different emulsifiers and oil inclusion on *proV* expression following thermal treatment, intra-group comparisons were conducted on emulsifier solutions and emulsions. The results showed that bacteria cultured in Triton X-100 exhibited significantly higher levels of *proV* expression compared to those cultured in Tween 20 ($P < 0.05$). Conversely, the lowest levels of *proV* expression were observed in Tween 80 solution (P < 0.05). On the other hand, among emulsion samples, a significant increase in *proV* expression was observed in Triton X-100 stabilized 60% emulsion compared to Tween 20 and Tween 80 stabilized 60% emulsion (P < 0.05). However, there was no statistically significant difference in *proV* expression between Tween 20 and Tween 80 stabilized emulsions (P > 0.05). The reason for the higher *proV* expression in Triton X-100 solution compared to Tween 20 and Tween 80 solutions was potentially due to differences in the ability of these emulsifiers to interact with cell membrane and affected the transport of essential nutrients, such as amino acids, to the cell. This difference in nutrient transport resulted in higher proline production, which is regulated by *proV*. Therefore, the higher *proV* expression observed in Triton X-100 solution revealed that more efficient in promoting nutrient uptake and proline synthesis compared to the other emulsifiers. However, further research is needed to confirm this hypothesis and to investigate the exact mechanism underlying the observed differences in *proV* expression between the different emulsifiers. Compared to emulsifier solutions and their corresponding emulsions, a significant decrease in *proV* expression was observed in groups containing Tween 20 and Triton X-100 with oil (P < 0.05). Therefore, the inclusion of oil in these emulsifier solutions may have a negative impact on *proV* expression, which could be attributed to changes in the microenvironment surrounding the bacterial cells (Martínez-Palou et al., 2013).

5.3.2.5 Expression of fadA

FadA plays a crucial role in the tricarboxylic acid cycle by breaking down long-chain fatty acids into acetyl-CoA to generate ATP (H. Li et al., 2012). Previous studies have suggested that the expression of *fadA* is downregulated in *Salmonella* under thermal treatment due to the degradation of fatty acids on the cell membrane (Fong et al., 2016). However, in some cases of desiccation and starvation, *fadA* has been observed to be upregulated (Deng, Li, & Zhang, 2012). This upregulation may be attributed to the increased demand for ATP to synthesize stress response proteins (Seeras, 2017).

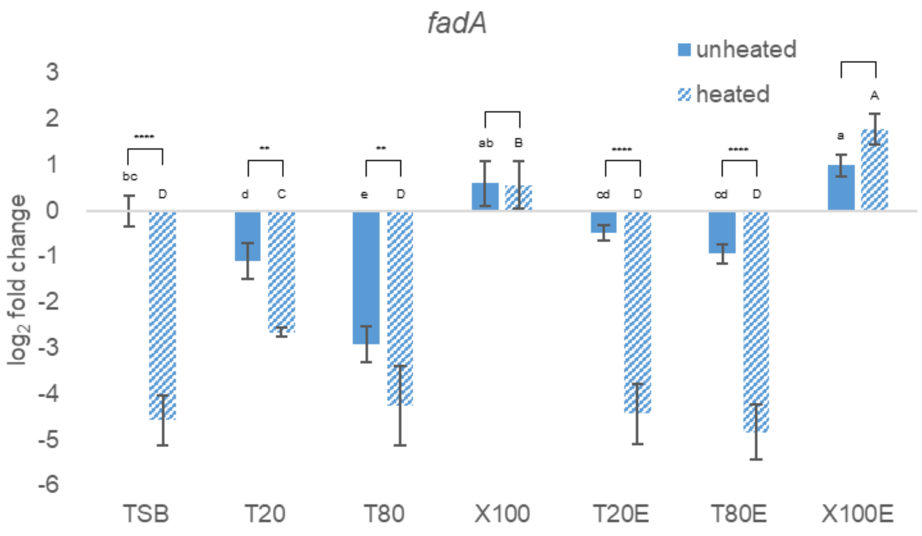

Figure 5-2E. Gene expression of *fadA*. Log$_2$ fold change in the relative expression of genes in *S.* Typhimurium in TSB, Tween 20 solution (T20), Tween 80 solution (T80), Triton X-100 solution (X100), Tween 20 stabilized 60% emulsion (T20E), Tween 80 stabilized 60% emulsion (T80E), and Triton X-100 stabilized 60% emulsion (X100E) with (orange bar) or without (blue bar) 45-minute thermal treatment at 55°C. Lower case letter labels (a, b, c, etc.) were used to indicate significant differences between groups without thermal treatment, upper case letter labels (A, B, C, etc.) were used to indicate significant differences between groups with thermal treatment, and the asterisks indicate significant differences before and after thermal treatment. The determination of significant difference was based on a two-way ANOVA. Groups with the same letter were not significantly different ($P > 0.05$), while those with different letters were significantly different ($P < 0.05$).

Figure 5-2E showed the expression of *fadA* after 20 hours of incubation at 37°C in different culture conditions. Bacteria cultured in emulsifier solutions and emulsions were compared to those cultured in TSB. The results indicated a downregulation of *fadA* in both Tween 20 and Tween 80 solutions ($P < 0.05$), while *fadA* expression was upregulated in the Triton X-100 stabilized 60% emulsion ($P < 0.05$). Downregulation of *fadA* suggested that Tween 20 and Tween 80 may not be efficient in promoting fatty acid metabolism, while the upregulation of *fadA* in Triton X-100 stabilized 60% emulsion implied that Triton X-100 in combination with oil may facilitate fatty acid metabolism and the production of ATP in bacteria.

To investigate the impact of different emulsifiers on *fadA* expression, comparisons were made between emulsifier solutions and emulsions over a 20-hour period. The results showed that Triton X-100 had the highest *fadA* expression, followed by Tween 20 and then Tween 80 ($P < 0.05$). Furthermore, *S*. Typhimurium cultured in Triton X-100 stabilized 60% emulsion displayed significantly higher *fadA* expression than those cultured in Tween 20 and Tween 80 stabilized 60% emulsion ($P < 0.05$). However, there was no significant difference between the *fadA* expression in Tween 20 and Tween 80 stabilized 60% emulsions ($P > 0.05$). On the other hand, *fadA* expression was only upregulated in Tween 80 stabilized 60% emulsion compared to Tween 80 solution. The results showed that the expression of *fadA* was significantly affected by the type of emulsifier used. Among the emulsifier solutions, Triton X-100 may be a more effective emulsifier for promoting fatty acid catabolism in bacteria. Moreover, bacteria cultured in Triton X-100 stabilized 60% emulsion the highest *fadA* expression among emulsions, which was consistent with Triton X-100 solution. Interestingly, no significant difference in *fadA* expression was observed between Tween 20 and Tween 80 stabilized 60% emulsions, indicating that these two emulsifiers may have similar effects on fatty acid catabolism. However, it should be noted that upregulation of *fadA* was detected in Tween 80 stabilized 60% emulsion when compared to Tween 80 solution. This result suggested that the emulsification of Tween 80 may enhance the catabolism of long-chain fatty acids, possibly due to the increased availability of fatty acids in the emulsion.

After 45 minutes of thermal treatment at 55°C, the *fadA* expression was compared within the groups. The results showed that the highest expression level of *fadA* was observed in *S*. Typhimurium cultured in Triton X-100 solution and Triton X-100 stabilized 60% emulsion, which was not significantly different from the expression level prior to the treatment ($P > 0.05$). Conversely, a downregulation of *fadA* was observed in the other groups ($P < 0.05$). This finding was consistent with previous studies, which have reported that thermal stress can lead to the downregulation of *fadA* in *Salmonella* (Fong et al., 2016).

Comparative analysis revealed upregulation of *fadA* in Tween 20 solution, Triton X-100 solution, and Triton X-100 stabilized 60% emulsion, as compared to TSB ($P < 0.05$). The results could be attributed to the bacterial stress response mechanism that aims to maintain energy production and cell survival under adverse conditions. The upregulation of *fadA*, which is involved in fatty acid metabolism, can generate energy that is crucial for bacterial survival when nutrients are scarce or when subjected to stress. Therefore, it is possible that the observed upregulation of *fadA* in the mentioned environments is a result of stress or nutrient scarcity, where fatty acids can serve as an alternative energy source in the absence of glucose or other nutrients (Yao & Rock, 2017).

To investigate the impact of different emulsifiers and oil inclusion on *fadA* expression after thermal treatment, a series of comparisons in emulsifier solutions and emulsions were conducted. Among the emulsifier solutions, *fadA* expression was highest in bacteria cultured in Triton X-100 and lowest in those cultured in Tween 80 ($P < 0.05$). In emulsions, *fadA* expression was significantly higher in bacteria inoculated in Triton X-100 stabilized 60% emulsion compared to those in Tween 20 and Tween 80 stabilized 60% emulsion ($P < 0.05$), with no significant difference between Tween 20 and Tween 80 stabilized 60% emulsion ($P > 0.05$). Higher *fadA* expression indicated more severe stress in Triton X-100 solution and Triton X-100 stabilized 60% emulsion compared to Tween 20 and Tween 80 included solutions and emulsions. It was potentially due to its high solubility and membrane-disrupting properties (Gooran, Yoon, & Jackman, 2022). In addition, downregulated *fadA* was observed in Tween 20 stabilized 60% emulsion ($P < 0.05$), while upregulation of *fadA* was detected in Triton X-100 stabilized 60% emulsion ($P < 0.05$) when compared to their corresponding emulsifier solutions. No difference was found between Tween 80 solution and Tween 80 stabilized 60% emulsion ($P > 0.05$). These results suggested that the effect of oil inclusion on *fadA* expression varied depending on the emulsifier type.

More severe stress was observed when oil was added to the Triton X-100 environment, while degradation of fatty acids on cell membrane was observed when oil was included in Tween 20 environment.

5.3.2.6 Expression of fabA

The heat sensitivity of bacteria under thermal treatment can be determined by their membrane fluidity, where a more rigid membrane displays greater heat resistance (Álvarez-Ordóñez, Fernández, López, Arenas, & Bernardo, 2008). The *fabA* gene encodes for 3-hydroxydecanoyl acyl carrier protein dehydratase, which is responsible for the biosynthesis of unsaturated fatty acids (Magnuson, Jackowski, Rock, & Cronan, 1993). Upregulation of *fabA* was observed under stressful environment, indicating increased membrane fluidity and lower phospholipid phase transition temperature of *S*. Typhimurium (Q. Wang, Cen, & Zhao, 2015; Yun, Liu, Zeng, & Han, 2016). In contrast, downregulation of *fabA* was detected after pulsed electric field treatment, which suggests a decrease in the biosynthesis of unsaturated fatty acids and an increase in heat resistance (Yun, Zeng, Brennan, & Han, 2016). Therefore, the regulation of *fabA* expression can impact the membrane fluidity of *S*. Typhimurium, which in turn affects the heat sensitivity of the bacteria.

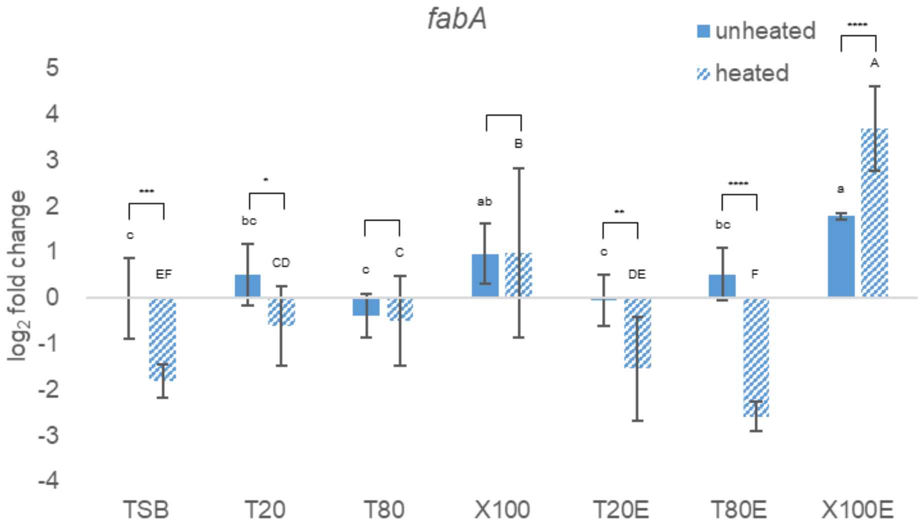

Figure 5-2F. Gene expression of *fabA*. Log_2 fold change in the relative expression of genes in *S.* Typhimurium in TSB, Tween 20 solution (T20), Tween 80 solution (T80), Triton X-100 solution (X100), Tween 20 stabilized 60% emulsion (T20E), Tween 80 stabilized 60% emulsion (T80E), and Triton X-100 stabilized 60% emulsion (X100E) with (orange bar) or without (blue bar) 45-minute thermal treatment at 55°C. Lower case letter labels (a, b, c, etc.) were used to indicate significant differences between groups without thermal treatment, upper case letter labels (A, B, C, etc.) were used to indicate significant differences between groups with thermal treatment, and the asterisks indicate significant differences before and after thermal treatment. The determination of significant difference was based on a two-way ANOVA. Groups with the same letter were not significantly different ($P > 0.05$), while those with different letters were significantly different ($P < 0.05$).

The expression of *fabA* was analyzed and presented in Figure 5-2F. Following a 20-hour incubation at 37°C, the level of *fabA* expression in *S*. Typhimurium cultured in emulsifier solutions and emulsions was compared to those cultured in TSB. Significantly increased *fabA* expression was detected in Triton X-100 containing samples ($P < 0.05$). Thus, Triton X-100 may enhance the biosynthesis of unsaturated fatty acids. The increase of unsaturated fatty acid may subsequently alter membrane fluidity in *S*. Typhimurium, which was consistent with previous studies (Allakhverdiev, Kinoshita, Inaba, Suzuki, & Murata, 2001; Vacaresse et al., 1999).

The effect of the emulsifier type on *fabA* expression was assessed by comparing the expression levels of *S*. Typhimurium cultured in emulsifier solutions and emulsions for 20 hours. It was found that lower *fabA* expression was observed in Tween 80 solution compared to Triton X-100 solutions ($P < 0.05$), while no difference was observed between Tween 20 and Triton X-100 solutions ($P > 0.05$). The lower *fabA* expression observed in Tween 80 solution may indicate higher membrane rigidity, which could contribute to greater heat resistance. This effect was consistent with previous study that mentioned the oleic acid released from Tween 80 can help to protect the cells by maintaining the membrane structure (Mohammad et al., 2021a). Additionally, Triton X-100 stabilized 60% emulsion showed the highest *fabA* expression among all emulsion samples ($P < 0.05$), indicating the combination of Triton X-100 and oil may have a significant impact on membrane fluidity and phospholipid phase transition temperature of *S*. Typhimurium, resulting in reduced heat resistance (Yegutkin, 1997).

Moreover, the presence of oil did not have a significant effect on *fabA* expression, as demonstrated by the comparison between emulsifier solutions and their corresponding emulsions ($P > 0.05$). Therefore, the type of emulsifier used may be a more critical factor in modulating membrane fluidity and heat resistance by altering the expression of *fabA*.

Following thermal treatment at 55°C for 45 minutes, a significant decrease in *fabA* expression was detected in *S*. Typhimurium cultured in TSB ($P < 0.05$) compared to untreated TSB. Similar outcomes were obtained for bacteria cultured in Tween 20 solution and Tween 20 and Tween 80 stabilized 60% emulsions, showing significant downregulation of *fabA* expression ($P < 0.05$). In contrast, bacteria cultured in Triton X-100 stabilized 60% emulsion exhibited significant upregulation of fabA compared with untreated emulsion sample ($P < 0.05$). The decrease in *fabA* expression in TSB aligned with previous research conducted by Yun et al. (2016), which reported downregulation of *fabA* in *S*. Typhimurium cultured at higher temperatures. The emulsifiers Tween 20 and Tween 80, in solution as well as emulsions, may have contributed to the decreased membrane fluidity, which can affect the biosynthesis of unsaturated fatty acids, thus increasing the heat resistance under these conditions. Conversely, the upregulation of *fabA* in Triton X-100 stabilized emulsion suggests that the mechanism in response to thermal stress involves an increase in membrane fluidity, which is consistent with previous studies on *fabA* expression under stressful conditions (Mykytczuk, Trevors, Leduc, & Ferroni, 2007; L.-H. Wang, Zeng, Wang, Brennan, & Gong, 2018). These studies have shown that increased unsaturated fatty acids in the membrane can enhance membrane fluidity, which is achieved by upregulating *fabA* while reducing both cyclopropane and saturated fatty acids under stressful conditions. This adaptation allows the bacteria to better cope with stress.

A comparison was made between samples containing emulsifiers and those cultured in TSB to investigate the effect of heat treatment on *fabA* expression. Except for Tween 20 and Tween 80 stabilized emulsions, *S*. Typhimurium showed upregulated *fabA* expression when inoculated in the remaining environments ($P < 0.05$). There was no significant difference in *fabA* expression observed in the Tween 20 stabilized 60% emulsion ($P > 0.05$), while the Tween 80 stabilized 60% emulsion exhibited downregulation of *fabA* expression compared to TSB ($P < 0.05$). The upregulation of *fabA* expression observed in most of the emulsifier-containing environments suggests that the presence of emulsifiers may enhance the ability of *S*. Typhimurium to adapt to the environment by increasing membrane fluidity. This could facilitate the movement of molecules across the membrane, including nutrient uptake and waste removal, which are important for bacterial growth and survival (Yoon, Lee, Lee, Kim, & Choi, 2015). Conversely, the combination of Tween 80 and oil may increase membrane rigidity, potentially leading to enhanced heat resistance in *S*. Typhimurium (Yun, Liu, et al., 2016).

To investigate the effect of emulsifier type on *fabA* expression following thermal treatment, various emulsifier solutions and emulsions were compared. The results demonstrated that bacteria cultured in Triton X-100 exhibited significantly higher levels of *fabA* expression compared to those cultured in

Tween 20 and Tween 80 solutions (P < 0.05). Among the emulsion samples tested, *fabA* expression levels were ranked highest to lowest in the order of Triton X-100, Tween 20, and Tween 80 stabilized 60% emulsion (P < 0.05). Triton X-100, which is known for its ability to solubilize membrane proteins and lipids, was found to induce the highest *fabA* expression levels, suggesting an increase in membrane fluidity that facilitates molecular transport and adaptation to thermal and other stress. This finding is consistent with the notion that harsher environments induce higher expression of genes related to membrane fluidity, as demonstrated by previous study (Yoon et al., 2015). In contrast, lower *fabA* expression levels observed in Tween 20 and Tween 80 solutions may be attributed to an increase in membrane rigidity, possibly due to the difference in characteristics of these emulsifiers. Specifically, the release of oleic acid from Tween 80 may contribute to a decrease in membrane fluidity upon application of heat energy in the system. The impact of oil inclusion on *fabA* expression was further investigated by comparing emulsifier solutions and their corresponding emulsions. Notably, downregulation of *fabA* expression was observed in Tween 80 stabilized 60% emulsion (P < 0.05), suggesting that the addition of oil may decrease membrane fluidity and induce a protective effect on *S*. Typhimurium. In contrast, upregulation of gene expression was observed in Triton X-100 stabilized 60% emulsion (P < 0.05), indicating that the presence of oil in the Triton X-100 environment may increase waste removal and nutrient uptake, potentially leading to alleviate stress on the bacterial cells. These findings revealed reactions between emulsifiers, oils, and bacterial membrane dynamics.

5.3.2.7 Expression of dnaK

The gene *dnaK* encodes for the chaperone protein Hsp70, which plays a crucial role in protecting other proteins from heat stress (Fong et al., 2016; Gruzdev et al., 2012). Mild thermal treatment has been shown to upregulate the expression of *dnaK* in *S*. Typhimurium (Berk, de Jonge, Zwietering, Abee, & Kieboom, 2005; Gruzdev et al., 2012), leading to increased production of Hsp70 and enhanced heat resistance in bacteria (Fong et al., 2016). Consequently, upregulation of *dnaK* confers a heat-protective effect on the bacteria (Fong et al., 2016). The DnaK protein, which is controlled by *dnaK* gene, specifically is involved in assisting in the folding of newly synthesized proteins, preventing the aggregation of misfolded or denatured proteins, and facilitating the disaggregation of protein aggregates (Agashe et al., 2004). It also plays a crucial role in protecting cells from thermal and oxidative stress, by stabilizing proteins and preventing their denaturation or aggregation under stress conditions (Katikaridis, Bohl, & Mogk, 2021). However, desiccation of peanut oil was found to decrease the expression of *dnaK* (Fong et al., 2016), and it has been suggested that different methods of desiccation can result in varying levels of *dnaK* expression (Abdelhamid et al., 2020; Deng et al., 2012). Moreover, the DnaK/DnaJ chaperone system has been identified as a regulator of *rpoH* expression, and higher expression of *rpoH* can increase the expression of *dnaK* (Takaya, Tomoyasu, Matsui, & Yamamoto, 2004). Furthermore, it has been reported that the upregulation of σ factor 32, induced by *rpoH* and other heat shock protein-related genes, can result in the downregulation of *dnaK* expression. (Da Silva, Simão, Susin, Baldini, Avedissian, & Gomes, 2003).

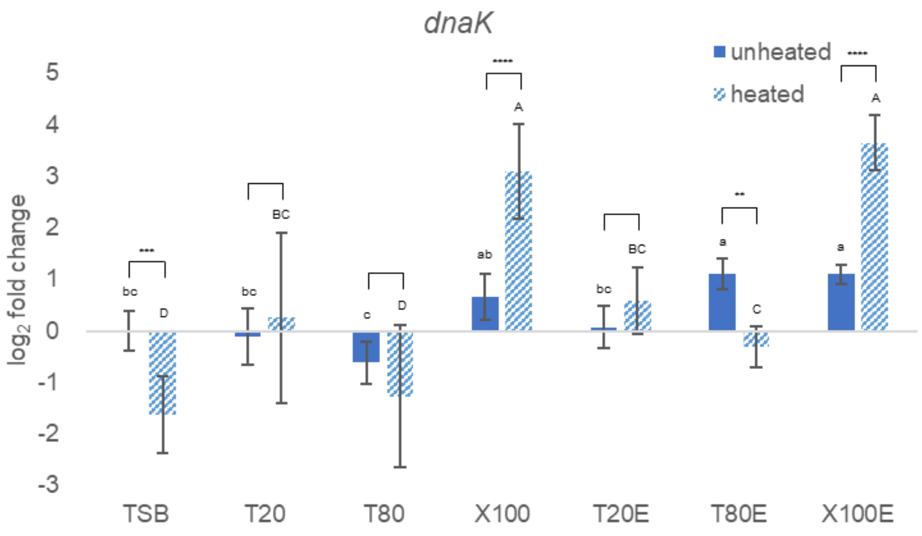

Figure 5-2G. Gene expression of *dnaK*. Log_2 fold change in the relative expression of genes in *S.* Typhimurium in TSB, Tween 20 solution (T20), Tween 80 solution (T80), Triton X-100 solution (X100), Tween 20 stabilized 60% emulsion (T20E), Tween 80 stabilized 60% emulsion (T80E), and Triton X-100 stabilized 60% emulsion (X100E) with (orange bar) or without (blue bar) 45-minute thermal treatment at 55°C. Lower case letter labels (a, b, c, etc.) were used to indicate significant differences between groups without thermal treatment, upper case letter labels (A, B, C, etc.) were used to indicate significant differences between groups with thermal treatment, and the asterisks indicate significant differences before and after thermal treatment. The determination of significant difference was based on a two-way ANOVA. Groups with the same letter were not significantly different ($P > 0.05$), while those with different letters were significantly different ($P < 0.05$).

Figure 5-2G illustrated the expression of *dnaK* in *S*. Typhimurium following 20 hours of incubation at 37°C in different emulsifier solutions and emulsions, compared to TSB. Upregulation of *dnaK* expression was observed in Tween 80 stabilized 60% emulsion and Triton X-100 stabilized 60% emulsion ($P < 0.05$). However, no significant difference of *dnaK* expression was found in the remaining environments when compared to TSB ($P > 0.05$). The observed upregulation of *dnaK* expression in the presence of Tween 80 and Triton X-100 emulsions may indicate that these emulsifiers and oil offer some degree of protection against stress. This response also suggests that these emulsions and oil may induce stress in the bacteria, thereby triggering the activation of *dnaK* expression as a protective response.

In order to examine how emulsifiers affect *dnaK* expression, *S*. Typhimurium was cultured in different emulsifier solutions and emulsions for a duration of 20 hours. Triton X-100 solution induced a significantly higher expression of *dnaK* compared to Tween 80 solution ($P < 0.05$). However, there was no significant difference between Triton X-100 solution and Tween 20 solution ($P > 0.05$). The upregulation of *dnaK* expression in the presence of Triton X-100 suggests that Triton X-100 may induce stress or other cellular responses that activate the *dnaK* gene to help cope with the stress by the activation of DnaK protein (Katikaridis et al., 2021). Furthermore, the expression of *dnaK* in *S*. Typhimurium was found to be lower in Tween 20 stabilized 60% emulsion when compared to both Tween 80 and Triton X-100 stabilized 60% emulsions ($P < 0.05$). Based on the results, it can be concluded that Tween 20 may have a weaker stress response compared to Tween 80 and Triton X-100 when used in emulsions. This could be due to the lower hydrophobicity of Tween 20, causing a weaker interaction with the cell membrane (Mandal, Gupta, & Moulik, 1985). This may result in a lower stress response in the bacterial cells, as they may not be as effectively shielded from the emulsion components as they would be in the presence of Tween 80 or Triton X-100. The effect of oil inclusion was examined, and the results showed that *S*. Typhimurium cultured in Tween 80 stabilized 60% emulsion had significantly higher *dnaK* expression compared to Tween 80 solution ($P < 0.05$). Previous research has indicated that the presence of oil can create additional stress in the environment, potentially triggering the activation of *dnaK* to assist the organism in coping with the stress (Ghoshal, Chuang, Zhang, & McLandsborough, 2022). Nevertheless, this effect was not observed in environments containing Tween 20 or Triton X-100.

Following a 45-minute thermal treatment at 55°C, a downregulation of *dnaK* expression was observed in *S*. Typhimurium cultured in TSB and Tween 80 stabilized 60% emulsion ($P < 0.05$), as shown by the comparison of the blue and orange bars within the group. In contrast, the expression of *dnaK* was upregulated in Triton X-100 solution and Triton X-100 stabilized 60% emulsion after thermal treatment ($P < 0.05$). A more comprehensive understanding on bacterial stress response could be achieved by considering the expression of *rpoH* alongside *dnaK*. As shown in figure 2B, the upregulation of *rpoH* in Tween 80 stabilized 60% emulsion after thermal treatment suggests an increase in σ factor 32, which is consistent with previous studies (Da Silva et al., 2003). This could explain the downregulation of *dnaK* observed in the same system. The downregulation of *dnaK* in TSB may not be solely attributed to increased *rpoH* expression but could be due to the presence of fewer stress factors in the system. It is possible that other genes may have been activated to alleviate some of the stress caused by heat. Further research is required to fully comprehend the underlying mechanism responsible for these observations. Moreover, the upregulation of *dnaK* in Triton X-100 included systems following heat treatment suggests an increased production of Hsp70 chaperone, which is associated with heat resistance (Fong et al., 2016; Gruzdev et al., 2012). Therefore, it is possible that heat resistance is present in both Triton X-100 included environments. This observation aligns with previous research findings, where the 5D value of *S*. Typhimurium in Triton X-100 solution was higher than in TSB, while in Triton X-100 emulsion, there was no significant difference compared to TSB (Ch. 2).

The effect of thermal treatment on *dnaK* expression was examined and compared to the control, TSB. The results showed that *dnaK* was upregulated in all emulsifier solution and emulsion samples except for Tween 80 solution ($P < 0.05$). This upregulation suggested that Tween 20, Triton X-100, and oil could increase response to thermal stress and promote appropriate growth temperatures. These findings are consistent with Berk et al. (2005), who reported that mild thermal treatment could induce *dnaK* upregulation. However, in contrast to the previous study, the presence of oil in the emulsion system did not provide any heat resistance after thermal treatment (Ch. 2). These findings suggest that

S. Typhimurium may exhibit other, potentially more significant, stress-related responses in the emulsion system.

The impact of emulsifier type on *dnaK* expression after thermal treatment was analyzed by comparing the expression levels within emulsifier solutions and emulsions. Among the emulsifier solutions, bacteria cultured in Triton X-100 had the highest expression of *dnaK*, followed by Tween 20 and Tween 80 solutions ($P < 0.05$). In emulsion solutions, Triton X-100 stabilized 60% emulsion had significantly higher *dnaK* expression than Tween 20 and Tween 80 stabilized 60% emulsions ($P < 0.05$), while there was no significant difference between the latter two ($P > 0.05$). The observation that the highest expression of *dnaK* was detected in Triton X-100 compared to Tween 20 and Tween 80 solutions implies that Triton X-100 could be a more effective emulsifier in enhancing bacterial stress tolerance. The observed higher expression of *dnaK* in Triton X-100 stabilized emulsion compared to Tween 20 and Tween 80 stabilized emulsions may also imply that Triton X-100 is more effective in improving bacterial stress resistance. Nevertheless, from previous research, no difference was found between emulsions stabilized with these emulsifiers (Ch. 2). The difference in *dnaK* expression between Tween 80 stabilized 60% emulsion and Tween 80 solution in the presence of oil was found to be significant, with higher expression in the emulsion ($P < 0.05$). The higher expression of *dnaK* in the emulsion system suggests the presence of oil may induce the heat shock protein Hsp70, which is known to confer protection against thermal stress. However, this effect was contrary to the previous study, where more heat resistance was observed in Tween 80 solution compared to Tween 80-stabilized 60% emulsion (Ch. 2). Thus, it is more possible that upregulation of *dnaK* may be attributed to its regulatory role in *rpoH* expression (Takaya et al., 2004).

5.3.2.8 Expression of ibpA

IbpA is a crucial member of the σ factor E and H regulon that plays a vital role in coping with thermal treatment (Roncarati et al., 2017). This gene encodes the small heat shock protein IbpA, which has been shown to possess RNA thermometer properties and exhibits higher expression levels under osmotic stress (Canals et al., 2019; Waldminghaus, Gaubig, Klinkert, & Narberhaus, 2009). The significance of *ibpA* in response to heat stress has been demonstrated by the growth defect observed in *ibpA* mutants at 40°C (Krajewski, Nagel, & Narberhaus, 2013). The upregulation of *ibpA* also confers the benefit of preventing endogenous protein aggregation induced by heat shock, thereby enhancing survivability under thermal treatment (Kuczynska-Wisnik et al., 2002).

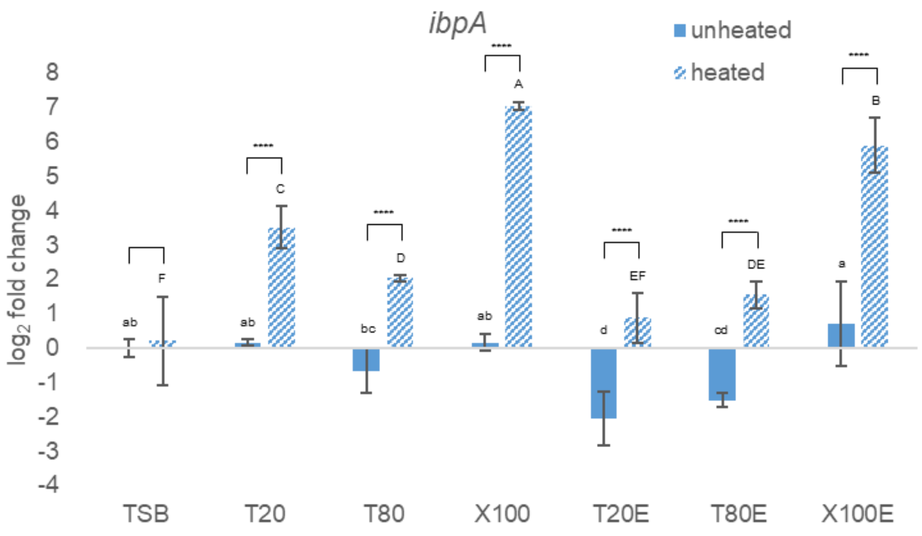

Figure 5-2H. Gene expression of *ibpA*. Log_2 fold change in the relative expression of genes in *S.* Typhimurium in TSB, Tween 20 solution (T20), Tween 80 solution (T80), Triton X-100 solution (X100), Tween 20 stabilized 60% emulsion (T20E), Tween 80 stabilized 60% emulsion (T80E), and Triton X-100 stabilized 60% emulsion (X100E) with (orange bar) or without (blue bar) 45-minute thermal treatment at 55°C. Lower case letter labels (a, b, c, etc.) were used to indicate significant differences between groups without thermal treatment, upper case letter labels (A, B, C, etc.) were used to indicate significant differences between groups with thermal treatment, and the asterisks indicate significant differences before and after thermal treatment. The determination of significant difference was based on a two-way ANOVA. Groups with the same letter were not significantly different (P > 0.05), while those with different letters were significantly different (P < 0.05).

Figure 5-2H showed the expression levels of *ibpA* in bacteria cultured in emulsifier solutions and emulsions after 20 hours of incubation at 37°C, with TSB as the reference. Notably, downregulation of *ibpA* was observed in Tween 20 stabilized 60% emulsion and Tween 80 stabilized 60% emulsion ($P < 0.05$). However, there was no significant difference in *ibpA* expression levels between all other samples and TSB ($P > 0.05$). This unexpected finding suggests that the regulation of *ibpA* expression under stress from emulsifiers and oil may be more complex than previously thought. Future studies could explore potential factors, such as the interaction between the emulsifier and other components in the culture medium, that may contribute to this downregulation effect.

To better understand the impact of different emulsifiers on the expression of *ibpA*, statistical comparison was performed among different emulsifier solutions and emulsions ed. No significant difference was found within emulsifier solutions, but a lower expression of *ibpA* was observed in Tween 20 and Tween 80 stabilized 60% emulsions compared to Triton X-100 stabilized 60% emulsion ($P < 0.05$). Furthermore, the inclusion of oil also had an effect on the expression of *ibpA*, with downregulation observed in Tween 20 stabilized 60% emulsions when compared to in Tween 20 solutions ($P < 0.05$). However, the downregulation effect observed requires further investigation to gain a more comprehensive understanding, as it has not been reported in any previous publications.

After being subjected to a 45-minute thermal treatment at 55°C, the expression of *ibpA* was upregulated in *S*. Typhimurium cultured in all emulsifier solutions and emulsions ($P < 0.05$), except for those cultured in TSB where no significant difference was observed (P > 0.05). The upregulation of *ibpA* in response to thermal stress is consistent with previous studies (Krajewski et al., 2013; Kuczynska-Wisnik et al., 2002). It is believed to enhance the protective effect of IbpA, which in turn helps to maintain the survivability of the bacteria under thermal treatment by preventing endogenous protein aggregation induced by heat shock. The lack of upregulation of *ibpA* in TSB may be due to the absence of emulsifiers, which could potentially provide a protective effect against thermal stress. Emulsifiers are known to stabilize emulsions and protect bacteria from thermal stress by creating a more favorable environment for bacterial survival, such as Tween 80. Therefore, the presence of emulsifiers in the other samples may have provided an additional layer of protection, leading to the upregulation of *ibpA*.

In order to examine how the type of emulsifier affects the expression of *ibpA* following thermal treatment, comparisons between various emulsifier solutions and emulsions. The results showed that Triton X-100 had the highest *ibpA* expression among emulsifier solutions, followed by Tween 20 and Tween 80 solutions (P < 0.05). Among emulsions, Triton X-100 stabilized 60% emulsion had the highest *ibpA* expression (P < 0.05), while no significant difference was observed between bacteria cultured in Tween 20 stabilized 60% emulsion and Tween 80 stabilized 60% emulsion (P > 0.05). The results showed that the presence of Triton X-100 resulted in a higher expression of IbpA protein in response to heat stress, while the presence of Tween 20 and Tween 80 resulted in lower expression of IbpA protein. The study also suggests that Triton X-100 is the most effective emulsifier for inducing heat response in *S*. Typhimurium. When comparing emulsifier solutions with their corresponding emulsions, a decrease in *ibpA* expression was observed in 60% emulsions stabilized with Tween 20 and Triton X-100, compared to their respective emulsifier solutions (P < 0.05). However, there was no significant difference in the *ibpA* expression of the Tween 80 group (P > 0.05). These findings indicate that the presence of oil in the emulsions may decrease the amount of IbpA protein in environments containing Tween 20 and Triton X-100, while it has no effect in Tween 80 environment. Thus, the protective effect was found to be lower in the emulsion when Tween 20 and Triton X-100 were used as emulsifiers. The results regarding Triton X-100 are consistent with a previous study that reported more protection in Triton X-100 solution compared to Triton X-100 emulsion (Ch. 2). On the other hand, Tween 20 had the opposite effect, where emulsifier solution showed no difference compared to emulsion, indicating IbpA may not be the main factor involved in environments containing Tween 20 (Ch. 2). Overall, these results are consistent with a previous study by Hews, Pritchard, and Rowley (2019), which reported upregulation of *ibpA* after thermal treatment.

5.3.2.9 Expression of ompC

The gene *ompC* encodes the outer membrane porin, OmpC, as reported by Arockiasamy et al. (2004). During *Salmonella* Typhimurium infection, OmpC is the primary antigen detected by human antibodies, making the expression of *ompC* an indicator of the bacteria's pathogenicity throughout the infection period (Muthukkaruppan, Nandakumar, & Palanivel, 1992). In response to severe environmental stress, bacteria may increase their pathogenicity for survival (Flint, Butcher, & Stintzi, 2016). However, higher *ompC* expression is also associated with increased passive diffusion of small molecules and wastes through the channel, which can be influenced by the presence of stress in the environment (Sugawara & Nikaido, 1994). As a result, measuring the expression of *ompC* may serve as a useful marker for assessing the level of stress experienced by the bacteria.

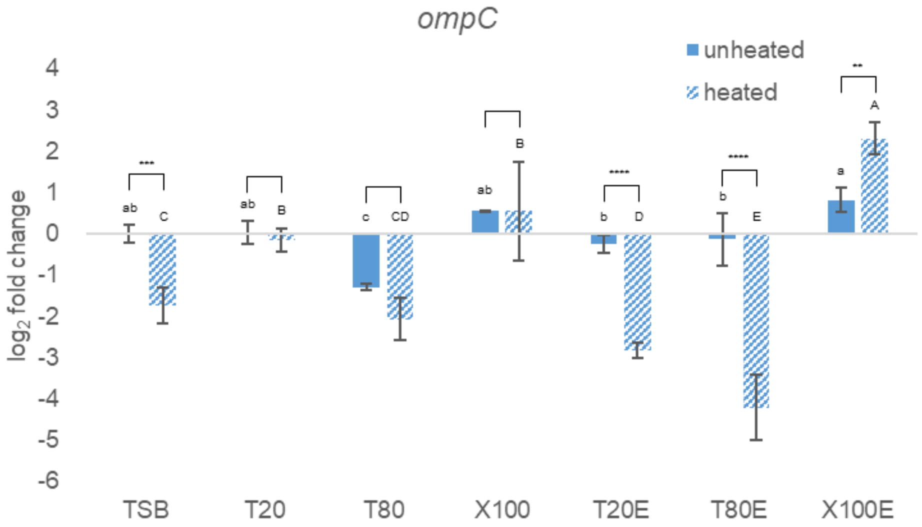

Figure 5-2I. Gene expression of *ompC*. Log2 fold change in the relative expression of genes in *S.* Typhimurium in TSB, Tween 20 solution (T20), Tween 80 solution (T80), Triton X-100 solution (X100), Tween 20 stabilized 60% emulsion (T20E), Tween 80 stabilized 60% emulsion (T80E), and Triton X-100 stabilized 60% emulsion (X100E) with (orange bar) or without (blue bar) 45-minute thermal treatment at 55°C. Lower case letter labels (a, b, c, etc.) were used to indicate significant differences between groups without thermal treatment, upper case letter labels (A, B, C, etc.) were used to indicate significant differences between groups with thermal treatment, and the asterisks indicate significant differences before and after thermal treatment. The determination of significant difference was based on a two-way ANOVA. Groups with the same letter were not significantly different ($P > 0.05$), while those with different letters were significantly different ($P < 0.05$).

Fig. 5-2I illustrated the expression of *ompC*, which was compared between bacteria cultured in emulsifier solutions and emulsions and those cultured in TSB after 20 hours of incubation at 37°C. The results showed a significant downregulation of *ompC* only in the *S*. Typhimurium cultured in Tween 80 solution ($P < 0.05$), indicating a potential negative impact on the pathogenicity of the bacteria. Lower pathogenicity can also be indicative of a less severe stress environment.

In order to understand the effect of the emulsifier type on *ompC* expression, the comparison of *Salmonella* cultured in emulsifier solutions for 20 hours was also performed. The results showed that *ompC* expression was significantly lower in Tween 80 solution compared to Tween 20 and Triton X-100 solutions ($P < 0.05$). The presence of Tween 80 resulted in low pathogenicity of *Salmonella*, indicating that the stress level in the environment was mild compared to that in the presence of Tween 20 and Triton X-100 solutions. On the other hand, the highest expression of *ompC* was detected in Triton X-100 stabilized 60% emulsion compared to Tween 20 and Tween 80 stabilized 60% emulsions ($P < 0.05$). Among emulsion samples, the combination of Triton X-100 and oil resulted in a more severe stress environment, as indicated by the higher expression of *ompC*. The inclusion of oil showed a difference in the Tween 80 group, with higher *ompC* expression observed in the emulsion. This suggests that the presence of oil induces more stress in the Tween 80 environment.

Following a 45-minute thermal treatment at 55°C, a considerable reduction in *ompC* expression was noted in *S*. Typhimurium cultured in TSB, as well as in Tween 20 and Tween 80 stabilized 60%

emulsions, with a statistically significant difference ($P < 0.05$). In contrast, upregulation of *ompC* was found in Triton X-100 stabilized 60% emulsion ($P < 0.05$). These findings are consistent with previous studies, which suggest that thermal treatment can lead to a decrease in pathogenicity and virulence (Dawoud et al., 2017; Sirsat et al., 2011). On the other hand, the combination of Triton X-100 with oil may have a protective effect on the bacteria, leading to an increase in pathogenicity even in a more severe stress environment, rather than being eliminated by thermal treatment. Interestingly, no difference in *ompC* expression was observed in emulsifier solutions under heat stress. This suggests that emulsifiers may have a protective effect on bacteria, preventing downregulation of *ompC* expression. Moreover, *S*. Typhimurium exhibited a change in *ompC* expression depending on the culture medium used. When compared to TSB, the bacteria cultured in Tween 20 and Triton X-100 solutions, as well as Triton X-100 stabilized 60% emulsion, showed an upregulation of *ompC* ($P < 0.05$). This suggests these environments provided a protective effect on the pathogenicity of the bacteria. In contrast, when *S*. Typhimurium cultured in Tween 20 and Tween 80 stabilized 60% emulsion, the bacteria showed a downregulation of *ompC* ($P < 0.05$), which indicated these conditions may present more stress due to heat energy.

The levels of *ompC* expression within emulsifier solutions and emulsions was compared to examine the effect of various emulsifiers on *ompC* expression following thermal treatment. Among emulsifier solutions, the bacteria cultured in Tween 80 had the lowest *ompC* expression ($P < 0.05$).

However, there was no significant difference between Tween 20 and Triton X-100 solutions ($P > 0.05$). The result suggests that individual Tween 80 provided limited protection on the pathogenicity of bacteria, while Tween 20 and Triton X-100 protected bacteria from thermal inactivation. The reason for this difference may be came from the different chemical structures of the emulsifiers. Tween 80 contains a higher proportion of unsaturated fatty acid chains, which are more susceptible to oxidation and can generate reactive oxygen species (ROS) that damage bacterial cells (Mohammad et al., 2021b). In contrast, Tween 20 and Triton X-100 have more stable chemical structures, which may provide better protection to bacterial cells from thermal stress. The levels of *ompC* expression in emulsions stabilized by different emulsifiers were compared. The results showed that the *ompC* expression level in the 60% emulsion stabilized by Triton X-100 was the highest, followed by Tween 20 and then Tween 80 ($P < 0.05$). These results suggest that the type of emulsifier used can have an impact on the expression of *ompC* in the presence of oil. Differences in molecular weight may help to explain the observed effects, as Triton X-100 (250.38 g/mol) had the lowest molecular weight, followed by Tween 20 (1227.54 g/mol) and then Tween 80 (1310 g/mol). Previous studies have shown that in emulsion systems, emulsifiers with lower molecular weights can provide better protection for bacteria (Ch. 4). Specifically, the use of whey protein hydrolysate (MW = 450-500 Daltons) to stabilize the emulsion resulted in a higher 5D value compared to emulsion stabilized with whey protein isolate (MW ~ 18,000 Daltons). The impact of oil on *ompC* expression after thermal treatment was investigated by comparing emulsifier solutions and the corresponding emulsions. The results showed that the 60% emulsions stabilized by Tween 20 and

Tween 80 had significantly lower levels of *ompC* expression compared to their respective solutions ($P < 0.05$), while the 60% emulsion stabilized by Triton X-100 had significantly higher levels of *ompC* expression compared to its solution ($P < 0.05$). These findings suggest that the influence of oil on *ompC* expression may vary depending on the type of emulsifier used. With oil, Tween 20 and Tween 80 were found to have a protective effect, while Triton X-100 induced more stress. This could be due to the different chemical properties of the emulsifiers, as Tween 20 and Tween 80 have higher hydrophilic-lipophilic balance (HLB) values, which make them better emulsifiers for oil-in-water emulsions, whereas Triton X-100 has a lower HLB value (Boyd et al., 1972). The differences in emulsifier properties can affect the stability and structure of the emulsion, which in turn can influence the expression of *ompC* in the presence of oil and thermal stress. However, further investigation is needed to fully understand the detail of the mechanism.

5.3.2.10 Summary

The target genes and their functions are summarized in Table 5-2. Genes such as *rpoE*, *rpoH*, and *ibpA* are involved in the activation of specific heat shock proteins after thermal treatment, while *otsB* and *proV* are related to stress detection through synthesis of trehalose and alternation of osmotic pressure. Fatty acid metabolism genes, *fadA* and *fabA*, provide evidence for membrane alternation. Additionally, *dnaK* plays two roles: regulating *rpoH* and protecting proteins on the cell membrane from stress. Lastly, *ompC* is another gene involved in detecting stress and the pathogenicity of *Salmonella* through the alteration of waste passive diffusion from channels on the membrane.

Table 5-2. Function of target genes in this study.

	Function	**Reference**
16s rRNA	Reference gene	(Castelijn et al., 2012)
rpoE	Regulation of stress response, including heat stress	(Humphreys et al., 1999)
rpoH	Regulation of heat shock response	(Kang et al., 2018)
otsB	Synthesis of sugar trehalose which served as a protectant against a variety of stress	(H. Li et al., 2012)
proV	Help bacteria survive from osmotic stress	(Z. Chen et al., 2017)
fadA	Fatty acid metabolism, specifically in beta-oxidation pathway that breaks down fatty acids for energy production	(Fong et al., 2016)
fabA	Fatty acid biosynthesis that catalyzes the formation of unsaturated fatty acids	(W. Chen et al., 2014)
dnaK	Fold and stabilize other proteins and protects them from stress-induced denaturation	(Fong et al., 2016)

ibpA	Small heat shock protein to protect proteins from aggregation and denaturation during heat stress	(Carroll et al., 2016)
ompC	Channel formation on the outer membrane of gram-negative bacteria, allowing the diffusion of small molecules and wastes	(Kollanoor Johny et al., 2017)

Table 5-3. Summary of the expression of target genes of *S*. Typhimurium in samples. G: 20 hr growth (compare with TSB), G (E): 20hr growth (compare with corresponding emulsion), T: 45 min thermal treatment at 55 °C (compare with sample before thermal treatment), T (TSB[T]): 45 min thermal treatment at 55 °C (compare with TSB after thermal treatment), T (E): 45 min thermal treatment at 55 °C (compare with corresponding emulsion).

Sample	Treatment	Genes								
		rpoE	*rpoH*	*otsB*	*proV*	*fadA*	*fabA*	*dnaK*	*ibpA*	*ompC*
TSB	G	-	-	-	-	-	-	-	-	-
	T	-	-	↓	↓	↓	↓	↓	-	↓
T20	G	-	-	-	↓	↓	-	-	-	-
	G (E)	↑	↑	-	-	-	-	-	↑	-
	T	-	↑	↓	↑	↓	↓	-	↑	-
	T (TSB[T])	↑	↑	↑	↑	↑	↑	↑	↑	↑
	T (E)	↑	↑	↑	↑	↑	-	-	↑	↑
T80	G	-	-	-	↓	↓	-	-	-	↓
	G (E)	-	-	-	-	↓	-	↓	-	↓
	T	-	↑	↓	-	↓	-	-	↑	-
	T (TSB[T])	-	↑	↑	-	-	↑	-	↑	-
	T (E)	↑	↑	↑	-	-	↑	↓	-	↑
X100	G	-	-	-	↓	-	↑	-	-	-
	G (E)	↓	-	↑	-	-	-	-	-	-
	T	↑	↑	↑	↑	-	-	↑	↑	-

	T (TSB[T])	↑	↑	↑	↑	↑	↑	↑	↑	↑
	T (E)	-	-	↑	↑	↓	↓	-	↑	↓
T20E	G	↓	↓	-	↓	-	-	-	↓	-
	T	↑	↑	↓	-	↓	↓	-	↑	↓
	T (TSB[T])	-	-	-	-	-	-	↑	↑	↓
T80E	G	-	-	-	↓	-	-	↑	↓	-
	T	↓	↑	↓	↑	↓	↓	↓	↑	↓
	T (TSB[T])	-	-	-	-	-	-	↑	↑	↓
X100E	G	↑	-	↑	↓	↑	↑	↑	-	-
	T	↑	↑	↑	↑	-	↑	↑	↑	↑
	T (TSB[T])	↑	↑	↑	↑	↑	↑	↑	↑	↑

Table 5-3 presented a concise summary of the expression and comparison of target genes, with color-coded indications for ease of interpretation. In comparison to TSB, *S*. Typhimurium cultivated in Tween 20 solution showed downregulation of *proV* and *fadA* after 20 hours of growth at 37°C. In Tween 80 solution, downregulation of *proV*, *fadA*, and *ompC* was observed. Conversely, in Triton X-100 solution, upregulation of *fabA* and downregulation of *proV* were detected, indicative of increased membrane fluidity. Among emulsion samples, the 60% emulsion stabilized with Tween 20 showed downregulation of *rpoE*, *rpoH*, *proV*, and *ibpA*. Meanwhile, the 60% emulsion stabilized with Tween 80 exhibited downregulation of *proV* and *ibpA*, and upregulation of *dnaK*. The 60% emulsion stabilized with Triton X-100 demonstrated downregulation of *proV*, and upregulation of *rpoE*, *otsB*, *fadA*, *fabA*, and *dnaK*. The upregulation of *dnaK* in the 60% emulsion stabilized with Tween 80 indicates the prevention of misfolded or denatured protein aggregation and the protection of newly synthesized protein folding. The upregulation of *rpoE* in the 60% emulsion stabilized with Triton X-100 suggests the alleviation of stress on the cell membrane. The upregulation of *otsB* and *fadA* in the same emulsion indicates an increased demand for metabolites and ATP to synthesize stress response proteins, thereby reducing stress on the bacterial cell. The upregulation of *fabA* signifies an increase in membrane fluidity, while the upregulation of *dnaK* provides protection for newly synthesized proteins.

Upon comparing the emulsifier solutions and their corresponding emulsions after 20 hours of growth at 37°C, the impact of oil inclusion on gene expression can be summarized. Presence of oil affected the expression of specific genes. Specifically, the 60% emulsion stabilized with Tween 20 showed downregulation of *rpoE*, *rpoH*, and *ibpA*, compared to the Tween 20 solution. The 60% emulsion stabilized with Tween 80, on the other hand, exhibited upregulation of *fadA*, *dnaK*, and *ompC*, compared to the Tween 80 solution. This suggested that the stress induced by the emulsifier, Tween 80, was mitigated by the presence of oil. This was supported by the upregulation of *fadA*, which indicated an increased demand for ATP to alleviate stress in the environment, upregulation of *dnaK*, which provided protection for newly synthesized proteins, and upregulation of *ompC*, which facilitated waste diffusion across the cell membrane. In the case of the 60% emulsion stabilized with Triton X-100, upregulation of *rpoE* and downregulation of *otsB* were observed, compared to the Triton X-100 solution. The increased expression of *rpoE* indicated activation of the stress relief mechanism by sigma factor E and provided evidence of stress reduction induced by the presence of oil in the Triton X-100 environment.

After being subjected to thermal treatment at 55°C, S. Typhimurium cultured in TSB showed a decrease in the expression of *otsB*, *proV*, *fadA*, *fabA*, *dnaK*, and *ompC*. In Tween 20 solution, the expression of *otsB*, *fadA*, and *fabA* was downregulated, while *rpoH*, *proV*, and *ibpA* were upregulated. These findings suggest that the sigma factor 32 was triggered, leading to the activation of the heat shock response. The increased expression of *proV* indicates that the environment was abundant in metabolites, and the upregulation of *ibpA* provides evidence of increased levels of heat shock protein, *IbpA*. In Tween 80 solution, downregulation of *otsB* and *fadA* was observed, along with upregulation of *rpoH* and *ibpA*, indicating that the heat shock response was again triggered by sigma factor 32. Additionally, the expression of heat shock protein, IbpA, was activated by gene expression. In Triton X-100 solution, the expression of *rpoE*, *rpoH*, *otsB*, *proV*, *dnaK*, and *ibpA* was upregulated. These findings suggest that the sigma factor was activated by *rpoH* and *rpoE*, leading to the production of IbpA. The upregulation of *otsB* and *proV* indicated that the environment was rich in metabolites, and the increased expression of *dnaK* indicated that newly synthesized proteins on the cell membrane were being protected. When subjected to thermal treatment, S. Typhimurium in Tween 20 stabilized 60% emulsion showed downregulation of *otsB*, *fadA*, *fabA*, and *ompC*, and upregulation of *rpoE*, *rpoH*, and *ibpA*. These findings suggest that the stress response mechanism was activated again, with the sigma factor being activated by *rpoE* and *rpoH*, leading to the production of heat shock protein, *IbpA*. In Tween 80 stabilized 60% emulsion, downregulation of *otsB*, *fadA*, *fabA*, *dnaK*, and *ompC* was observed, while the expression of *rpoH*, *proV*, and *ibpA* was upregulated. The upregulation of *proV* suggests that the environment contained a plentiful supply of metabolites, whereas the downregulation of *otsB* implies that the bacteria either lacked of the ability to efficiently eliminate metabolites or did not require their removal. In Triton X-100 stabilized 60% emulsion, the expression of all target genes, except for *fadA*, was upregulated, indicating that this environment was the most severe for bacteria to survive and grow. However, the regulatory mechanisms were activated by the target genes helped the bacteria to cope with the stresses. The absence of a difference in *fadA* expression suggests that ATP may need to be used to progress other stress dealing mechanisms.

Compared to S. Typhimurium cultured in TSB after thermal treatment, cultures in Tween 20 solution, Triton X-100 solution, and Triton X-100 stabilized 60% emulsion exhibited upregulation of all target genes, indicating the presence of protective regulatory mechanisms. In contrast, Tween 80 solution showed upregulation of *rpoH*, *otsB*, *fabA*, and *ibpA*, suggesting a need to remove metabolites and increased membrane fluidity to facilitate this waste removal. Upregulation of *fabA* and *otsB* suggests a role in metabolite removal, while *rpoH* and *ibpA* indicate a heat shock response. Both Tween 20 and Tween 80 stabilized 60% emulsions resulted in downregulation of *ompC* and upregulation of *dnaK* and *ibpA*. The upregulation of *dnaK* without a corresponding increase in *rpoH* expression suggests the involvement of the DnaK/DnaJ chaperone system in the regulation of *rpoH*. As a result, *ibpA* was still observed to be upregulated, but *rpoH* was not upregulated compared to TSB after thermal treatment.

The effect of oil presence on the regulation of target genes was investigated after thermal treatment. The results showed that the presence of oil in Tween 20 stabilized 60% emulsion led to downregulation of *rpoE*, *rpoH*, *otsB*, *proV*, *fadA*, *ibpA*, and *ompC* compared to Tween 20 solution, indicating greater regulatory mechanisms in the absence of oil. However, there was no significant difference in *fabA* expression, suggesting that membrane fluidity was not affected. In contrast, Tween 80 stabilized 60% emulsion showed downregulation of *rpoE*, *rpoH*, *otsB*, *fabA*, and *ompC*, and upregulation of *dnaK* compared to Tween 80 solution, indicating that heat shock response was regulated by *dnaK* in the presence of oil. Therefore, heat resistance was less in Tween 80 stabilized emulsion compared to Tween 80 solution. Similarly, Triton X-100 stabilized 60% emulsion showed downregulation of *otsB*, *proV*, and *ibpA*, and upregulation of *fadA*, *fabA*, and *ompC* when comparing with Triton X-100 solution, suggesting decreased metabolites, synthesis of heat shock protein, and increased membrane fluidity with oil. Consequently, heat resistance was lower in Triton X-100 emulsion than in Triton X-100 solution. These findings indicate that emulsifier solutions without oil, especially in Tween 80 and Triton X-100 environments, provide greater protection to bacteria, which is consistent with the results of a previous study (Ch. 2).

Based on multidimensional scaling and hierarchical cluster analysis, Figure 5-3 to 5-5 showed samples displayed similarity. Three clusters were identified: (a) all samples before thermal treatment, except for Tween 80 solution, (b) Tween 80 solution before thermal treatment, TSB, Tween 20, Tween 80 solutions, and their corresponding emulsions after thermal treatment, and (c) Triton X-100 environment after thermal treatment. In summary, only Tween 80 solution showed variance in bacterial gene expression after 20 hours incubation at 37 °C, and after thermal treatment, Triton X-100 environments exhibited a difference in bacterial gene expression compared to the other samples. Furthermore, Figure 5-6 displayed a cluster summary with additional detail on the gene expression data in this study.

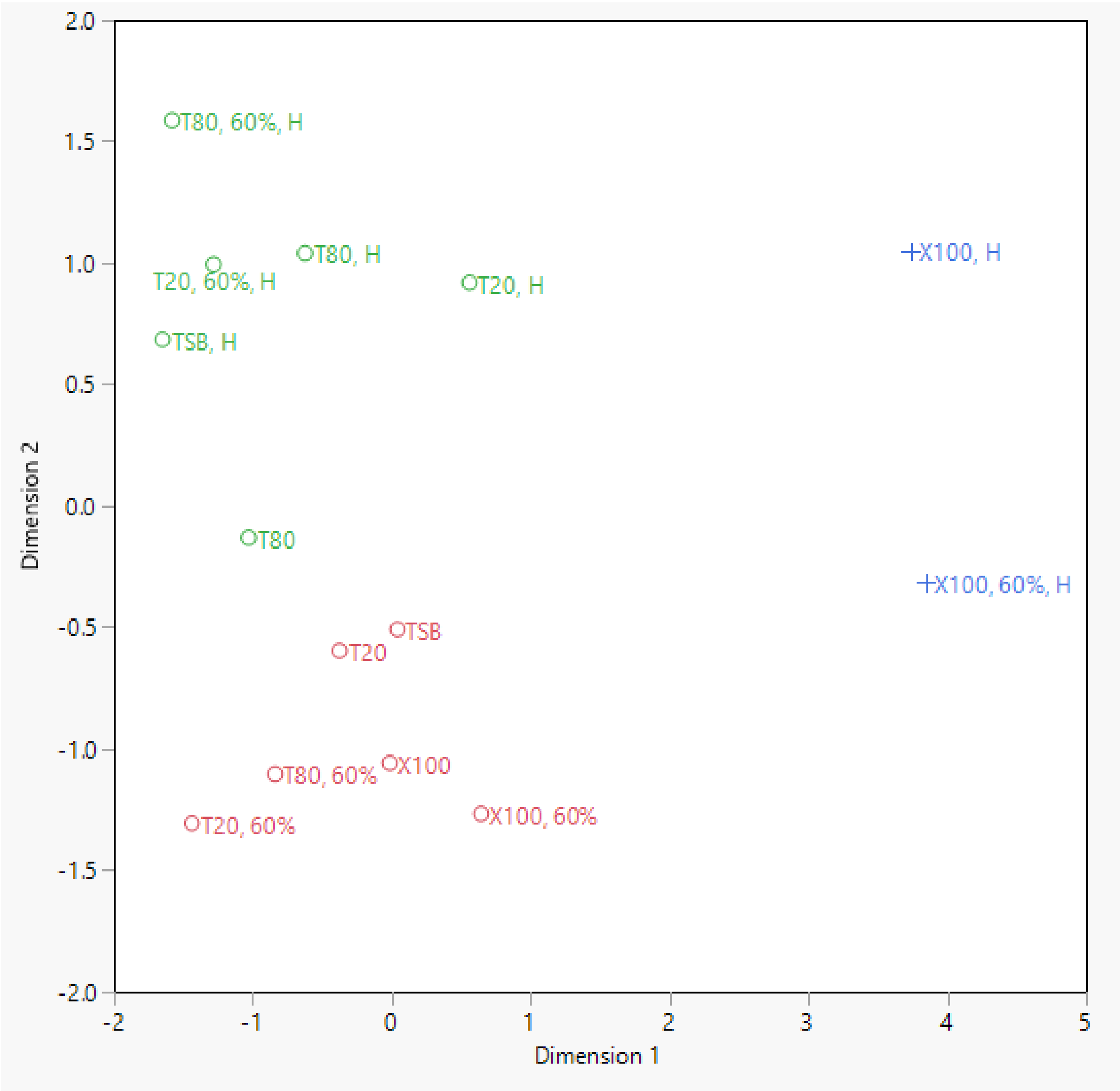

Figure 5-3. Multi-dimension scaling plot based on selected stress-related gene expression data among all samples, including TSB, Tween 20, Tween 80, Triton X-100 solutions and emulsions, before and after thermal treatment at 55 °C.

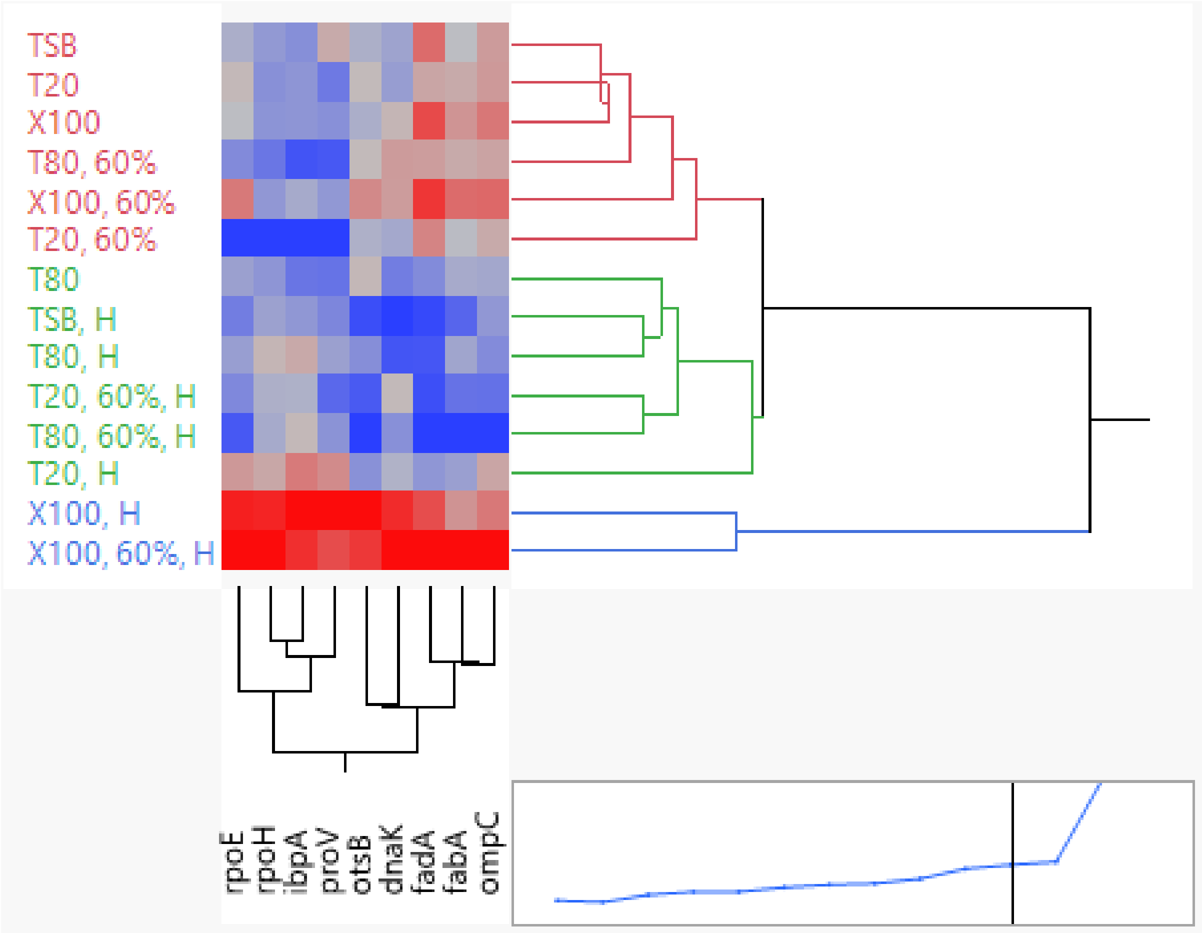

Figure 5-4. Hierarchical cluster analysis based on selected gene expression among all samples in this chapter.

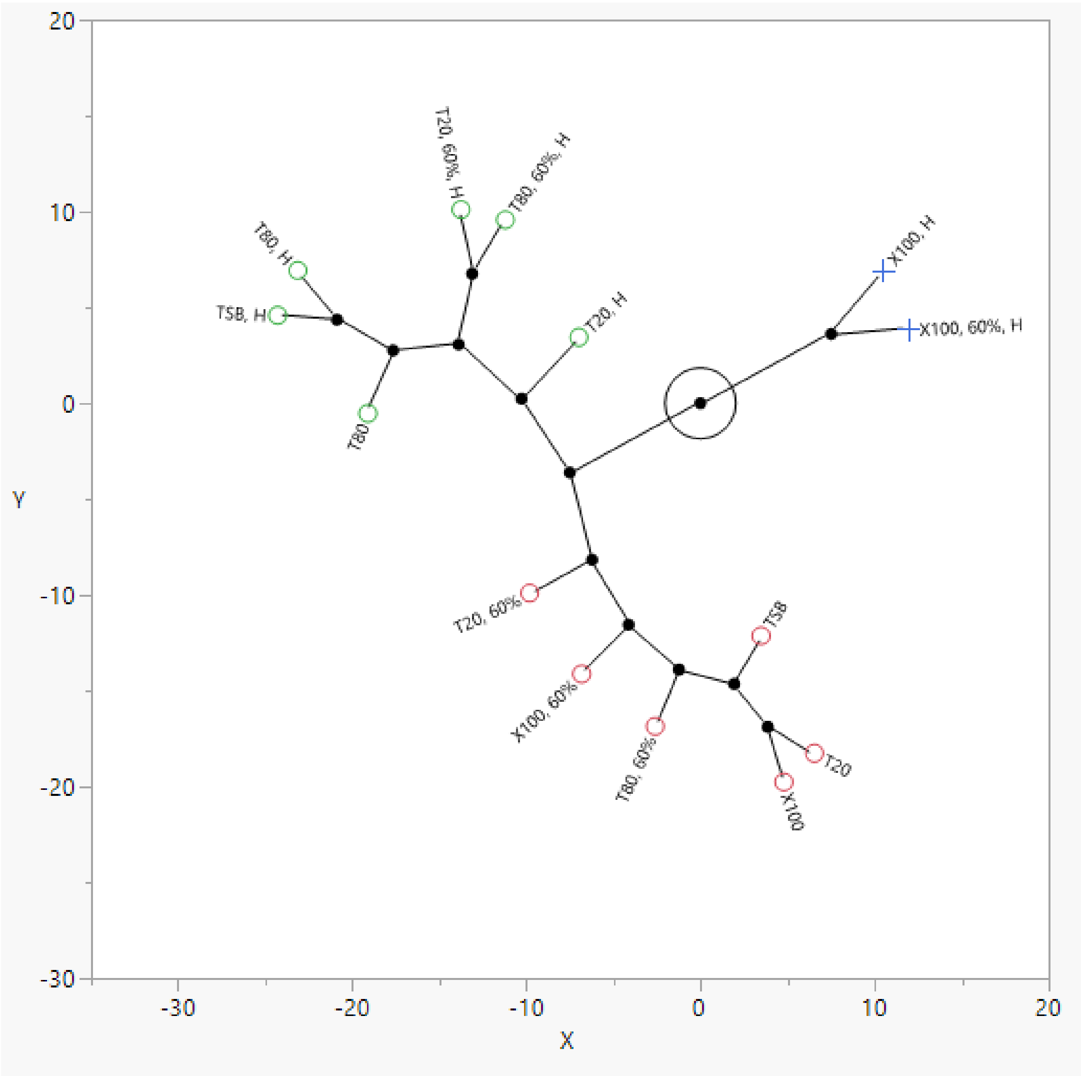

Figure 5-5. Constellation plot based on hierarchical cluster analysis data. Three different clusters were observed.

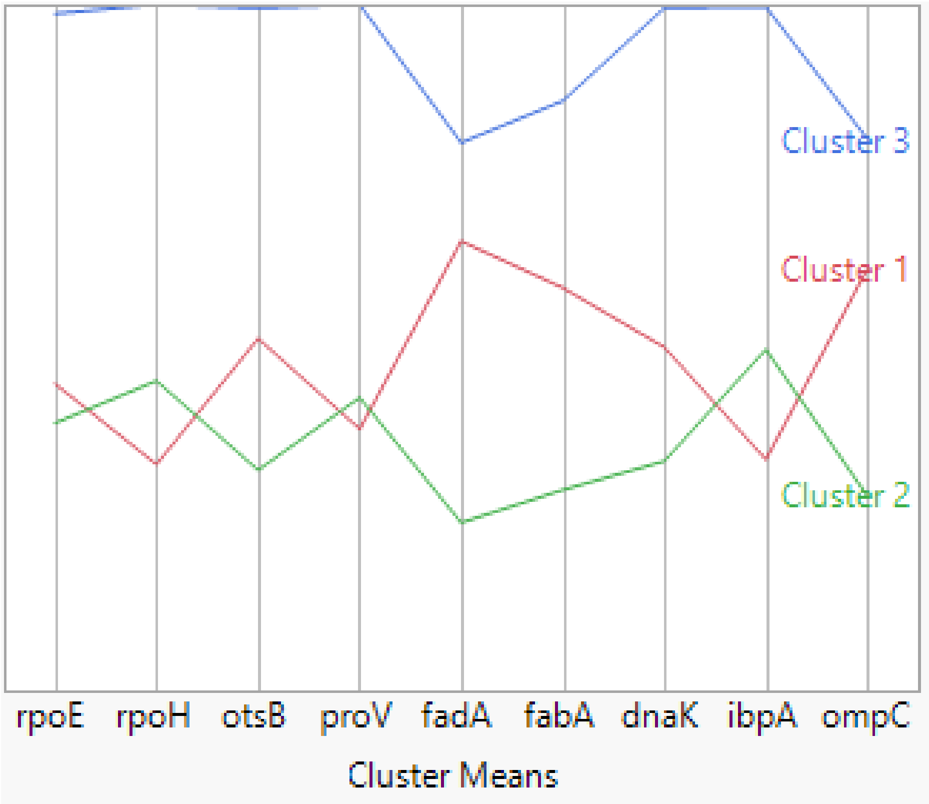

Figure 5-6. Cluster summary from the hierarchical cluster analysis data. Cluster 1: TSB, Tween 20 solution, Triton X-100 solution, Tween 20 emulsion, Tween 80 emulsion, and Triton X-100 emulsion before thermal treatment. Cluster 2: Tween 80 solution before thermal treatment, and TSB, Tween 20 solution, Tween 80 solution, Tween 20 emulsion, Tween 80 emulsion after thermal treatment. Cluster 3: Triton X-100 solution and emulsion after thermal treatment.

5.4 Conclusion

This study analyzed the effects of emulsifier type and oil inclusion on *Salmonella* growth and inactivation in specific conditions by examining the expressions of selected stress-related genes (*rpoE*, *rpoH*, *otsB*, *proV*, *fadA*, *fabA*, *dnaK*, *ibpA*, *ompC*). The findings indicated variations in the expression of each gene in *S*. Typhimurium before and after thermal treatment, depending on the type of emulsifier used compared to TSB. Notably, *ibpA* was upregulated in all emulsifier environments, suggesting that IbpA is synthesized in emulsifier environments regardless of the presence of oil. Contrary to previous research, the study found that emulsifier solutions without oil presented more regulatory mechanisms compared to those with oil, indicating that oil did not offer protective effects after thermal treatment. Additionally, increased expression of *fabA* in Triton X-100 stabilized 60% emulsion was observed, indicating poor heat resistance due to increased membrane fluidity. Overall, the results of this study provide insights into the mechanisms concerned with the effects of emulsifier type and oil inclusion on *Salmonella* growth and inactivation. By gaining a better understanding of the effects of these ingredients, the food industry can develop more effective strategies to ensure food safety. Future studies should explore the potential of using other stress-related genes as biomarkers or other emulsifier type and oil type for *Salmonella* growth and inactivation in emulsion.

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6 Chapter 6 Overall conclusions and future work

6.1 Overall conclusions

In this dissertation, the complexity of bacterial behavior in heterogeneous systems was highlighted, specifically in oil-in-water emulsions. The findings demonstrated that the physicochemical properties of emulsifiers and oil fraction played important roles in bacterial growth and inactivation in these systems, with temperature also being a crucial factor. In chapter 2, the presence of oil had no protective effect against bacterial thermal inactivation, while certain emulsifiers, such as Tween 80 and Triton X-100, provided significant protection against inactivation at 55°C. Chapter 3 further demonstrated the importance of temperature in bacterial growth and inactivation. In addition, the impact of emulsifier type and oil fraction became more pronounced at higher temperatures, highlighting the need for deeper understanding of the physicochemical properties of emulsifiers. Further details concerned with this aspect were discussed in next chapter, investigating the effect of molecular weight and surface charge of emulsifiers on bacterial growth and inactivation in emulsions. The results showed that molecular weight and surface charge of emulsifiers had significant effects on bacterial behavior in emulsions, with lower molecular weight and more positive surface charge generally leading to slower growth rates and higher resistance to thermal inactivation. The last chapter combined these findings to develop a comprehensive understanding of bacterial behavior in heterogeneous systems, highlighting the importance of considering multiple variables when studying bacterial growth and inactivation under these complex environments. In summary, this dissertation revealed the importance of considering emulsifier type, oil content, temperature, and molecular weight, and surface charge of emulsifier when studying bacterial behavior in these complex systems. By providing insights into the underlying mechanisms that govern bacterial behavior in heterogeneous environments, it showed important implications for food safety. Additionally, a deeper understanding of the effects of emulsifiers and oil content in emulsion systems can lead to the development of effective strategies for controlling bacterial growth and inactivation in emulsion-based food products. Such strategies could be crucial in ensuring the safety and quality of emulsion-based products and reducing the risk of foodborne illnesses.

6.2 Future work

Based on the findings presented in this dissertation, there are several avenues for future research on bacterial growth and inactivation in heterogeneous systems. Firstly, it would be beneficial to investigate the effect of other emulsifiers and their combinations on bacterial behavior in oil-in-water emulsions. Secondly, molecular weight and surface charge of emulsifiers have been shown to play a crucial role in bacterial growth and inactivation, but there are many other properties of emulsifiers that could influence bacterial behavior, such as their composition, HLB value, and hydrophobicity. Examining a broader range of emulsifiers would allow for a more comprehensive understanding of the molecular mechanisms underlying bacterial behavior in heterogeneous systems. Thirdly, further studies could be conducted to evaluate the impact of emulsifier type and oil fraction on the growth and inactivation behavior of Gram-positive bacteria. This would provide a more comprehensive understanding of the stress-related mechanism in a heterogeneous environment. Finally, the findings presented in this dissertation could be extended to real-world applications, such as food processing and preservation. Developing effective strategies for controlling bacterial growth and inactivation in heterogeneous systems is important for ensuring the safety and quality of food products. Therefore, future research could focus on applying the findings of this study to real-world scenarios, such as investigating the effectiveness of different emulsifiers and processing parameters for controlling bacterial growth in food products.