

ABSTRACT

Title of Dissertation: INVESTIGATION INTO THE IMPACT OF
FOOD MATRIX ON BACTERIAL SURVIVAL
DURING GASTRIC DIGESTION

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Over the years, food safety research often focused on the bacterial survival during food processing and storage, whereas physiological studies extensively explored the host-pathogen interaction in gastrointestinal tract. There is a need to understand the intermediate step on pathogen survival during gastric digestion and the potential impact from its food carrier. This study utilized water-in-oil (W-O) and oil-in-water (O-W) emulsion as well as deionized water (DI) as the fundamental model food matrices to study the potential protection by food matrix during simulated gastric digestion. Using *Salmonella enterica* subsp. *enterica* serovar Typhimurium as a sample foodborne pathogen, this study investigated the survival kinetics of bacteria using various models of simulated gastric digestion. In a simplified static pH simulated gastric digestion model, inoculated W-O and O-W emulsion matrices were challenged with simulated gastric fluid (SGF) containing HCl and pepsin with mixing using a stomacher for two hours. W-O emulsion showed significant protection of *Salmonella* survival compared to O-W emulsion and DI water. This protective effect appeared to be matrix dependent regardless of the inoculation location of

Salmonella (in dispersed phase vs. in continuous phase). Within the same emulsion type, inoculating *Salmonella* in water phase or oil phase did not show significant difference in its survivability during simulated gastric digestion.

The study was then extended to an improved gastric digestion model where the chyme pH dropped from 4.0 to 1.5 over three hours, and the chyme mixing was achieved by an orbital shaker. In addition, the new SGF was modified to be HCl solution with pepsin, amano lipase A, mucin and NaCl. Under this digestion condition, there was no significant difference in *Salmonella* survival between W-O emulsion, O-W emulsion, and DI water. Moreover, the dispersed-continuous phase ratio of emulsion composition also showed no impact on *Salmonella* survival. The simulated gastric digestion model setup was also further analyzed including the role of individual digestive enzyme, the pH impact, and the mechanical mixing approach. In the dynamic pH simulated gastric digestion model, partial activity from lipase accelerated the disruption of emulsion structure for both W-O and O-W emulsion matrices. Mild mixing using an orbital shaker also showed difference in *Salmonella* survival compared to vigorous mixing using a stomacher.

Lastly, this study expanded from using *Salmonella* as the single bacteria strain into a tailored natural microbiome community. Natural microbiome communities from Golden Delicious (GD) and Empire (EP) apples were manually enriched using bacteria culturing broth at pH 5 and pH 7, respectively. The enriched apple microbiome was then collected and analyzed using 16S rRNA sequencing to study the microbial composition. With a significant decrease in Alpha diversity, the culturable apple microbiome was successfully enriched from less than 3 log CFU/ml to more than 8 log CFU/ml. There was no known foodborne human pathogens detected in the enrichment, and the most abundant genera appeared to be potential plant growth promoting bacteria. The collected apple microbiome was then inoculated in various food matrices to study its

survivability during dynamic pH simulated gastric digestion including DI water, apple sauce (AS), chicken puree (CK), sweet potato puree (SP), and W-O emulsion. The enriched apple microbiome showed remarkably high survivability in W-O emulsion throughout the full three-hour digestion treatment. CK also exhibited moderate protective effect compared to SP at the same condition. There was no significant difference between DI and AS on bacterial survivability. In addition, the apple microbiome enriched at two pH levels (5 & 7) showed similar bacteria inactivation kinetics.

In conclusion, this study revealed the potential impact from food matrix on bacterial survival during simulated gastric digestion. W-O emulsion offered significant protection of certain bacteria strains or communities in specific simulated gastric digestion models. The parameters in gastric digestion models also affected bacterial survival. Future work should focus on exploring the potential impact from other types of food matrices, expanding the microbial survival study into other bacterial strains as well as a more complex microbiome community, and further comparing the various gastric digestion models.

INVESTIGATION INTO THE IMPACT OF FOOD MATRIX ON BACTERIAL
SURVIVAL DURING GASTRIC DIGESTION

by

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Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2024

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Foreword

Throughout my Ph.D. study, I often dreamed about the moment of writing my final dissertation, especially the Foreword, Dedication, and Acknowledgement sections. However, it became one of the most difficult pieces when I started to write it. I have been studying at the University of Maryland from 2014. During the past eleven years at UMD, I finished my undergrad and master's programs, and now I am about to finish my Ph.D. study. In my life so far, I spent one-third of my time at UMD, and it is the most memorable decade to me.

I always thought I am ordinary but fortunate enough to have these education opportunities, life experiences, and interaction with all the good people. I was fortunate when I received the offer from UMD when I was a sophomore at South China Agriculture University, so I was able to start my journey at UMD as a “2+2” student. I was fortunate when I got the intern volunteer opportunities to work at Dr. Yi Chen's lab and Dr. Rohan V. Tikekar's lab when I was in junior and senior years. I started to explore my interests in scientific research and developed determination for pursuing graduate degrees. I was fortunate when I got the chance to work with our department Student Program Director Sara Kao from junior year and was exposed and involved in various departmental affairs. I was fortunate when I got the opportunity of studying with Dr. Robert L. Buchanan for my master's study. I started to systematically develop my scientific mind of thinking and research skills. I was fortunate when I got the chance to continue working with Dr. Rohan V. Tikekar for my Ph.D. study. During Ph.D. study, I continued growing towards an independent researcher where I was able to design and perform multiple research projects independently, and I also polished my critical thinking and writing skills. I was also fortunate to have my parents supporting my study throughout the past eleven years. When I looked

back on these experiences, I truly appreciate all nice and supportive people I have met and interacted with. I always think I am an ordinary person, and I could not actually recognize my strengths and talents. In every single step, I was fortunate to interact with an encouraging and considerate person to lead me move forward. I would think the achievement I have accomplished so far is largely contributed by all gentle people I have met. I appreciate every one of them, and I also appreciate the lucky fate of bringing me to them.

Besides research experiences, I also started to recognize and learn about myself deeper during my Ph.D. study. I was raised up in a culture of working hard to meet other people's expectation and do my own things by myself. I still follow this mindset and habitual pattern, but I started to realize that things can be done in different ways. My Ph. D. study has extensively shaped my personality and I believe it will have an impact on the rest of my life. As I get older, I may increasingly recognize the meaning of this Ph.D. study experience in my entire life.

The world is vast, and we will meet again if fate allows.

Dedication

I would like to dedicate my dissertation work to my parents, Wu Gao and Houyu Su. Their tremendous love and supports help me grow into who I am today. I could not accomplish my achievements so far without their infinite understanding and supports.

I would like to dedicate the dissertation to my academic advisor, Dr. Rohan V. Tikekar. As one of the most considerate and supportive advisors I ever knew, he always provides constructive guidance and advisement on my study. He supervised me on my scientific research progress, mentored me on career and networking development, and guided me on communication and teaching. He always maintains a positive and calm attitude, keeps his curiosity to research, has a thoughtful mindset of considering other people. All of these influenced me growing towards a dedicated independent researcher.

I would like to also dedicate this work to my cat, whose name is “Paper”. I adopted him in October 2020, and he accompanied me during my Ph.D. study and brought me numerous of joys. He is always quiet, friendly, and gentle to me, and I wish him always healthy and happy.

Finally, to all my friends and colleagues both in the U.S. and in China, for their great supports for this work.

Acknowledgements

I would like to take this opportunity to sincerely thank my committee members for spending their precious time and utilizing their expertise to give me constructive guidance. A special thanks to my academic advisor and my committee chairman, Dr. Rohan V. Tikekar. I truly appreciate your time, patience as well as all the advises and encourages throughout this entire process working with me. I appreciate Dr. Robert L. Buchanan for being my master's advisor and continuing being on my Ph. D. committee even after you retired. I appreciate Dr. Gail Bornhorst at UC Davis for providing extensive guidance and knowledge on the entire digestion component throughout the study. I appreciate Dr. Ryan A. Blaustein for tremendous help on 16S rRNA sequencing and substantial data analysis. I appreciate Dr. Diana N. Obanda for the guidance and knowledge on gut microbiome and gastrointestinal digestion components. I appreciate Dr. Stephanie Lansing for the advises and comments from an outside reviewer standpoint and for allowing me to use the instruments and supplies in your lab.

I would like to also acknowledge Dr. Nitin Nitin at UC Davis and Dr. Ryan A. Blaustein for your innovative ideas and feedback on the study in Chapter 4.

At last, I would also like to thank my current and past lab mates, Kevin Tarwa, Stratton Wimsatt, Abraham Montemayor, Dr. Andrea Gilbert-Eckman, Dr. Julie Haendiges, Dr. Qiao Ding, Dr. Shawn Tsai, Dr. Qingyang Wang, Dr. Hongchao Zhang and Dr. Aprajeeta Jha for all their help and supports during this project.

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List of Abbreviations

O/W: oil-in-water

W/O: water-in-oil

DI: deionized water

TSB: tryptic soy broth

TSA: tryptic soy agar

SGF: simulated gastric fluid

BPW: buffered peptone water

PBS: phosphate buffer solution

GD: Golden Delicious apple

EP: Empire apple

AS: apple sauce

SP: sweet potato puree

CK: chicken puree

Chapter 1: Literature review and statement of problem

1.1 The linkage between foodborne diseases and potential impact from food matrices

Over the past few decades, foodborne diseases continued to be prominent public health issues. From 2010 to 2020, there were 9,052 foodborne outbreaks happened in the U.S., which caused 154,866 illnesses, 10,430 hospitalizations and 261 deaths (CDC, 2022). Among the foodborne outbreaks that occurred in the past, a large number of cases were linked with bacterial contamination, and most of them were associated with living microorganisms presented in foods and survived during digestion to cause diseases by infection and toxico-infection (Bhunia, 2008). On the other hand, foods generally are complex in terms of the chemical composition, physical properties, and various processing and handling methods. Due to the complexity, it can provide the habitat for bacterial growth and survival, and essentially act as a vehicle to transport the bacteria into human body. In addition, the pathogenic bacteria can come from various sources during production, packaging, and distribution; and the microorganisms are usually able to survive well because of the nutrients, water, and appropriate pH in the food. When the foodborne bacteria enter human body by food consumption, they would typically face a challenging and complex environment. There are multiple sections in the digestive tract including mouth, esophagus, stomach, small intestine (duodenum, jejunum and ileum), and large intestine (colon) (Álvarez-Ordóñez et al., 2011). Each of the sections is a unique environment for bacteria. In addition, the bacterial cells, especially pathogens, presented in complex food commodities can interact with the food matrices and survive through these various stressors in the digestive tract. Using emulsion as a model system as well as other common food matrices, this dissertation investigated how *Salmonella* and other natural microbiome survived in a complex and heterogeneous food matrix

during digestion and categorize how the matrix properties and digestion treatment affected their survival.

1.2 Gastrointestinal digestion and the antimicrobial stressors to pathogens

Human digestion process is considered to start from oral processing to reduce particle size, followed by gastric processing to initiate chemical and enzymatic breakdown, small intestinal processing to hydrolyze macromolecules and absorb nutrients, and lastly ending with fermentation and water removal in the colon (Boland, 2016). From the bacterial standpoint, the first set of major lethal and sub-lethal stresses they encounter during digestion is located in the stomach. From the host perspective, gastric processing has multiple functions including storage of food, shearing and mixing the food, as well as acid and enzymatic hydrolysis of some food components (Guerra et al., 2012; Somaratne et al., 2020). As for physiological structure, human stomach is categorized into three main regions: the fundus, the body, and the antrum. During gastric digestion, the secretion of gastric acids into stomach body is triggered by bolus entering the stomach and accumulating at fundus. At this point bolus accumulated at fundus is not homogeneously mixed with the digesta in bottom stomach body and antrum (Boland, 2016). When the bolus moves to stomach body and antrum, it is sheared and mixed with gastric acid at approximately pH 2 containing pepsin and lipase. It is also possible that the amylase from saliva continues to be effective on the carbohydrates for short time depending on the acid generation and digesta mixing rates (Boland, 2016). Human studies revealed the heterogenous environment in stomach in terms of pH levels (Koziolek et al., 2015). After the consumption of a standard meal, the stomach pH level first rises to 4 or 5 because of the accumulation of food in stomach and the buffering capacity of food. The pH levels would then decrease fairly consistently by 1 pH unit per hour during the digestion of food. The pH change correlates to the gastric emptying time which is determined by

the individual differences, the consumed food composition and particle size, and other factors that are not well known (Minderhoud et al., 2004; Hrair P. Simonian et al., 2005).

The digesta emptied from stomach then passes through pylorus and moves into small intestine for the next stage of digestion. Anatomically, there are three regions in small intestine: duodenum, jejunum, and ileum. Duodenum, as the first section of small intestine, is critical since the pH level of digesta usually increases from acidic to nearly neutral pH. The pancreas secretes bicarbonate salts that neutralizes the acidity of digesta and provides a proper environment to pancreaticin which includes a range of hydrolytic enzymes (Boland, 2016). Bile salts are the major constituent that are synthesized in the liver from cholesterol, stored and concentrated in the gall bladder in between the meals, and released after food intake into the duodenum (Boland, 2016; Guerra et al., 2012). The major digestive function of bile salts is emulsifying the lipids and breaking them down into small droplets for further digestion, which is essentially acting as a detergent. The digesta continuously passes along the duodenum and is mixed by peristalsis. The jejunum and ileum are the locations for digesta to be further hydrolyzed by digestive enzymes and some gut microbiota although the microbiota level is significantly lower than colon (Cecchini et al., 2013; Evaldson et al., 1982; Montoya et al., 2015; Wang et al., 2005). The small molecules produced from the digestive hydrolysis are absorbed in the small intestine with the large surface area and the structure of villi and microvilli (Helander & Fändriks, 2014). Although the intestinal environment is milder compared to stomach, there are also sub-lethal stresses to pathogenic bacteria. Based on the function and characteristics of bile salts, its primary antimicrobial mechanism is based on targeting bacterial cell membranes. Besides destroying the cell membranes, they can also cause secondary structure formation in RNA, induce DNA damage, and misfolding or denaturation of proteins (Begley et al., 2005; Gunn, 2000; Merritt & Donaldson, 2009). In

addition, high levels of bile salts presented in small intestine may also create osmotic shock to bacterial cells, which is equivalent to 0.3 M sodium chloride and other ionic species (Chowdhury et al., 1996). The low oxygen level in small intestine may not be a sub-lethal stress for a majority of foodborne pathogens since they are typically facultative aerobes and facultative anaerobes. However, the low oxygen level in the environment usually triggers other responses and switches the metabolic pathways that may increase the virulence expression (Green et al., 2009). Lastly, the commensal gut microbiome in a healthy individual tends to occupy the surface area in small intestine, alter intestinal morphology and angiogenesis, help on maturation of immune system, and essentially prevent the colonization and growth of pathogenic bacteria (Matsuki & Tanaka, 2014; Woo et al., 2019).

1.3 Simulated gastric digestion models and the current studies related to *Salmonella* survivability

Over the years, scientists have conducted digestion studies with *in vitro* models, animal models and human volunteers. Infogest, an international network of excellence on the fate of food in the gastrointestinal tract, have developed a standardized static model and asemi-dynamic model (Brodkorb et al., 2019; Minekus et al., 2014; Mulet-Cabero et al., 2020). These models were widely used in studying the food and nutrients digestibility and bioavailability.

The standardized static model was designed according to an international consensus (Brodkorb et al., 2019; Minekus et al., 2014). The protocol categorizes the standard simple method for simulating oral, gastric, and intestinal digestion. At each step of digestion, the food samples are mixed with digestive fluids at a constant ratio and the digestion treatment is proceeded at static pH levels. The general flow diagram is showed in Figure 1-1A. In this protocol, the oral phase digestion is needed for dilution consistency purposes regardless the food is liquid or solid. Oral digestion step is mixing the food with simulated salivary fluid at 1:1 (w/w) ratio. Salivary amylase

is optional and simulated mastication should be included for solid and semisolid food. The oral phase lasts for up to 2 min at 37 °C at pH 7. The bolus from oral phase is then diluted with simulated gastric fluid (SGF) on a 1:1 ratio (v/v). Pepsin should be involved, and gastric lipase is recommended in this step. The chyme is incubated at 37 °C with proper agitation at pH 3 for 2 hours. After that, the gastric chyme is then diluted with simulated intestinal fluid on a 1:1 ratio (v/v), bile salt and pancreatic enzymes are included. The simulated intestinal digestion takes 2 hours at 37 °C at pH 7 with proper mixing.

The dynamic and semi-dynamic digestion models are more appropriate to simulate the digestion kinetics compared to static models. Under an international consensus, the COST Action INFOGEST also developed a standardized semi-dynamic *in vitro* digestion model that simulates adult upper gastrointestinal tract digestion process (Mulet-Cabero et al., 2020). The flow diagram of the INFOGEST semi-dynamic model is outlined in Figure 1-1B. The major differences compared to the static models are the inclusion of critical kinetic aspects in the gastric digestion stage. In semi-dynamic model, the gastric pH is gradually acidified, fluid and enzymes are secreted over time, and gastric emptying activity is also considered. Prior to the digestion, a separate experiment is needed to determine the food buffering capacity from initial pH to 2.0. In the actual simulated gastric digestion process, HCl, SGF and digestive enzymes are gradually added to the gastric chyme throughout the treatment until the final pH reached to 2.0. Moreover, gastric emptying is included in this semi-dynamic model in which the gastric chyme is emptied into intestinal digestion phase on a constant rate throughout the digestion process. In practice, this is achieved by manually transfer an aliquot from the bottom of gastric digestion vessel at each designated emptying time point.

Figure 1-1A: Flow diagram of the complete INFOGEST 2.0 digestion method (Brodkorb et al., 2019).

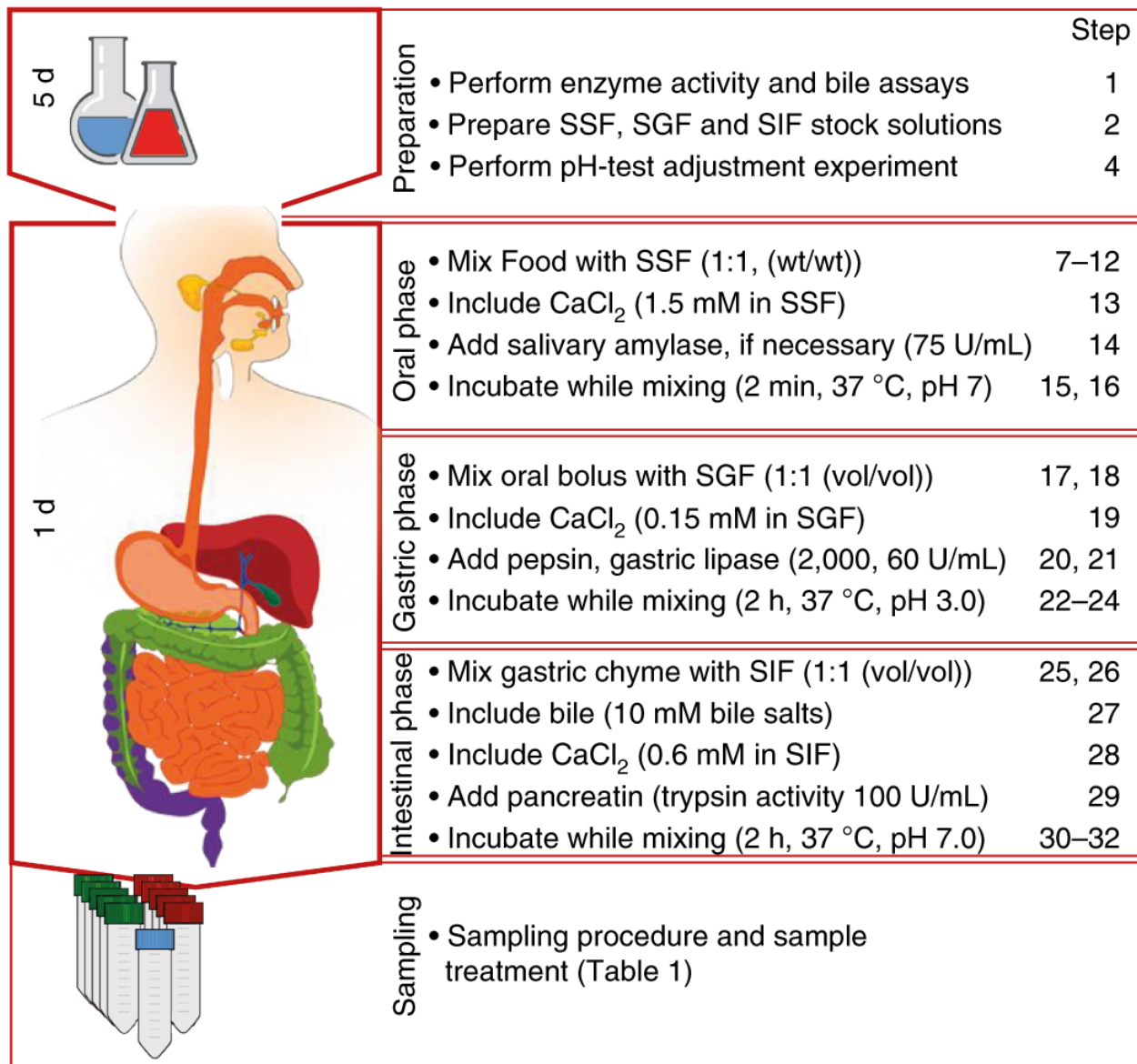
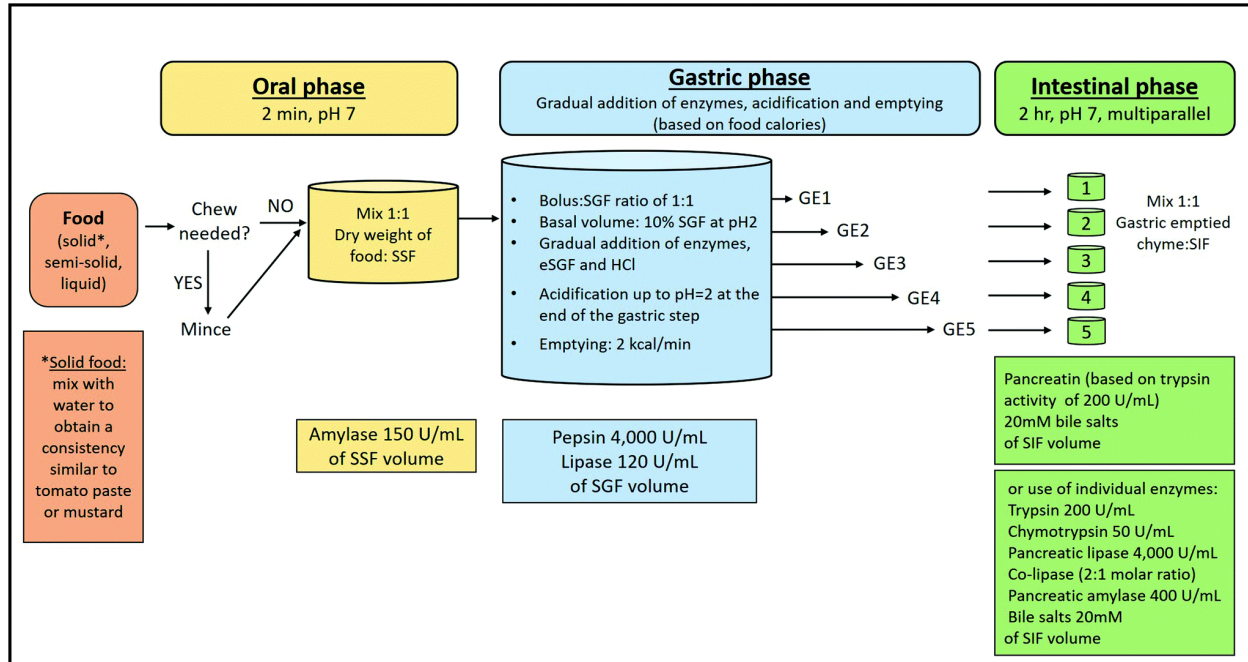


Figure 1-1B: Flow diagram of the INFOGEST semi-dynamic model (Mulet-Cabero et al., 2020).



Some studies on *Salmonella* survival during digestion were designed with some modifications of these models: Table 1-1 summarizes some significant findings of selected *in vitro* digestion studies. With modification of the *in vitro* models and the following studies, this dissertation is designed to better investigate the mechanisms of how emulsion impact *Salmonella* survival during simulated gastric digestion.

Table 1-1: Summary of the study approaches and key findings of selected studies on *Salmonella* survival during *in vitro* gastric digestion model.

Study approach	Key findings	Citation
Using a static gastric digestion model with porcine pepsin at pH 2.0 to 3.0 to	Biofilms and prolonged dry storage offer cross-protection to survival in simulated gastrointestinal conditions.	(Aviles et al., 2013)

study <i>Salmonella</i> survival with prolonged dry storage.	Increased expression of stress response genes (<i>rpoS</i> and <i>otsB</i>) was observed to be correlated with survival.	
Using a semi-dynamic model with pepsin to study pre-treated <i>Salmonella</i> Typhimurium PT4 survival in chicken breast meat.	Resistant cells of <i>S. Typhimurium</i> PT4 showed higher survival rates ($p < 0.05$) than control cells at the end of the in vitro digestion. The cells that survived the sequential stress exposure showed higher tolerance ($p < 0.05$) to acidic conditions during continuous exposure (182 min) to simulated gastrointestinal conditions.	(Melo et al., 2017)
Using a static gastric digestion model with porcine pepsin at pH 2.0 to 3.0 to study <i>Salmonella enterica</i> Serotype Tennessee survival in peanut butters with different fat contents and a_w (high fat, high a_w ; high fat, low a_w ; low fat, high a_w ; low fat, low a_w).	Exposures to increased fat content and decreased a_w both were associated with a protective effect on the survival of <i>Salmonella</i> Tennessee in the simulated gastric fluid compared with control cells.	(Aviles et al., 2013)
Using a static gastric digestion model with pepsin at acidic pH to study potential of lactic acid bacteria cell-	In simulated swine digestion, <i>Salmonella</i> counts were reduced ≥ 1.78 log CFU g ⁻¹ in the digesta containing selected cell-free supernatants.	(Evangelista et al., 2021)

free supernatants on reducing <i>Salmonella</i> population.		
Using a semi-dynamic model with pepsin to study <i>Salmonella</i> antibiotic resistance on chicken breast meat during gastrointestinal digestion.	After the <i>in vitro</i> digestion, <i>Salmonella</i> had significant higher resistance to multiple antibiotics including ciprofloxacin, ceftriaxone, ampicillin, and sulfamethoxazole-trimethoprim. In some cases, <i>Salmonella</i> switched from susceptible to resistant to antibiotics.	(De Sales et al., 2018)
Using a static gastric digestion model with pepsin and mucin at pH 2.5 to study <i>Salmonella</i> survival during gastrointestinal digestion.	It proved the gastrointestinal tract system (GIT) developed in this study allows quantification of pathogen survival and attachment/invasion into human intestinal mucosal cells.	(Wijnands et al., 2017)

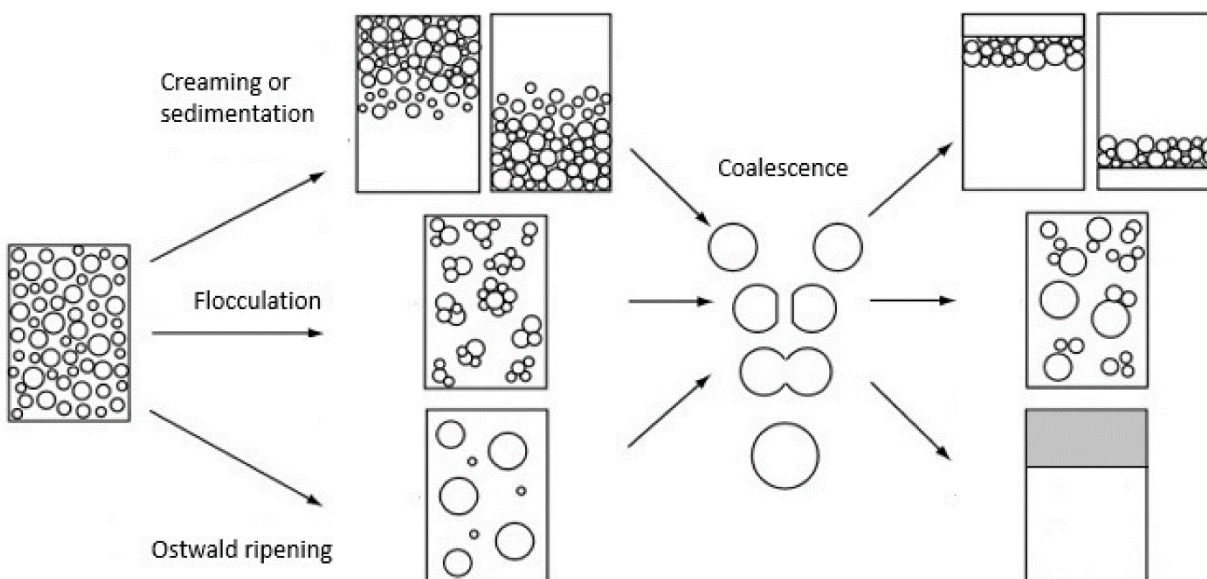
1.4 Food emulsion matrices

By definition, an emulsion consists of two immiscible liquids (usually oil and water), with one of the liquids being dispersed as small spherical droplets in the other (McClements, 2004). A system that consists of oil droplets dispersed in an aqueous phase is called an oil-in-water (O-W) emulsion, for example, milk, cream, salad dressings, beverages, soups, and sauces. On the other hand, a system that consists of water droplets dispersed in an oil phase is called a water-in-oil (W-O) emulsion, for example, margarine and butter. Multiple emulsions such as oil-in-water-in-oil (O/W/O) and water-in-oil-in-water (W/O/W) are also used in food industry, and the fine emulsion structure can block the external environmental stresses and keep the encapsulated matter intact,

thus, double emulsion and encapsulation are widely used in the food industry as delivery system for nutrients and probiotics (Mao & Miao, 2015; Marefati et al., 2021; Shima et al., 2009; Singh et al., 2018; Van Der Ark et al., 2017). This dissertation will mainly focus on conventional O-W and W-O emulsion since their structure and chemical composition are less stable during gastric digestion.

The process of emulsion formation is known as homogenization. Stabilizers are usually added to the emulsions to prevent the droplets merging with their neighbors and essentially cause phase separation (Hasenhuettl, 2008; McClements, 2004; Serdaroğlu et al., 2015). Stabilizers are categorized as emulsifiers, texture modifiers, weighting agents, and ripening inhibitor (McClements, 2004). Emulsifiers are surface-active molecules that adsorb to the surface of freshly formed droplets during homogenization, forming a protective layer that prevents the droplets from coming close enough together to aggregate. Texture modifiers are the agents that enhance emulsion stability by hindering the movement of the droplets. Weighting agents are usually added to the dispersed phase to decrease the density differential between dispersed droplets and the surrounded continuous phase. Lastly, ripening inhibitors are the nonpolar agents that added to oil phase to prevent small droplets redepositing into large particles (i.e., Ostwald ripening). The reason of adding these stabilizers is that a food emulsion may naturally lose its stability in various ways including creaming, sedimentation, flocculation, coalescence, and phase inversion (Figure 1-2). The addition of stabilizers can effectively prevent these instability tendencies.

Figure 1-2: Breakdown mechanisms of an emulsion (Holmberg et al., 2002).



Emulsion matrices are complex in various ways. The properties of a dispersed system cannot be fully derived from its chemical composition, since they also depend on physical structure (Walstra & van Vliet, 2007). Besides oil phase and water phase, the oil-water interface is also a special structure to consider since it is extremely thin but represents a large surface area and controls the physiochemical stability of emulsions to a great extent (Berton-Carabin et al., 2018). From chemical perspective, aqueous phase and oil phase may contain various water-soluble and lipid-soluble ingredients, respectively. A mixture of surface-active components (i.e., emulsifiers) can include proteins, polysaccharides, phospholipids, surfactants, alcohols, and molecular complexes that are presented in the interfacial region (Berton-Carabin et al., 2014; McClements, 2004). In a typical food emulsion, a small portion of emulsifier is sufficient to cover the surface of the dispersed droplets while a large portion of emulsifier stays in the continuous phase (Berton-Carabin et al., 2018; Berton-Carabin et al., 2014). The excessive emulsifier or thickening agent may cause physical destabilization which is categorized as droplet flocculation (Dickinson, 2009; Guzey & McClements, 2006). Moreover, the thin interface is a dynamic region where the

composition and structure may change over time (Monahan et al., 1993), and droplet coalescence may occur naturally since it is thermodynamically favorable (Berton-Carabin et al., 2018). Therefore, the dispersed phase is not completely isolated although it is physically separated into small droplets in the continuous phase.

As for the gastrointestinal digestion of emulsion, it has gained increasing interests in the past few decades. In human stomach, besides the gastric acid breakdown of food matrix by enzymatic activity and acid hydrolysis, the low pH environment may change the electrical charge on the food components in the emulsion, including proteins, polysaccharides, lipid droplets, minerals, and others. The response of an emulsion to these changes is highly dependent on the nature of the emulsifier used to stabilize this emulsion (Infantes-Garcia et al., 2021; McClements, 2018). Furthermore, the presence of proteases and lipases can digest the emulsifiers and change the composition of interfacial region and the dispersed droplets may no longer be protected against aggregation (McClements & Li, 2010a, 2010b; Singh et al., 2009). Food emulsions would be further digested in small intestine in which the remaining macronutrients including starches, proteins, and lipids would be broken down and absorbed by the epithelium cells. A significant physical change in small intestine is the formation of colloidal structures (“mixed micelles”) which consist of micelles, vesicles, and liquid crystals formed from bile salts, phospholipids, free fatty acids, and monoacylglycerols, and other minor lipophilic components, such as oil-soluble vitamins and nutraceuticals (Porter et al., 2007). Moreover, the nature of these colloidal structures depends on the nature of the food material ingested (Hong & Salentinig, 2022; Ye, 2021). Before moving to colon, most food emulsions are typically fully digested and absorbed after passage through the stomach and small intestine. However, an appreciable fraction may reach the colon for certain systems that are fabricated with indigestible food components (Du Le et al., 2020; McClements,

2004; Mudgil & Barak, 2013). Lastly, the gut microbiome presented in colon would continue the breakdown action through fermentation.

1.5 *Salmonella enterica* subsp. *enterica* serovar *Typhimurium*

Salmonella enterica subsp. *enterica* serovar Typhimurium is a facultative anaerobic Gram-negative, rod-shape bacterium that is generally 2-5 microns long by 0.5-1.5 microns wide and motile by peritrichous flagella (Andino & Hanning, 2015). *Salmonella* is a pathogenic bacterium that is often linked with foodborne outbreaks: it caused 1,491 outbreaks, 38,105 illnesses, 6,057 hospitalizations and 51 deaths from 2010 to 2020 in the United States (CDC, 2022). It can grow at temperatures from 8 to 45 °C, pH values from 4.0 to 9.5, and water activity levels from 0.94 and above (Chlebicz & Śliżewska, 2018). These characteristics enable *Salmonella* growth in various sources and environments and its survival in even more harsh conditions. Over the years, multi-drug resistance genes have been typically identified from the *Salmonella* strains isolated from foodborne outbreaks (de Melo et al., 2021; Fàbrega & Vila, 2013; Godínez-Oviedo et al., 2020; Jajere, 2019; Nadi et al., 2020). In addition, environmental stressors can trigger other stress responses, including acid tolerance, thermal resistance, resistance to low water activity environment, antimicrobial compounds, and cross-protection among these factors.

Some stress responses are essential for *Salmonella* to survive during manufacturing, storage, and human digestion. One of the well-studied stress responses is acid tolerance response, which is characterized as a strong resistance to acidic attack after growth in mildly acidic environments (Foster & Hall, 1990). This stress response in *Salmonella* cells is easily developed in food industry as organic and some inorganic acids are widely used in foods and create a mild acidic environment (Alvarez-Ordóñez et al., 2014; Lang et al., 2021; Wu et al., 2021). A previous study had found that acid-adaptation to different acids (acetic, citric, lactic, and HCl) at different

mild acidic levels (pH at 6.4, 5.4, and 4.5) effectively induced acid resistance response in extreme pH environment (pH 2.5) (Alvarez-Ordóñez et al., 2009). The acid resistance also induces cross-protection to heat, low water activity, antibiotics, and other environmental stresses (Wu et al., 2021). The acid-adapted *Salmonella* Typhimurium showed significantly higher heat resistance at mild heating temperatures (50, 54, and 58 °C) (Alvarez-Ordóñez et al., 2009). Pre-adaptation to some acids (pH 5.5), especially more lipophilic acid such as acetic acid, were also found to induce *Salmonella* cross-protection to osmotic stresses (NaCl and KCl) (Greenacre & Brocklehurst, 2006). In addition, acid-resistant *Salmonella* Typhimurium cells also displayed resistance to antibiotics including amoxicillin, ampicillin, clavulanic acid, streptomycin, tetracyclines, cephalosporins, trimethoprim and other common antibiotics (Bacon et al., 2003; D'amico et al., 2014; Hughes et al., 2010). Exposure to salt is also able to induce cross-protection to other environmental stressors. NaCl-adapted *Salmonella* showed higher heat resistance at 60 °C (Cui et al., 2019; Kang et al., 2018). Kang and Seo (2018) also found the exposure to combined acid and salt stresses ultimately decreased the *Salmonella* heat resistance. Exposure of salt may decrease *Salmonella* resistance to antibiotics (Kang & Seo, 2020; Yoon et al., 2013). When *Salmonella* exposed to low level of salt followed by higher level of salt, it significantly increased susceptibility to eight antibiotics. Heat-treated *Salmonella* Typhimurium had showed a higher resistance to salt but lower resistance to acidic condition (Kim et al., 2021). *Salmonella* stored under refrigerated temperature exhibited higher acid resistance to simulated gastric fluid (Yuk & Schneider, 2006). The final outcomes of the stress response are usually dependent on the stressor and its dosage.

The resistance to environmental stresses may also affect *Salmonella* virulence. Transcriptomic results revealed that *Salmonella* with heat stress responses also had altered virulence gene expression (Sirsat et al., 2011). The changes were generally related to an

enhancement of adhesions to intestinal Caco-2 cells, resulting in an increased virulence and higher infection probability in the host. The exposure to salt has proved to induce Sip proteins expression which was a virulence factor that releases through Type III secretion system (Mizusaki et al., 2008). On the other hand, acid tolerance response, as a complex phenomenon, was also found to be linked with expression of virulence factors (Gahan & Hill, 1999) with numerous chromosomal loci being shared between sensing-responding to acid stress and virulence. A mutant *Salmonella* Typhimurium strain that lacked the ability to induce acid tolerance response, had significantly lower virulence in mouse typhoid model (Portillo et al., 1993). Therefore, the *Salmonella* Typhimurium cells with induced resistance to environmental stresses can survive better in the environment and likely more virulent to human.

To sum, the presence of acids, salts, antimicrobial compounds, and the course of processing that is commonly applied to the food commodities can alter *Salmonella* resistance to environmental and digestive stressors, as well as enhance its virulence. This dissertation is designed to investigate these critical factors in emulsion matrices such as milk and peanut butter during gastric and intestinal digestion.

1.6 Statement of problem

The extensive literature review revealed that:

- 1) When *Salmonella* is presented in food, the complexity of food may protect *Salmonella* against digestive stresses. Several foodborne outbreaks have linked with *Salmonella* contamination in food emulsions. It is possible that the food emulsions provide a protective effect to the pathogenic bacterial survival in gastrointestinal tract that results in foodborne diseases.

- 2) It is remarkably challenging to simulate gastric digestion *in vitro*. There are multiple well-established simulated digestion models but all with specific limitations. Due to the accessibility of resources and equipment, the simulated digestion studies may not be performed at an optimal condition. The modifications in digestion treatment parameters may significantly impact the bacterial survivability during digestion.
- 3) The majority of gastric digestion studies related to microbiology have focused on host-pathogen interaction, beneficial microbe delivery, and survival of a single bacterial strain. There are major gaps in comprehensively understanding the bacteria (potentially pathogen)-food matrix-gastric digestion interaction. Moreover, there is also lack of extensive studies on complex microbiome community that people consumed via natural food.

To address these gaps, the overall objectives of this project were to 1) analyze the impact of emulsion matrices on *Salmonella* survivability during simulated gastric digestion, 2) categorize the role of emulsion composition and the parameters of digestion system on *Salmonella* survivability, and 3) expand the investigation of food matrix impact from a single bacterial strain to a complex microbiome community.

1.7 Specific objectives

- 1) Evaluation of impact of emulsion matrix on survival of *Salmonella* during simulated gastric digestion.

Hypothesis: *Salmonella* inactivation kinetics from W-O emulsion is significantly lower than O-W emulsion during simplified static pH simulated gastric digestion as the W-O emulsion structure and large content of oil may create barrier to *Salmonella* cells.

- 2) Investigation on *Salmonella* survival in emulsion matrix during simulated gastric digestion
– a case study of dynamic pH gastric digestion and the impact of digestive enzymes

Hypothesis: *Salmonella* inactivation kinetics from W-O and O-W emulsions during dynamic pH simulated gastric digestion is higher compared to that obtained from static pH simulated digestion as the digestion treatment time is extended and the pH is lower in dynamic pH simulated gastric digestion.

- 3) Investigation on survival of natural microbiome in emulsion and other food matrices during simulated gastric digestion.

Hypothesis: W-O emulsion and other high-fat food matrices provides significant protection to natural microbiome community during dynamic pH simulated gastric digestion.

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Chapter 2: Emulsion matrix affects *Salmonella* survival during simulated gastric digestion

Publication status

Gao, Z., & Tikekar, R. V. (2023). Emulsion matrixes affect *Salmonella* survival during simulated gastric digestion [10.1039/D3FO00796K]. *Food & function*, 14(15), 6864-6876.

<https://doi.org/10.1039/D3FO00796K>

Abstract

Salmonella enterica subsp. *enterica* serovar Typhimurium is an emerging prominent foodborne pathogen worldwide. While its acid resistance and its pathogenicity have been investigated over the years, there is necessity to systematically study how food matrices impact *Salmonella*'s resistance to environmental stresses and survivability in digestive tract. In this study, coarse water-in-oil (W-O) emulsion and oil-in-water (O-W) emulsion matrices were inoculated with *Salmonella* in oil and water phase, respectively. The emulsion matrices were then challenged with simulated gastric acid (pH 2 HCl solution with 3g/L pepsin) facilitated with stomacher mixing at 37 °C, and samples were taken at designated time points for bacterial counts. The survival curves suggested W-O emulsion offered significant protective effect against simulated gastric digestion, which had 1.55 ± 0.61 log (CFU/ml) reduction in 60 minutes. However, the same type of protection was not observed in O-W emulsion, and it showed 4.54 ± 0.69 log (CFU/ml) reduction in 60 minutes. As for the acid resistance of *Salmonella*, there was no significant difference between water phase and oil phase inoculation. In addition, the protective effect could be mainly attributed to the W-O emulsion structure but not simply from the high viscosity of W-O emulsion matrix. Furthermore, the results also indicated more than 16.3% of bacterial cells were present in oil phase of the W-O emulsion, which was critical to *Salmonella* survival. In conclusion, our results revealed an increased health risk of W-O emulsion against gastric digestion when it is contaminated with foodborne pathogens.

2.1 Introduction

Salmonellae are medically important pathogens for both humans and animals (Andino & Hanning, 2015), and are one of the leading causes of foodborne diseases in the United States resulting an estimated 1 million illnesses and 400 deaths annually in the United States (Chiller, 2019). Salmonellae have been linked with both water-in-oil (W-O) emulsion foods including peanut butter/paste and chocolate, and oil-in-water (O-W) emulsion foods including milk, ice cream, and mayonnaise (CDC, 2021). Among all of the subspecies and serovars of Salmonellae, *Salmonella enterica* subsp. *enterica* serovar Typhimurium is one of the top serovars that cause foodborne outbreaks. Some common food vehicles include chicken, leafy greens, and peanut butter (Andino & Hanning, 2015). There were numerous research projects conducted on *Salmonella* in the last few decades on its virulence factors and resistance to processing and environmental stresses. It has been clearly demonstrated that *Salmonella*'s acid resistance could be induced by acid shock, acid pre-adaptation, and desiccation, and it may help the organism survive during food processing and cause human salmonellosis (Altendorf et al., 2009; Bang, 2000; Bearson, 1998; Chakraborty & Kenney, 2018; Kown, 2000; Lee, 1994; Perez et al., 2010; Waterman, 1998; Wood, 1999; Yuk & Schneider, 2006). During the period, *Salmonella*'s virulence mechanisms have been extensively investigated (Cheng et al., 2019; Valdez et al., 2009).

Considering the complexity of food structure and composition, some protective effect on *Salmonella* survival might occur during processing and human digestion. In order to better understand the overall route of foodborne contamination, there is a need to connect the *Salmonella* resistance, with complexity of food, and digestive stresses. Emulsion is specifically selected for this study because of its unique structure and composition. Fine emulsion structure can block the outside stresses and keep the encapsulated matter intact, thus, double emulsion and encapsulation

are widely used in the food industry as delivery system for nutrients and probiotics (Mao & Miao, 2015; Marefati et al., 2021; Shima et al., 2009; Singh et al., 2018; Van Der Ark et al., 2017). The emulsion structure can protect the internal material against gastric acid stress and slowly release in the intestine. On the contrary, if internal phase is contaminated with pathogens, the emulsion structure can also protect them from gastric digestion. To better characterize the possible protective effect of emulsion on *Salmonella* during gastric digestion process, simple water-in-oil (W-O) and oil-in-water (O-W) coarse emulsion models were chosen to mimic the common food emulsion matrices instead of fine emulsion that is commonly used as delivery system. Besides the structural effect, the involvement of large amount of oil could offer unique protection to bacteria (Trimble et al., 2020). The presence of lecithin and/or other emulsifiers may also protect pathogens by neutralizing the bactericidal compounds (Gamble et al., 2017; Yegin et al., 2019). In addition, the possible factors that may contribute to the protective effect were also investigated, including inoculation phase, viscosity of the matrices, and the possible migration of bacterial cells between the phases in emulsion.

2.3 Materials and method

Bacteria cultures. *Salmonella enterica subsp. enterica* serovar Typhimurium GFP (ATCC 14028GFP) was used throughout the study. Working cultures were sub-cultured monthly on tryptic soy agar (TSA) (Catalog No. BD 236920, BD Difco™, San Jose, CA) plates supplemented with 100 µg/mL ampicillin (Catalog No. AAJ6097714, Thermo Scientific™, Waltham, MA) and stored at 4 °C.

Lecithin and other emulsifiers. Lecithin from Soybean (Catalog No. L0023500G, TCI America, Inc., Portland, OR) was used for preparation of water in oil (W-O) emulsion. Sunflower lecithin Sunlipon®65 kindly donated by Perimondo (New York, NY) was used for preparation of

oil in water (O-W) emulsion. Span 60 (Catalog No. S006225G, TCI America, Inc., Portland, OR), Span 80 (Catalog No. S006025G, TCI America, Inc., Portland, OR), Tween 20 (Catalog No. MP1TWEEN201, MP Biomedicals, Santa Ana, CA), and Tween 80 (Catalog No. ICN103117080, MP Biomedicals, Santa Ana, CA) were used for phase migration study.

Simulated gastric acid. Simulated gastric juice was prepared based on previously reported formulations (Aviles et al., 2013; Lian, 2003). The simulated gastric acid solution was prepared using sterile DI water and 6N HCl solution with final pH level at 2.0 ± 0.01 . Then 3g/L pepsin powder (Catalog No. AAJ6167909, Thermo Scientific™, Waltham, MA) was suspended in the acid solution to make the simulated gastric acid. The prepared solution was stored in 4 °C refrigerator and used within 48 hours.

Preparation of water phase and oil phase inoculated with *Salmonella* Typhimurium.

Using a sterile inoculation loop, a single colony was picked from working culture TSA plate and transferred to a 15-ml centrifuge tube containing 10 ml of Tryptic Soy Broth (TSB) (Catalog No. BD211825, BD Bacto™, San Jose, CA) with 100 µg/mL Ampicillin. Inoculated broth was incubated at 37 °C for 20-24 hours and then centrifuged at 7000 rcf for 10 minutes. Supernatant was decanted and the pellet was re-suspended in 10 ml of 0.1% buffered peptone water (BPW), which served as the inoculated water phase for subsequent emulsion preparation. To prepare inoculated oil phase, the inoculated broth was centrifuged at 7000 rcf for 10 minutes after the incubation at 37 °C for 20-24 hours. The cell pellet was then collected using a sterile inoculation loop and spread on a sterile petri dish in the biosafety cabinet. After the cells had completely dried, 15 ml of peanut oil (Ahold™ Peanut Oil purchased from Giant Food stores) was aseptically transferred on to the petri dish and the dried cells were scraped off from petri dish using a sterile

stainless-steel spatula. The oil was then collected into a sterile test tube for preparation of emulsion in the same day.

Preparation of W-O, O-W emulsion and water phase control. **W-O:** 27 ml of inoculated or uninoculated peanut oil was transferred to a 50-ml centrifuge tube as well as 0.6 g of soybean lecithin (2% w/v), followed by vortexing to dissolve the soybean lecithin. 3 ml of prepared water phase with or without *Salmonella* Typhimurium was then added to the tube and homogenized at 24,000 rcf for 90 seconds using a handheld homogenizer (VWR International, LLC., VDI 250, Radnor, PA). The final product had a gravy-like texture with an apparent viscosity of 20,000 cP as measured by the viscometer (Brookfield Engineering Laboratories, Inc., KU-1, Middleboro, MA). **O-W:** 3 ml of inoculated or uninoculated BPW and 24 ml of sterile DI water were transferred to a 50-ml centrifuge tube as well as 0.9 g of sunflower lecithin (3% w/v), followed by vortexing to dissolve the sunflower lecithin. Three milliliters of prepared peanut oil with or without *Salmonella* Typhimurium was added to the tube and then homogenized at 24,000 rcf for 90 seconds using the same disperser as above. The final product had a milk-like texture with a viscosity level less than 2000 cP as measured by the viscometer. **Water phase control:** 27 ml of sterile DI water and 3 ml of inoculated BPW were transferred to a 50-ml centrifuge tube, and then homogenized at 24,000 rcf for 90 seconds using the same homogenizer. The prepared emulsion (W-O and O-W) and water phase control were stored at room temperature in dark for 24 hours before experiment.

Simulated digestion. The simulated digestion system was prepared by transferring 4 ml of simulated gastric acid and 4 ml of 24-hour emulsion of culture or water phase control to five sample bags (Catalog No. 01-812-120, Nasco Whirl-Pak™, Madison, WI), respectively. The final pH of the mixture was 2.54 ± 0.1 . The remaining emulsion was utilized to measure initial bacterial

population. The five sample bags were immediately closed with the air expelled and aligned in stomacher (Seward Ltd., Stomacher® 80 Biomaste, London, United Kingdom) that was equilibrated in $35 \pm 1^\circ\text{C}$ incubator. The stomacher was then started at low level, infinite mode, and a separate timer was initiated at the same time. At each designated sampling time (5 min, 30 min, 60 min, 90 min, and 120 min), one bag was removed from the stomacher. 0.1 ml of sample was directly taken from the stomacher bag, serially diluted in 0.1% buffered peptone water (Catalog No. R452672, Thermo Scientific™, Waltham, MA) and plated on TSA plates with spiral plater (Neutec Group Inc., Nr 10003700/113, Farmingdale, NY) with E50 mode. The plates were dried at room temperature for 10 minutes, and then inverted and incubated at 37°C for 20-24 h. Plates were enumerated using an automatic plate counter (Neutec Group Inc., Nr 10006021/182, Farmingdale, NY), and the counts were converted to $\log_{10}$ values. The high viscosity of W-O emulsion created difficulty in estimating a highly accurate bacterial level at the initial level. Therefore, the W-O data was normalized with respect to bacterial population after 5 minutes of stomaching, whereas O-W data was normalized respect to initial population.

Examination of diffusion of simulated gastric acid in emulsion without stomacher. 10 ml of simulated gastric acid and 10 ml of W-O emulsion were transferred into five sterile 125-ml separation funnels, respectively. The remaining emulsion was used to measure initial bacterial population. The five separation funnels were immediately transferred into the $35 \pm 1^\circ\text{C}$ incubator and a separate timer was started. One separation funnel was removed from the incubator at each designated sampling time points (5 min, 30 min, 60 min, 90 min, and 120 min). Emulsion sample from the top was obtained using micropipette and the bottom valve was opened to obtain water phase sample. The samples were serially diluted using 0.1% buffered peptone water, plated and enumerated in the same manner that described above.

Estimation of migration of *Salmonella* between oil and water phases and the role of emulsifier. To verify whether bacterial cells remain in the inoculated phase or migrate between phases in emulsion, *Salmonella* cells were inoculated in water and oil phase separately and evaluated for their possible migration. Inoculated oil phase was prepared following the method described above without overnight storage. The oil was then centrifuged at 100 rpm for 1 minute and only the supernatant oil was used for testing. Under the biosafety cabinet, nine 100 mm petri dishes were used for this experiment. Three petri dishes were loaded with sterile DI water and inoculated peanut oil; three petri dishes were loaded with sterile DI water supplemented with 3% sunflower lecithin (w/v) and inoculated peanut oil; three petri dishes were loaded with sterile DI water and inoculated oil supplemented with 2% soybean lecithin (w/v). Samples were taken from top oil layer and bottom water layer, and then serial diluted and enumerated following same manner described above. Following the same logic, water-to-oil migration was examined using inoculated water and uninoculated oil with or without soybean and sunflower lecithin. Furthermore, these oil-to-water and water-to-oil migration experiments were extended to four more synthetic emulsifiers including Tween 20, Tween 80, Span 60, and Span 80.

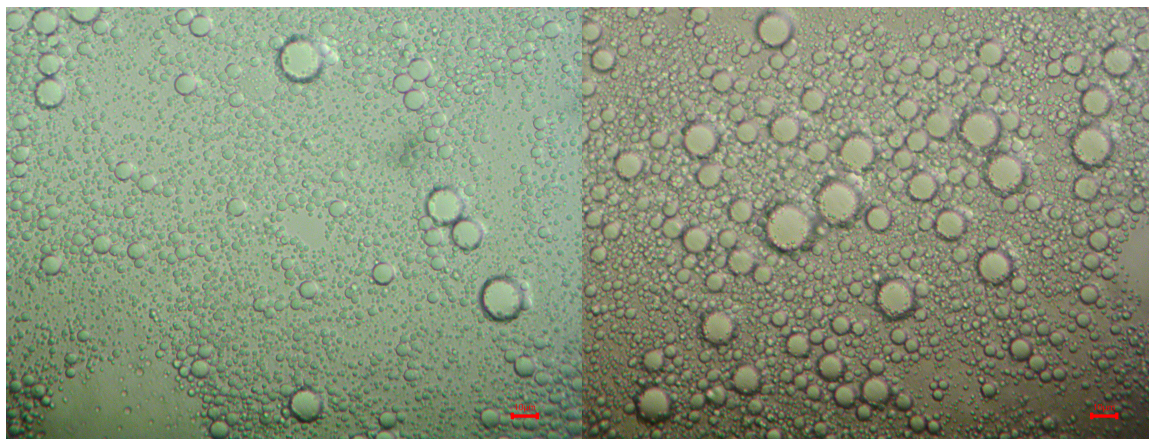
Data analysis. The collected data were imported into Excel spread sheets and the survivor curves were plotted. All data points below the lower detection limit (LDL) were assigned a value of $0.5 \times 200 = 100 = 2 \log$ and then used for normalizing. The survivor levels at certain time points were compared with a one-way analysis of variance and Tukey honest significant difference test in Excel.

2.4 Results

Before simulated digestion study, the nature of the two emulsion matrices in terms of their particle/droplet size was investigated (Figure 2-1A). Microscopic pictures of uninoculated W-O

emulsion (left) and O-W emulsion (right) demonstrated a similar particle size range of the dispersed phase in the two emulsion matrices. The particles in both emulsion matrices range from approximately 1 to 18 μm , with more smaller droplets in W-O emulsion and more larger droplets in O-W emulsion. Since they were coarse emulsions, we did not perform a more detailed particle size analysis.

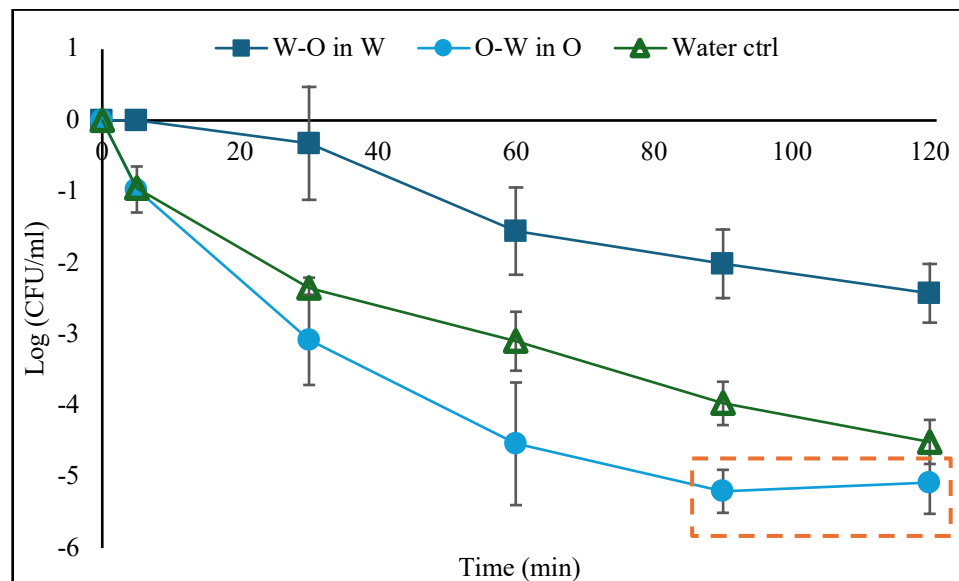
Figure 2-1A: Microscopic pictures of uninoculated W-O emulsion (left) and O-W emulsion (right). The scale bars in the figure are 10 μm .



The first hypothesis tested in this study was that the presence of emulsion structure would protect *Salmonella* in the dispersed phase and the type of emulsion can influence its survivability. Figure 2-1B shows the bacterial inactivation within two emulsions, O-W and W-O emulsions during simulated gastric digestion. When the bacteria were inoculated in the dispersed phase (water) of the W-O emulsion, minimal (<0.5 log CFU/mL) bacterial inactivation was observed in the first 30 minutes of simulated gastric digestion. However, subsequently, the bacterial inactivation accelerated and at the end of 120 minutes of digestion, 2.4 ± 0.41 log CFU/ml total reduction was achieved. In contrast to this, when the bacteria were inoculated in the dispersed phase (oil) of the O-W emulsion, simulated gastric digestion resulted in a steady decline of bacterial population and 5.08 ± 0.44 log CFU/ml total reduction was achieved after 120 minutes of digestion. This level of inactivation was significantly ($P < 0.01$) higher than that accomplished in

W-O emulsion. When the bacteria were inoculated simply in the water and added to simulated gastric digestion (water control), a log-linear inactivation was observed and over 5 log CFU/mL of total inactivation was achieved. Interestingly, at 60-minutes, this level of inactivation was significantly different from that accomplished in O-W emulsion with bacteria in dispersed phase (oil), compared to the 60-minute-reduction ($P < 0.05$). These results demonstrated obvious protection from the W-O emulsion structure when *Salmonella* was inoculated in the same dispersed phase (water) compared to water control. Unlike W-O, when *Salmonella* was inoculated in dispersed phase of O-W emulsion, the emulsion structure surprisingly had minimal effect on the inactivation level from simulated digestion.

Figure 2-1B: Survival kinetics of *Salmonella* inoculated in the dispersed phases in emulsion models during simulated gastric digestion. (Dash box: Data values below lower detection limit.)



To demonstrate that the inactivation was a result of exposure to the simulated gastric acid, two additional control experiments were conducted. In the first experiment, W-O emulsion with bacteria inoculated in the water phase was placed in a separation funnel on top of the simulated gastric fluid, without mixing (Figure 2-1C). Bacterial levels were then measured in both the

emulsion and gastric juice phases periodically. The set-up enabled us to measure the impact of mixing and potential diffusion between emulsion and gastric juice on bacterial inactivation. At 5 minutes, a small increase in bacterial population in gastric acid phase was observed likely because the gastric acid at the bottom contained a small number of emulsion droplets that had not floated to the top. Overall, there was no significant inactivation in emulsion during the 120-minute simulated digestion treatment, which indicated acid could not diffuse into the dispersed phase of W-O emulsion and inactivate the bacterial cells without agitation. Also, the bacterial cells presented in the dispersed phase of W-O emulsion did not diffuse out of the emulsion and transfer to simulated gastric acid when stomacher mixing was absent. The second control experiment was designed to isolate the possible effect of acid and emulsifier (Figure 2-1D). Simulated gastric acid was replaced with DI water for digestion treatment. There was no inactivation observed in both O-W and W-O emulsion and it demonstrated that the inactivation showed in Figure 2-1A was achieved predominantly by stomacher mixing with simulated gastric acid. W-O emulsion exhibited a slight increase in bacterial population during the treatment, likely because the emulsion was more homogeneously mixed with DI water during continued stomacher mixing and was therefore detected more easily. In addition, the role of emulsifier, sunflower lecithin, was studied individually by supplementing it into inoculated water phase solution. Soybean lecithin was not studied in this experiment because of its poor water solubility. In general, the addition of sunflower lecithin did not change the slopes of inactivation curves ($P>0.05$).

Figure 2-1C: Enumeration of *Salmonella* inoculated in the dispersed phase in W-O emulsion and carried the digestion treatment without stomacher mixing.

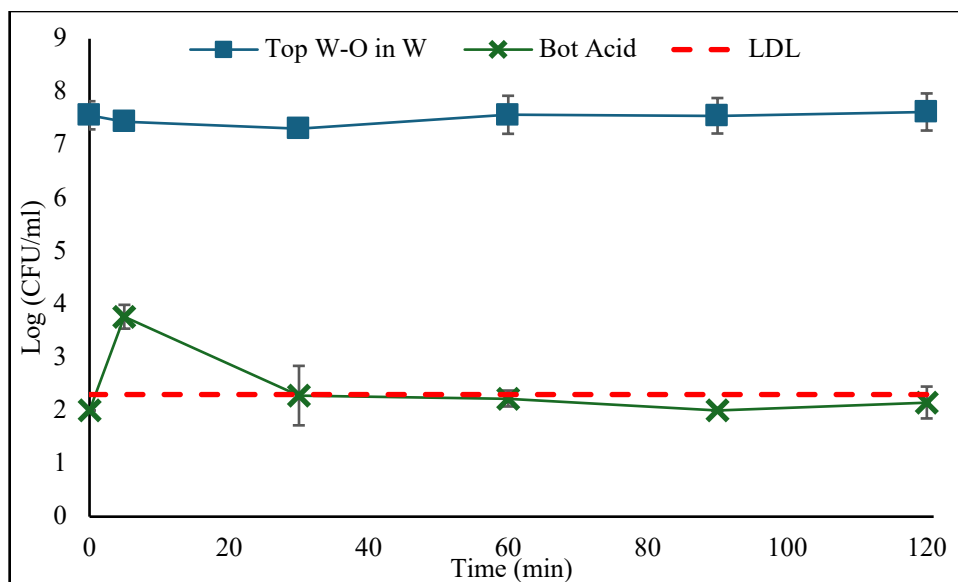
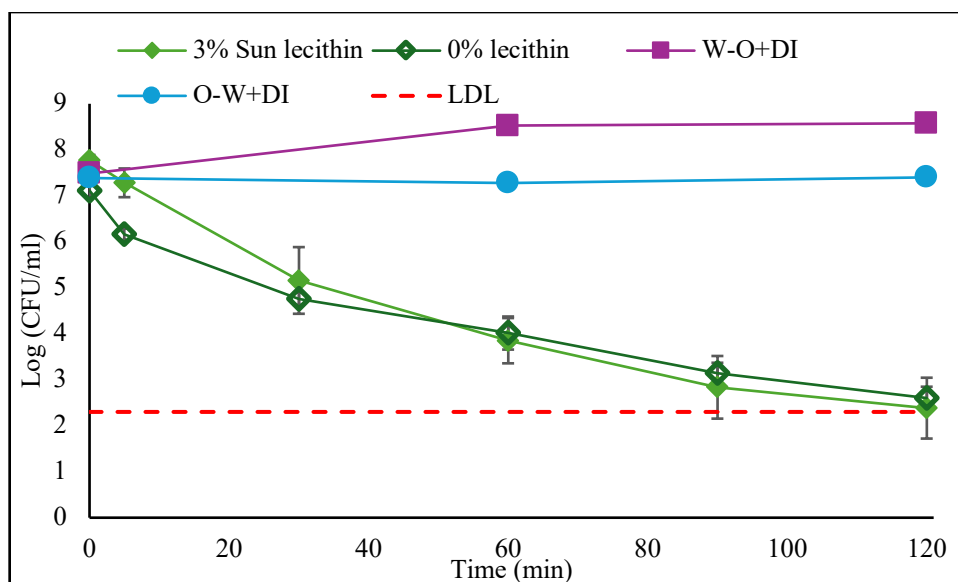


Figure 2-1D: Survival kinetics of multiple controls. 3% Sun lecithin: *Salmonella* inoculated in DI water containing 3% of sunflower lecithin and challenged with simulated gastric acid. 0% lecithin: *Salmonella* inoculated in DI water and challenged with simulated gastric acid. W-O+DI: *Salmonella* inoculated in dispersed phase of W-O emulsion and challenged with DI water in replace of simulated gastric acid. O-W+DI: *Salmonella* inoculated in dispersed phase of O-W emulsion and challenged with DI water in replace of simulated gastric acid.



The second hypothesis was that *Salmonella* inactivation during simulated gastric digestion would differ based on its location of inoculation, either in the dispersed phase or the continuous phase. Figure 2-2A illustrates the comparison between bacterial inactivation when *Salmonella* was inoculated in the water or oil phase of the W-O emulsion and figure 2-2B illustrates the phenomenon in O-W emulsion. In general, W-O emulsion showed protection to *Salmonella* in both phases against acid stress. However, when *Salmonella* was inoculated in continuous oil phase, there was a rapid drop of approximately 1.4 log (CFU/ml) that occurred in the first 5 minutes of treatment, whereas a slight increase for 0.4 log (CFU/ml) was observed when *Salmonella* was inoculated in dispersed water phase in the first 5 minutes. (Raw data not shown.) In O-W emulsion, *Salmonella* exhibited identical inactivation manner as water control when it was inoculated in continuous water phase. When the bacterial cells were inoculated in dispersed oil phase, it had similar level of reduction at 60 minutes compared to *Salmonella* in water phase ($P>0.05$) but higher reduction compared to water control ($P<0.05$). These results indicated that *Salmonella* inactivation patterns were similar within one emulsion matrix, regardless of whether it was in the dispersed phase or continuous phase.

Figure 2-2A: Survival kinetics of *Salmonella* inoculated in different phases in W-O emulsion model during simulated gastric digestion.

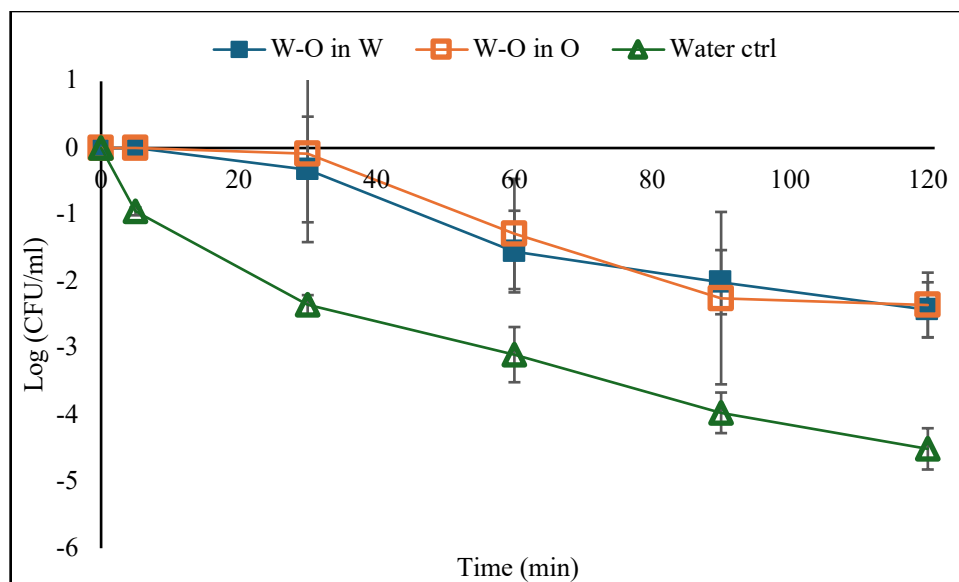
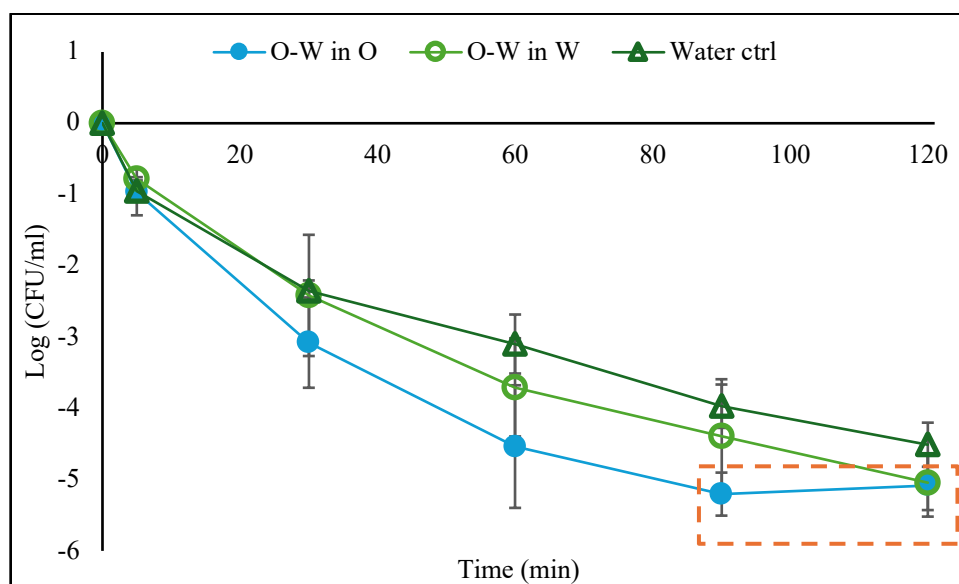


Figure 2-2B: Survival kinetics of *Salmonella* inoculated in different phases in O-W emulsion model during simulated gastric digestion. (Dash box: Data values below lower detection limit.)



With the assumption that *Salmonella* would behave differently in water versus oil, we took a closer look at its survival in the same water/oil phase but in different emulsion matrices. When *Salmonella* was inoculated in water phase (Figure 2-3A) or in oil phase (Figure 2-3B), the inactivation curves showed distinct pattern associated with the type of emulsion matrix. The W-O emulsion type showed strong protective effect over the O-W emulsion for both water phase and

oil phase bacterial inoculation. Moreover, bacteria inoculated in oil phase of the O-W emulsion was indeed more susceptible to digestive stress compared to water control at 60-min reduction ($P<0.05$).

Figure 2-3A: Survival kinetics of *Salmonella* inoculated in water phase in different emulsion models during simulated gastric digestion.

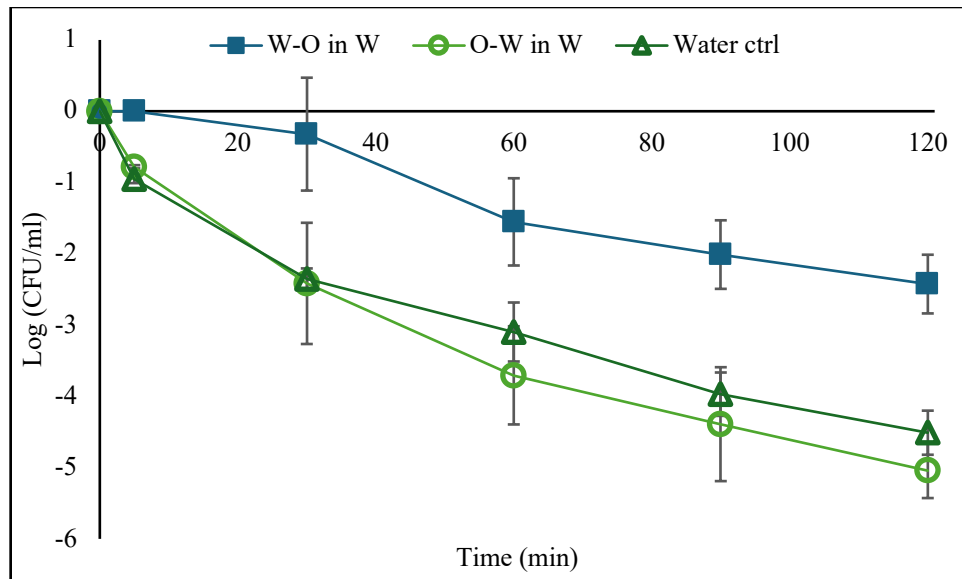
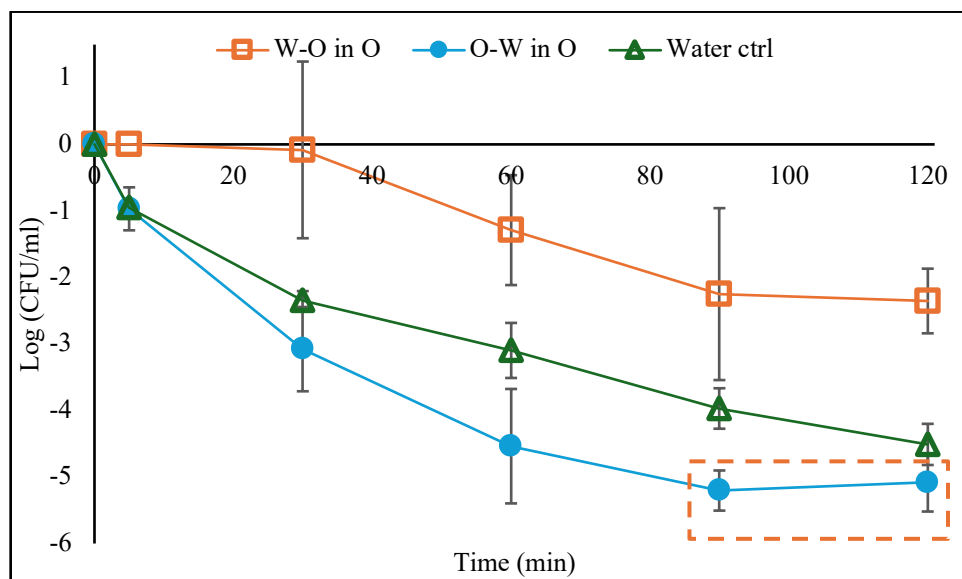


Figure 2-3B: Survival kinetics of *Salmonella* inoculated in oil phase in different emulsion models during simulated gastric digestion. (Dash box: Data values below lower detection limit.)



To further investigate the differences in *Salmonella* survival in the two emulsion matrices, four additional experiments were conducted to better categorize the simulated digestive system and emulsion characteristics. Considering the distinct texture characteristics in the two emulsion matrices, O-W emulsion was thickened with 0.67% (w/v) xanthan gum at the end of homogenization step to match its viscosity to that of W-O emulsion (Figure 2-4A). Surprisingly, the thickened O-W emulsion with bacteria inoculated in dispersed oil phase showed same inactivation pattern as of regular O-W with bacteria in dispersed phase for the first 60 minutes ($P>0.05$), and it was significantly different compared to W-O emulsion with bacteria inoculated in dispersed water phase ($P<0.05$). The next step was to study whether the overnight storage of *Salmonella* cells in different phases would modify its stress responses and resistance during simulated gastric digestion. The bacterial cells from prepared water phase and oil phase were harvested by centrifugation after overnight storage. The harvested cells were resuspended in DI water and then immediately challenged with simulated digestion treatment. In figure 2-4B, the *Salmonella* stored in pure DI for 24 hours and the ones stored in pure peanut oil for 24 hours showed similar survivability in terms of their 60-minute reduction ($P>0.05$). O-W emulsion was also taken into comparison considering its low viscosity and high similarity with water. When *Salmonella* cells were inoculated in pure oil phase for overnight storage, the survival curves against simulated stomach digestion showed similar trends with O-W emulsion, regardless of whether it was inoculated in water phase or oil phase ($P>0.05$).

Figure 2-4A: Survival kinetics comparison of *Salmonella* inoculated in modified emulsion models during simulated gastric digestion.

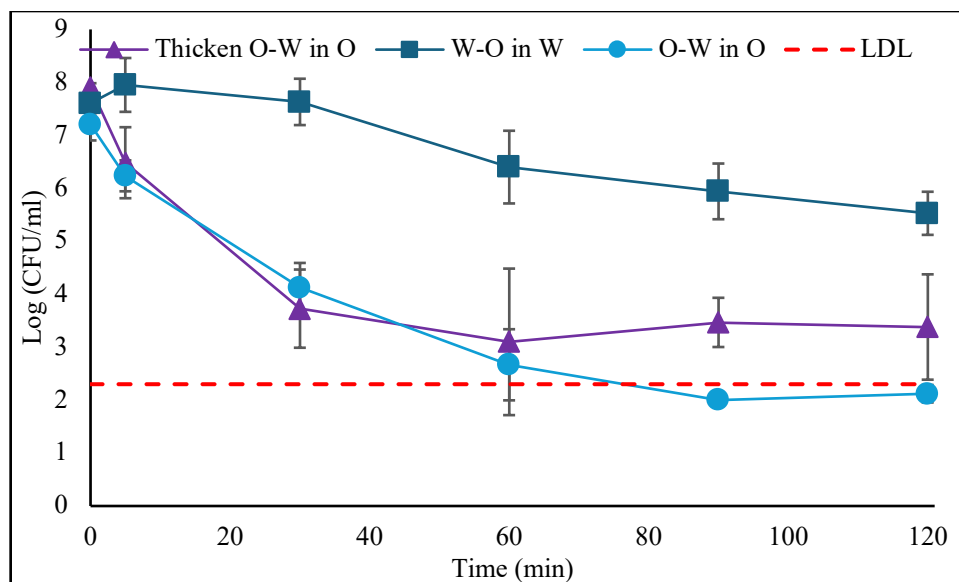
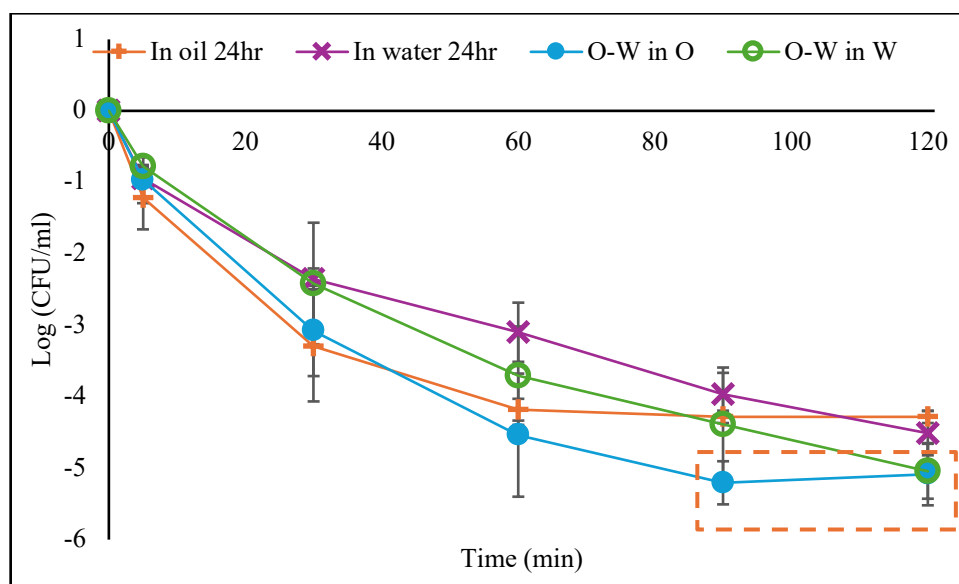


Figure 2-4B: Survival kinetics of *Salmonella* pre-adapted to oil and water for 24 hours and challenged with simulated gastric digestion compared with O-W emulsion. (Dash box: Data values below lower detection limit.)



Lastly, we categorized the possibility of *Salmonella* migrating between the oil phase and the water phase in the two types of emulsion models used in this study. With an initial *Salmonella* level in the oil phase at 4.14 ± 0.49 log (CFU/ml), majority of cells migrated into sterile water after 24 hours with or without emulsifiers and slightly grew in some cases (Table 2-1A). After 24-hour

storage at room temperature, the bacteria level of oil phase in all three groups reached to lower detection limit or below it, and the water phase increased to 3.27 ± 0.56 log (CFU/ml) in the group without lecithin, 5.36 ± 0.38 log (CFU/ml) in 3% sunflower lecithin group, and 5.64 ± 1.25 log (CFU/ml) in 2% soybean lecithin group. On the other hand, with an initial *Salmonella* level in the water phase at 4.85 ± 0.49 log (CFU/ml), majority of cells remained in water phase (Table 2-1B). After 24-hour of room temperature storage, bacterial level remained at 4.81 ± 0.07 log (CFU/ml) in the water phase in the group without lecithin, and there was no bacterial cell migrated to oil phase. In the group of water with supplementation of sunflower lecithin, bacteria slightly grew to 6.04 ± 0.05 log (CFU/ml) in water phase and only minimal bacterial cells migrated to oil phase. Interestingly, there was a water-to-oil migration observed with the presence of soybean lecithin. The bacteria level increased overnight, and 16.3% of total bacterial cells were detected in oil phase. In general, the presence of lecithin emulsifiers did not block *Salmonella* migration between two phases within the emulsion but promote bacteria migration in some cases. To better understand whether the migration is emulsifier specific, we expanded this experiment with a few synthetic emulsifiers: Span 60, Span 80, Tween 20, and Tween 80 at same levels as the lecithin emulsifiers. Similarly, with an initial *Salmonella* level in the oil phase at 6.82 ± 0.02 log (CFU/ml), majority of cells migrated into water phase within 24 hours in all emulsifiers (Table 2-2A). 2% Span 60 group exhibited an exceptionally high level of *Salmonella* in oil phase after 24 hours of storage, however, it is also worth noting that it had a substantially low solubility in oil that is expected to affect the cell migration from oil phase to water phase. When *Salmonella* was inoculated in water phase at 5.33 ± 0.02 log (CFU/ml), the bacterial cells all remained in the water phase after 24 hours of storage in all four of emulsifiers (Table 2-2B).

Table 2-1A: *Salmonella* migration between water phase and oil phase supplemented with lecithins with initial inoculation in oil after 24 hours. (Lower detection limit: 2.30 log (CFU/ml).)

	Oil phase (Log (CFU/ml))	Water phase (Log (CFU/ml))
No lecithin	Not detected	3.27 ± 0.56
2% soybean lecithin	2.40 ± 0.70	5.64 ± 1.25
3% sunflower lecithin	2.30 ± 0.52	5.36 ± 0.38

Table 2-1B: *Salmonella* migration between water phase and oil phase supplemented with lecithins with initial inoculation in water after 24 hours. (Lower detection limit: 2.30 log (CFU/ml).)

	Oil phase (Log (CFU/ml))	Water phase (Log (CFU/ml))
No lecithin	Not detected	4.81 ± 0.07
2% soybean lecithin	5.00 ± 0.23	5.71 ± 0.15
3% sunflower lecithin	2.56 ± 0.45	6.04 ± 0.05

Table 2-2A: *Salmonella* migration between water phase and oil phase supplemented with synthetic emulsifiers with initial inoculation in oil after 24 hours. (Lower detection limit: 2.30 log (CFU/ml).)

	Oil phase (Log (CFU/ml))	Water phase (Log (CFU/ml))
2% Span 60	4.55 ± 0.93	4.92 ± 1.11
2% Span 80	2.74 ± 0.35	6.26 ± 0.89
3% Tween 20	2.74 ± 0.76	6.29 ± 0.48
3% Tween 80	3.05 ± 1.32	5.98 ± 0.46

Table 2-2B: *Salmonella* migration between water phase and oil phase supplemented with synthetic emulsifiers with initial inoculation in water after 24 hours. (Lower detection limit: 2.30 log (CFU/ml).)

	Oil phase (Log (CFU/ml))	Water phase (Log (CFU/ml))
2% Span 60	Not detected	5.14 ± 0.09
2% Span 80	Not detected	4.94 ± 0.76
3% Tween 20	Not detected	5.25 ± 0.39
3% Tween 80	Not detected	4.93 ± 0.02

2.5 Discussion

The impact of emulsion on *Salmonella* survival during simulated gastric digestion was analyzed in this study. There was noticeable difference between the two different types of emulsion, regardless of whether *Salmonella* was inoculated in water/oil phase or dispersed/continuous phase. The emulsifiers used in two emulsion matrices were both lecithins, although from different origins (soybean and sunflower). There were a few studies demonstrated the antimicrobial neutralizing effect of lecithins (Gamble et al., 2017; Yegin et al., 2019), but the results in Figure 2-1D indicated there was no such effect in our emulsion models. As a result, emulsifier is less likely to be the cause of the two distinct survival patterns. The bacterial migration experiment elucidated that the presence of emulsifier does not prevent *Salmonella* cells from migrating from oil phase to water phase regardless of the original inoculation phase. Considering the exponentially higher surface area in the actual emulsion matrices, thinner lecithin layer between water and oil phase, and facilitated by homogenization, the rate of diffusion may be significantly more in the emulsion than that observed in this study. Thus, it is likely that majority

of bacterial cells were actually present in the water phase of W-O and O-W emulsion regardless of the phase in which they were initially inoculated. Also, it compensates for the difference in inoculation methods. Even though *Salmonella* cells were inoculated in oil in the form of oil-suspended dehydrated cells, after the overnight storage, they are expected to be mostly rehydrated because of the oil-to-water migration. Therefore, the difference in their survivability during digestion is less likely to be associated with the cell status at the initial inoculation stage. In addition, soybean lecithin enables bacterial cells to migrate to oil phase in W-O emulsion, and this migration is expected to be strengthened in the actual W-O emulsion. The migration to oil is critical in this study as it prevents the contact between bacterial cells and gastric juice. Soybean lecithin is obtained as a by-product in the production of oil from soybeans with various composition depending on the history of soybeans themselves and the method used for their extraction (Rydhag & Wilton, 1981). It is possible that the soybean lecithin used in this study contains certain substance that interacts with *Salmonella* cell surface to reduce the polarity of the cell and promote its migration to oil phase. Compared to sunflower lecithin and other four synthetic emulsifiers (Tween 20, Tween 80, Span 60, and Span 80), this unique phenomenon was only observed with soybean lecithin that is used for W-O emulsion, and it is expected to significantly contribute to the unique *Salmonella* survival pattern observed in W-O emulsion.

Due to the natural hydrophilicity of bacteria and other possible environment stressors, bacteria inoculated in the oil phase of either emulsion had lower counts compared to those inoculated in the water phase prior to the gastric digestion treatment, and we assume most of the cells had migrated to water phase in the matrix. When *Salmonella* was inoculated in high-water, low-fat O-W emulsion, the inoculation location did not affect the survivability as the two survival curves had similar pattern, and it is also consistent with the possibility that bacteria were primarily

localized in the water phase of the emulsion, even though they were inoculated in the oil phase. In low-water, high-fat W-O matrix, however, the raw data displayed a quick drop of bacterial count at the initial 5 minutes of oil inoculation while the water inoculation had a slight increase. After normalization using the 5-minute data, the survival curves from oil phase and water phase in W-O emulsion were identical. Considering the high viscosity and hydrophobicity of W-O matrix, it is reasonable to observe a slight increase in bacteria count at the early stage of digestion. The unexpected decrease witnessed in oil inoculation may be attributed to another reason: the inoculation step in oil created desiccation stress and resulted in an injured subpopulation that is more susceptible to gastric digestion. Thus, a quick drop of bacterial counts was observed in the first 5 minutes when *Salmonella* was inoculated in oil phase of W-O emulsion matrix.

Both W-O and O-W emulsions were formulated with 10% of dispersed phase and 90% of continuous phase, and it creates disparate local water activity inside of the emulsion. It has been well demonstrated in numerous studies that in reduced water activity environment, *Salmonella enterica* are generally more resistant and induce cross protection to thermal treatment and acid stress (Aviles et al., 2013; Kataoka et al., 2014; Kieboom et al., 2006). The presence of high level of oil in W-O emulsion offers a large area of low water activity local environment, and soybean lecithin offers the potential for bacterial cells to migrate into oil phase. Thus, the high possibility of contacting with stress environment may trigger bacterial stress response. The *ompR* gene is the response regulator of a two-component system known to regulate the osmotically controlled genes *ompC* and *ompF*, which encode two major outer membrane proteins (Bang, 2000; Chakraborty & Kenney, 2018). And Bang et al. also showed that *ompR* regulates the stationary-phase acid tolerance response of *Salmonella enterica* serovar Typhimurium (Bang, 2000). Thus, the low water activity local environment may also contribute to the high acid resistance of *Salmonella* in W-O

matrix. In addition, high fat content has been shown to be associated with heat and acid tolerance of *Salmonella enterica* (D'Aoust, 1977, 2000; Waterman, 1998; Werber et al., 2005). The data acquired from our study also demonstrates the protective effect may be linked to high fat content in the system since it is only observed in W-O emulsion that contains 90% oil. Waterman et al. proposed that the organisms may be trapped in hydrophobic lipid moieties and protected from the acid stress (Waterman, 1998). Soybean lecithin used for W-O emulsion in our study is expected to support bacterial cells staying in oil phase, which may largely contribute to its high survivability during gastric digestion. On the other hand, another set of experiments was conducted to evaluate the possible impact of peanut oil in these matrices. When *Salmonella* was stored in pure peanut oil for 24 hours and then extracted into DI water for simulated digestion, it did not show enhanced resistance to acid stress. However, bacteria's stress response tends to be quite rapid, so this inactivation curve may not reflect its complete expression and responses during its overnight storage in oil. On the other hand, the extraction process from oil to water and the preparation for digestion treatment can create abrupt osmotic downshifts. And these osmotic downshifts can cause cells to lyse if the elastic limits of the membrane and the sacculus are exceeded (Altendorf et al., 2009; Wood, 1999). This may be part of the reason that the initial *Salmonella* levels of all oil phase inoculations were slightly lower than water phase inoculations (data not shown): a subpopulation of bacteria was inactivated during the abrupt osmotic stress downshifts, and the survival curves of simulated digestion only revealed the remaining cells. After normalization of data which excluded the sensitive subpopulation, prior storage in pure peanut oil did not change the acid survivability of remaining *Salmonella* cells. Lastly, fat content also added viscosity of the matrices, however, the protective effect observed in W-O emulsion could not be reproduced in O-W emulsion by simply increasing the viscosity. It had been discussed previously that *Salmonella* cells could

migrate from oil phase to water phase in these matrices. Considering the size and polarity of hydrogen ion, it could ultimately diffuse into the internal water phase and inactivate *Salmonella* with the assistance of stomacher mixing. The presence of large amount of fat created a barrier for the internal water phase and disabled hydrogen ion's rapid access to bacteria. During the simulated gastric digestion, it drastically delayed the accumulation of hydrogen ions in the internal dispersed phase. This explains low level of inactivation in the first 30 minutes of incubation with gastric acid followed by a more reasonable inactivation pattern in the next 90 minutes: W-O emulsion achieved 2.10 ± 0.55 log CFU/ml reduction and the water phase control exhibited 2.16 ± 0.31 log CFU/ml reduction.

Majority of the studies that focused on *Salmonella* survivability in acidic environment used a milder condition at pH above 2.0 (Karthikeyan et al., 2019; Koziolk et al., 2015; Lian, 2003; Marefati et al., 2021; Perez et al., 2010; H. P. Simonian et al., 2005; Van Der Ark et al., 2017; Yuk & Schneider, 2006), while we illustrated a more severe situation. In the actual human digestion with consumption of high-caloric and high-fat meal, the mean gastric pH value measured 30 minutes after the meal was around 4.5 and then decreased fairly consistently until approximately 4 h, when the baseline level of pH 1 was reached (Koziolk et al., 2015). Simonian et al. also demonstrated in a human study that the fatty dinner could elevate proximal and mid/distal gastric pH to 4.9 ± 0.4 and 4.0 ± 0.4 , respectively, and the buffering effect of the high-volume fatty meal to pH >4 could last for 150 minutes (H. P. Simonian et al., 2005). As for the gastric mixing, both diffusion and external force contribute to the process depending on the substance(s) and the location of stomach (Bornhorst, 2017). In the distal stomach region, where there would be external movement and agitation from phasic contractions, diffusion may play a limited role in the mixing process. In contrast, in the proximal stomach region, where portions of the meal may remain for

longer periods of time experiencing only tonic contractions, contact with gastric secretions may occur primarily through diffusion processes (Bornhorst, 2017). In current model study, we created a more severe environment than human stomach, with inclusion of higher acidity and more vigorous mixing action. Compared to human gastric digestion, the simulated gastric acid in the current study did not include lipase which is important to lipid digestion. Our O-W emulsion model contained only 10% of oil and the bacterial inactivation behaves similarly to the water control. The W-O emulsion model contained extremely high amount of oil and it was digested for only 2 hours. Considering the efficacy of the enzymatic reaction, lipase may slightly enhance the overall bacterial inactivation in W-O emulsion, but the significant protective effect may still be expected. Human gastric digestion is complicated dynamics in terms of the acidity transition in different locations, gastric mixing in combination with diffusion, and enzymatic activity with interaction with food composition. On the other hand, the simplified emulsion matrices in this study were made by one-step dispersion with essential ingredients only. Compared to real food emulsions, our emulsion models are different in terms of the fat content and composition, presence of stabilizers and antimicrobial agents and processing methods. More research is needed to better understand the interaction between human gastric digestion and food emulsion matrices, and how *Salmonella* could be released and/or survived in these dynamics.

In conclusion, the coarse W-O emulsion matrix created in this study with 10% of water and 90% of oil gives significant protective effect to *Salmonella* Typhimurium against simulated digestion stresses compared to O-W emulsion and water phase control without emulsion structure. The protective effect may be primarily attributed to the barrier effect created by the high fat content in the matrix, and it was enhanced by soybean lecithin emulsifier as it enables bacterial cells to migrate from water phase to oil phase. This protective effect was observed only in W-O emulsion

but not O-W emulsion, regardless of the initial inoculation location in both emulsion matrices. Considering the distinct features of this simulated digestion system and the actual human stomach digestion process, it is expected that *Salmonella* entrapped in emulsion can have significantly higher survivability in human gastric digestion process.

The overall results from this objective revealed the *Salmonella* inactivation kinetics in W-O and O-W emulsion matrices during static pH simulated gastric digestion. It would be beneficial to further investigate this phenomenon in an improved dynamic pH simulated gastric digestion model with inclusion of lipase. With the changes in pH level throughout the digestion treatment, the enzymatic activities may change, and the acid stress to bacterial cells also increases over time. It is expected to obtain different inactivation kinetics results compared to that observed in this chapter. Thus, the project continued to the next objective using dynamic pH simulated gastric digestion model.

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Chapter 3: Investigation on *Salmonella* survival in emulsion matrix during simulated gastric digestion – a case study of dynamic pH gastric digestion and the impact of digestive enzymes

Abstract

Water-in-oil (W-O) emulsions and oil-in-water (O-W) emulsions are common food matrices in food production and there are often foodborne outbreaks associated with emulsion foods. Previous study had shown distinct inactivation rates of *Salmonella enterica* subsp. *enterica* serovar Typhimurium in different types of emulsion matrices during simulated gastric digestion in a static pH scenario. The current study continued to investigate the emulsion matrix impact on bacterial survival during gastric digestion in a dynamic pH case. The full stomach digestion scenario was simulated by 1:1 mixing the inoculated emulsions with simulated gastric fluid (SGF, pH 1.8 HCl solution containing pepsin, lipase, gastric mucin and NaCl), agitating for 3 hours and adjusting the chyme pH over time from 4.0 to 1.5. Under the current digestion treatment parameters, *Salmonella* survivor levels in all matrices experienced a slight drop (<1.32 Log CFU/ml) in the first 90 minutes at pH 4.0 and 3.0. The inactivation continued in the next 90 minutes at pH 2.0 and 1.5, achieving total inactivation range from 4.76 to 5.90 Log CFU/ml. In addition, the type of emulsion (W-O vs. O-W) and the dispersed to continuous phase ratio (1:9 vs. 4:6) did not display a difference in the *Salmonella* survival kinetics, and they were generally comparable to DI water control group. The current study also revealed the significance of lipase and pepsin enzymatic activities and their correlation with substrate pH in disrupting emulsion structure and inactivating bacteria. These findings are discussed in the context using a static

digestion pH level and proper mechanical mixing of chyme (stomacher vs. orbital shaker) to study bacterial survival.

3.1 Introduction

In the previous study (Chapter 2), we demonstrated the protective effect from W-O emulsion to *Salmonella* Typhimurium against simulated gastric digestion treatment compared to O-W emulsion and water phase control at a static pH level with vigorous mixing (Gao & Tikekar, 2023). The experimental setup assumes an empty stomach scenario where the person consumes a small amount of food on an empty stomach. The emulsion-based food quickly enters the stomach and mixes with gastric acid, but the limited amount of food consumption does not considerably increase the chyme pH thus it was digested at a low static pH level. Considering the previous study was limited to a specific case, there is a need to study this potential food matrix effect in a generalized scenario. Therefore, the current study was designated mainly to simulate a full stomach digestion case in which assuming the person consumed the emulsion food in a large quantity, such as a full serving of milkshake or ice cream or consumed the emulsion food along with a full meal. In order to have comprehensive comparison with the previous study, the current study continued using the same *Salmonella enterica* subsp. *enterica* serovar Typhimurium strain, the same composition and preparation approach for W-O and O-W emulsions but modified the simulated gastric digestion treatment.

When a person consumes a high-caloric and high-fat meal, the pH value of the stomach environment tends to reach 4.5 on average, and it continues to drop as the digestion progresses until the baseline pH reaches 1.0. The whole process lasts around 4 h (Koziolek et al., 2015). One of the most significant requirements to simulate a full stomach condition is control of control the

chyme pH level over time. In the current study, the dynamic change of pH was achieved by manually adjusting pH level at specified time points to accomplish the overall pH decline throughout the simulated digestion treatment. Specifically, the chyme pH was immediately adjusted to 4.0 once the digestion was initiated and maintained at this level for 30 minutes; it was then adjusted to 3.0 and 2.0 at 30 minutes and 90 minutes, respectively. Lastly, the digestion was ended with 30 minutes treatment at pH 1.5. During the gastric digestion treatment, stomach peristaltic waves are achieved by agitation using an orbital shaker, which is aligned with the standardized *in vitro* digestion method (Brodkorb et al., 2019; Mulet-Cabero et al., 2020).

Aside from pH changes and mechanical forces, the other critical components of simulated gastric digestion treatment are the simulated gastric fluid (SGF) and the enzymes. Pepsin is one of the most critical enzymes in gastric fluid that hydrolyzes peptide bonds and breaks down protein molecules (Schreiber et al., 2006; Zhu et al., 2006). The other important digestive enzyme is gastric lipase which acts at the lipid-water interface to emulsify and hydrolyze lipids (Acevedo-Fani & Singh, 2022; Carey et al., 1983). Due to the accessibility issues with gastric lipase from animal sources, amano lipase A from *Aspergillus niger* was used. The optimal pH of this lipase is 4 to 7 and the optimal temperature range is 40 to 50 °C, whereas the experiments were operated at 37 °C in this project (Siódmiak et al., 2022). In the previous study, lipase was not included in SGF since the chyme pH was a fixed value at 2.54 ± 0.1 (Gao & Tikekar, 2023), where amano lipase A was unlikely to be active. However, in the dynamic pH model studied in this objective, it was plausible that the lipase would remain active in early stages of digestion and could affect emulsion structure, therefore, this potential effect was also evaluated.

With the improved dynamic pH gastric digestion treatment setup, the overall objectives of this phase of study are to a) investigate the potential impact from dynamic pH of digestion

treatment on *Salmonella* survivability b) to compare the possible difference in bacterial survival kinetics between emulsion type (W-O vs. O-W) and the ratio of dispersed phase to continuous phase, and c) to comprehensively understand the role of mechanical mixing and individual enzyme of gastric digestion on emulsion disruption and bacterial inactivation.

3.2 Materials and method

Bacteria cultures. *Salmonella enterica* subsp. *enterica* serovar Typhimurium GFP (ATCC 14028GFP) was used throughout the study. Working cultures were sub-cultured monthly on tryptic soy agar (TSA) (Catalog No. BD 236920, BD Difco™, San Jose, CA) plates supplemented with 100 µg/mL ampicillin (Catalog No. AAJ6097714, Thermo Scientific™, Waltham, MA) (TSAA) and stored at 4 °C.

Lecithin emulsifiers. Lecithin from Soybean (Catalog No. L0023500G, TCI America, Inc., Portland, OR) was used for preparation of water in oil (W-O) emulsion. Sunflower lecithin Sunlipon®65 kindly donated by Perimondo (New York, NY) was used for preparation of oil in water (O-W) emulsion.

Simulated gastric fluid. Simulated gastric fluid (SGF) was prepared following the formula from Swackhamer et al (Swackhamer et al., 2019). The base of simulated gastric fluid was pH 1.8 ± 0.02 HCl solution using sterile DI water and 6N HCl. Then 6.67 mg/ml of pepsin powder (300 U/g, MP Biomedicals, CAS: 9001-75-6), 1.50 mg/ml of gastric mucin (Porcine stomach - Type II, Sigma-Aldrich, CAS: 84082-64-4), 3.33 mg/ml of Amano Lipase A (120,000 U/g, Sigma-Aldrich, CAS: 9001-62-1), and 8.78 mg/ml of NaCl (Fisher Scientific, CAS: 7647-14-5) were suspended in the acid solution to make the final simulated gastric fluid. The prepared SGF was stored in 4 °C refrigerator and used within 48 hours.

Preparation of *Salmonella* Typhimurium inoculum. Using a sterile inoculation loop, a single colony was picked from working culture TSA plate with ampicillin and transferred to a 15-ml centrifuge tube containing 10 ml of Tryptic Soy Broth (TSB) (Catalog No. BD211825, BD Bacto™, San Jose, CA) with 100 µg/mL Ampicillin (TSBA). Inoculated broth was incubated at 37 °C for 20-24 hours and then centrifuged at 7000 rcf for 10 min. Supernatant was decanted and the pellet was re-suspended in 10 ml of 0.1% buffered peptone water (BPW) (Catalog No. R452672, Thermo Scientific™, Waltham, MA). The re-suspended culture was then served as the inoculum for subsequent emulsion preparation.

Preparation of W-O, O-W emulsion matrices and water phase control. W-O emulsion: 27 ml of peanut oil (Ahold™ Peanut Oil purchased from Giant Food stores) was transferred into a 50-ml centrifuge tube as well as 0.6 g of soybean lecithin (2% w/v), followed by vortexing to fully dissolve the soybean lecithin. Two ml of sterile DI water with 1 ml of prepared *Salmonella* Typhimurium inoculum were then added to the tube and homogenized at 24,000 rcf for 90 seconds using a handheld homogenizer (VWR International, LLC., VDI 250, Radnor, PA). The final product had a gravy-like texture with an apparent viscosity of 20,000 cP as measured by the viscometer (Brookfield Engineering Laboratories, Inc., KU-1, Middleboro, MA). The end-product was W-O emulsion in 1:9 dispersed-continuous phase ratio. As for 4:6 dispersed-continuous phase ratio, the formula was modified to 18 ml of peanut oil with 0.6 g of soybean lecithin, 11 ml of sterile DI water plus 1 ml of *Salmonella* inoculum, and the rest of the procedures was kept the same. The apparent viscosity of 4:6 ratio emulsions was not measured because the water and oil content in two emulsions are comparable. **O-W emulsion:** 26 ml of sterile DI water were transferred into a 50-ml centrifuge tube as well as 0.9 g of sunflower lecithin (3% w/v), followed by vortexing to fully dissolve the sunflower lecithin. 1 ml of prepared *Salmonella*

Typhimurium inoculum and 3 ml of peanut oil were added to the tube and then homogenized at 24,000 rcf for 90 seconds using the same disperser as above. The final product had a milk-like texture with a viscosity level less than 2000 cP as measured by the viscometer. This process prepared O-W emulsion in 1:9 dispersed-continuous phase ratio. To prepare 4:6 dispersed-continuous phase ratio of O-W emulsion, the formula changed to 17 ml of sterile DI water with 0.9 g of sunflower lecithin, 12 ml of peanut oil plus 1 ml of *Salmonella* inoculum, and the rest of formula and process were kept the same. **Water phase control:** 29 ml of sterile DI water and 1 ml of prepared *Salmonella* Typhimurium inoculum were transferred to a 50-ml centrifuge tube, and then homogenized at 24,000 rcf for 90 seconds using the same homogenizer. The prepared emulsion (W-O and O-W) and water phase control were stored at room temperature in the dark for 24 hours before simulated gastric digestion.

Simulated gastric digestion. All of the bacterial survival digestion experiments were conducted in triplicated cups simultaneously. Before the gastric digestion, the prepared SGF and emulsion or water phase control were transferred to an incubator adjusted to $37 \pm 1^{\circ}\text{C}$ and preheated for 30 min. The simulated gastric digestion system was prepared by transferring 10 ml of SGF and 10 ml of 24-hour emulsion or water phase control to three of 100 ml-sample cups, respectively. The remaining emulsion was utilized to measure the initial bacterial population levels. The pH of the chyme mixture was immediately adjusted to 4.0 ± 0.1 using 1N NaOH and placed onto an orbital shaker in the incubator that was equilibrated in $37 \pm 1^{\circ}\text{C}$. The orbital shaker was operated at 100 rpm and a separate timer was initiated at the same time. At 30, 90, and 150 min, the pH of the chyme was adjusted using HCl to 3.0, 2.0 and 1.5, respectively. The overall pH adjustment added less than 1.5 ml of total liquid volume in the chyme and had negligible effect on the enzyme concentration in chyme. At 30, 90, 150, and 180 min, 0.1 ml of the chyme sample was

directly taken from the upper half of each sample cup before the pH adjustment. In some cases, 120 min samples were also taken in the same manner. All samples were serially diluted in 0.1% BPW and plated on duplicate TSA plates with spiral plater (Neutec Group Inc., Nr 10003700/113, Farmingdale, NY) with E50 mode. The plates were dried at room temperature for 10 min, and then inverted and incubated at 37 °C for 20-24 h. Plates were enumerated using an automatic plate counter (Neutec Group Inc., Nr 10006021/182, Farmingdale, NY), and the counts were converted to log₁₀ values.

Emulsion structure disruption. W-O and O-W emulsions were prepared without bacterial inoculation and supplemented with 0.01g/ml of Oil Red O (Catlog No. 14419, Cayman Chemical, Ann Arbor, MI) in the oil phase. The hypothesis was that the simulated gastric digestion gradually disrupted the emulsion structure, and the entrapped Oil Red O would be released from the emulsion and float on top of the chyme. The prepared emulsions were digested in the same manner described above using SGF with or without enzymes (pepsin, amano lipase A, and gastric mucin). At 30, 90, 120, 150, and 180 min, 0.2 ml of the O-W emulsion chyme sample was directly taken from the bottom of each sample cup and the absorbance at 492 nm was measured using a SpectraMax M5e plate reader (Molecular Devices LLC, Sunnyvale CA).

Data analysis. During gastric digestion experiment, T₀ samples were collected from the original inoculated emulsions. Considering the equal portion mixing of emulsion and SGF at the beginning of digestion, the initial bacterial population levels obtained directly from emulsion sample were divided into half and then used as T₀ bacterial levels for data analysis. The collected bacterial population data were normalized with respect to T₀ bacterial population, calculated for the means and standard deviation, and the survivor curves were plotted. All data points below the lower detection limit (LDL) were assigned a value of half of LDL: $0.5 \times 200 = 100 = 2 \log$ and

then used for normalizing. The survivor levels at specified time points were compared with a one-way analysis of variance and Tukey honest significant difference test at $p = 0.05$.

3.3 Results

Using the dynamic pH digestion treatment, we first compared the *Salmonella* survival in W-O and O-W emulsions at different dispersed-continuous phase ratios. According to the previous study, W-O emulsion offers significant protection to *Salmonella* survival during simulated gastric digestion in a simplified static pH digestion model (Gao & Tikekar, 2023). Following the same logic, bacterial survival experiments were conducted in a dynamic pH scenario, where the SGF contains varieties of enzymes and pH drops from 4.0 to 1.5 over the 3-hr digestion treatment (Figure 3-1). In general, there was no significant difference observed in bacterial inactivation rates between W-O and O-W emulsion types as well as their dispersed-continuous phase ratios, and the survivor levels were not statistically different from water phase control. The exceptions occurred in 4:6 ratio W-O emulsion, which had statistically highest survivor levels at 90 min and lowest survivor levels at 180 min ($p < 0.05$), but the actual difference was within 1 log CFU/ml.

In order to better understand the potential stressors in the SGF to *Salmonella* cells in different emulsion matrices, the following experiments were conducted by simply replacing SGF with HCL solution and adjusting the pH in the same manner (Figure 3-2). Consistent to Figure 3-1, the bacterial survivor levels decreased as the pH dropped over time. However, with the absence of gastric digestive enzymes, 1:9 W-O emulsion showed strong protection at the final stage of digestion, where the survivor levels at 180 min were significantly higher than the rest four groups ($p < 0.05$). *Salmonella* cells digested in DI water suspension exhibited the lowest surviving levels at the end of digestion compared to the four emulsion matrices ($p < 0.05$). Regardless the presence or absence of digestive enzymes, the total bacterial inactivation throughout the 3-hr simulated

gastric digestion fell in the range of 4.61 to 5.23 log CFU/ml for both W-O and O-W emulsion types and both 4:6 and 1:9 ratios, except the strong protective effect observed in 1:9 W-O emulsion without digestive enzymes.

Figure 3-1. Survival kinetics of *Salmonella* inoculated in W-O and O-W emulsion matrices at different dispersed-continuous phase ratios during dynamic pH simulated gastric digestion with SGF. Each data point represents mean $\pm$ standard deviation (SD). (Dash box: data values below lower detection limit)

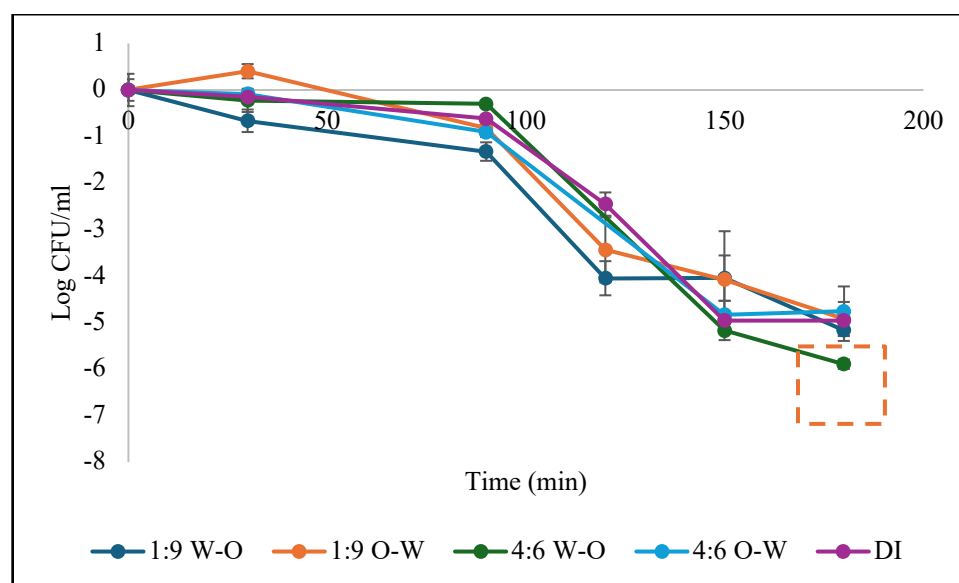
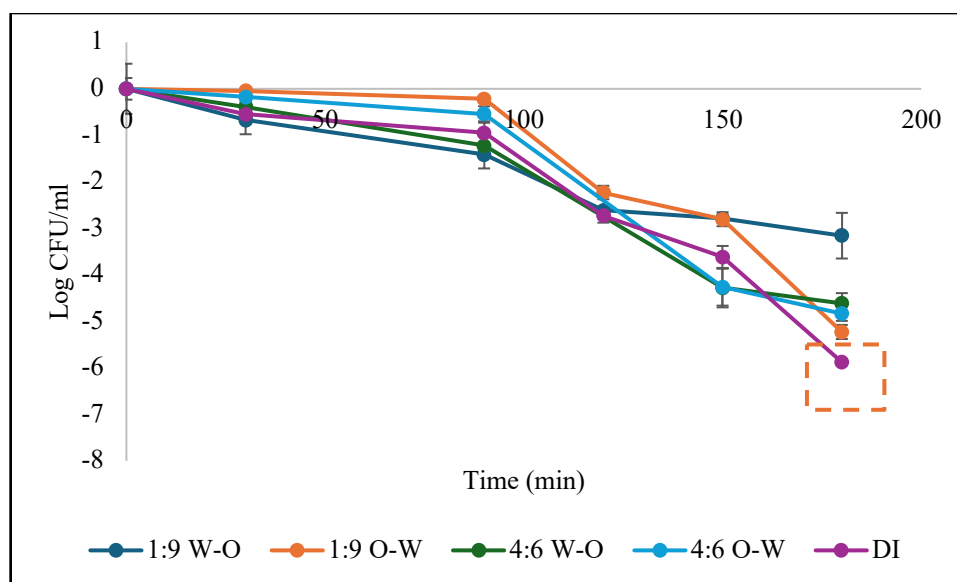


Figure 3-2. Survival kinetics of *Salmonella* inoculated in W-O and O-W emulsion matrices at different dispersed-continuous phase ratios during dynamic pH simulated gastric digestion with pure HCl without addition of any digestive enzyme. Each data point represents mean $\pm$ standard deviation (SD).



Next, we investigated the role of individual digestive enzyme on bacterial inactivation in two emulsion matrices during gastric digestion. Inoculated W-O emulsion was digested using SGF lacking a specific component of the SGF such as lipase (SGF-lipase), pepsin (SGF-pepsin), or mucin (SGF-mucin) (Figure 3-3A). In the first 90 min, the chyme pH was maintained at 4.0 for 30 min and 3.0 for the next 60 min, there was no different in the T_{30} and T_{60} inactivation between the absence or presence of the digestive enzymes ($p > 0.05$). When the pH dropped to 2.0 at 90 min, we started to observe the impact from digestive enzymes. According to the total inactivation level following the entire digestion treatment, removing mucin did not change the *Salmonella* survival kinetics in W-O emulsion compared to full profile of SGF ($p > 0.05$). On the other hand, when lipase or pepsin was removed from the SGF, there was a significantly higher inactivation of *Salmonella* after the conclusion of the digestion process compared to that in full profile SGF ($p < 0.05$) indicating that removal of these enzymes had a protective effect on *Salmonella*. Similarly, inoculated O-W emulsion was also digested with modified formula of SGF to study the impact of digestive enzymes on *Salmonella* survival (Figure 3-3B). In the first 120 min of digestion, the survivor levels of pH 1.8 HCl group were the highest compared to O-W digested with complete or

modified recipe of SGF ($p<0.05$). Similar with W-O emulsion, when mucin was removed from SGF, the total inactivation at each sampling point was comparable with regular SGF group ($p>0.05$). However, removing lipase induced higher inactivation at T_{120} compared to regular SGF ($p<0.05$). Removing pepsin resulted in higher inactivation at both T_{90} and T_{120} ($p<0.05$). Lastly, the same SGF comparison study was conducted on *Salmonella* inoculated in DI water (Figure 3-3C). When *Salmonella* was inoculated in DI water, removing lipase, pepsin or mucin particularly enhanced the inactivation up to more than 5-log reduction. At 120 min, full profile of SGF and HCl solution only induced 2.45 and 2.73 log CFU/ml of inactivation at 120 min, respectively.

Figure 3-3A. Survival kinetics of *Salmonella* inoculated in W-O emulsion during dynamic pH simulated gastric digestion with complete SGF or modified recipes. Each data point represents mean $\pm$ standard deviation (SD).

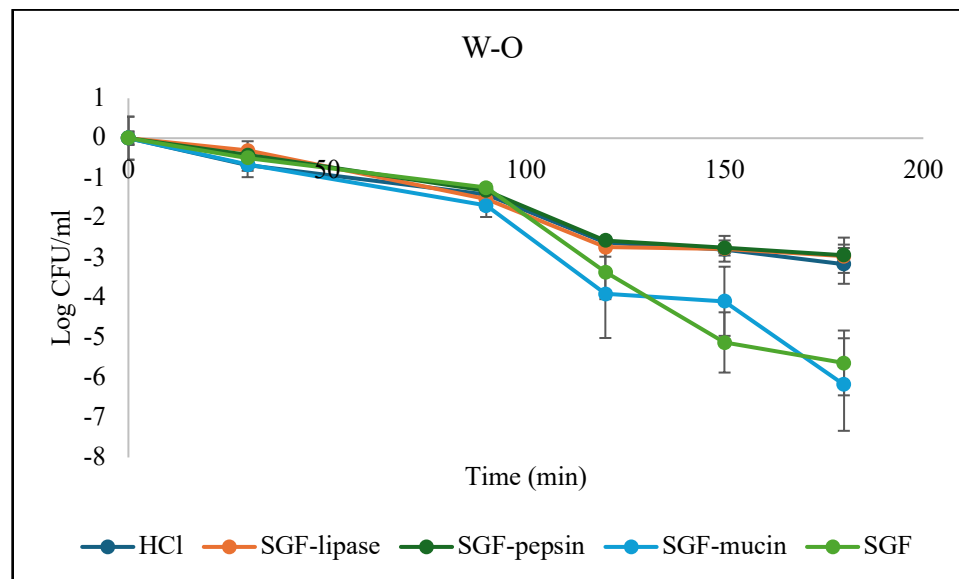


Figure 3-3B. Survival kinetics of *Salmonella* inoculated in O-W emulsion during dynamic pH simulated gastric digestion with complete SGF or modified recipes. Each data point represents mean $\pm$ standard deviation (SD).

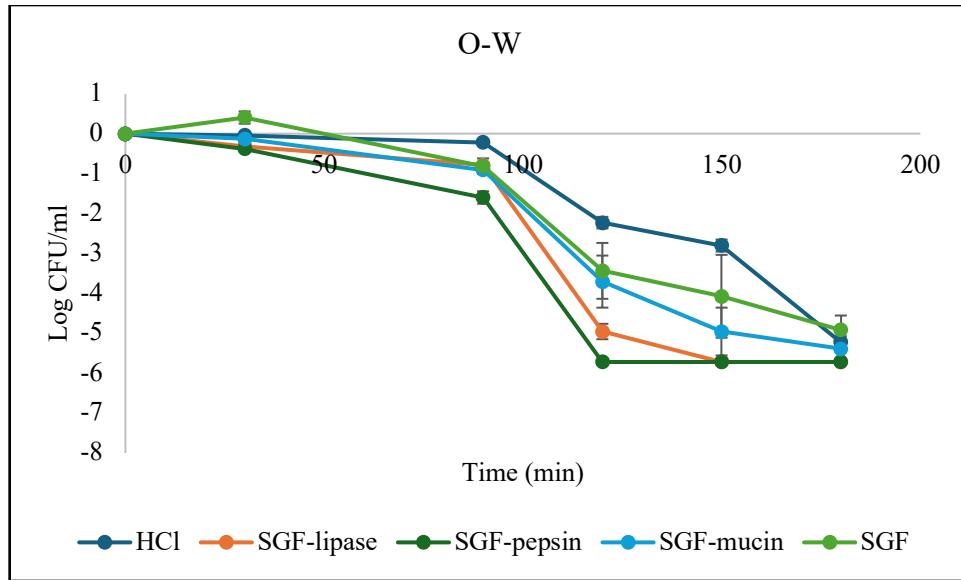


Figure 3-3C. Survival kinetics of *Salmonella* inoculated in DI water during dynamic pH simulated gastric digestion with complete SGF or modified recipes. Each data point represents mean $\pm$ standard deviation (SD).

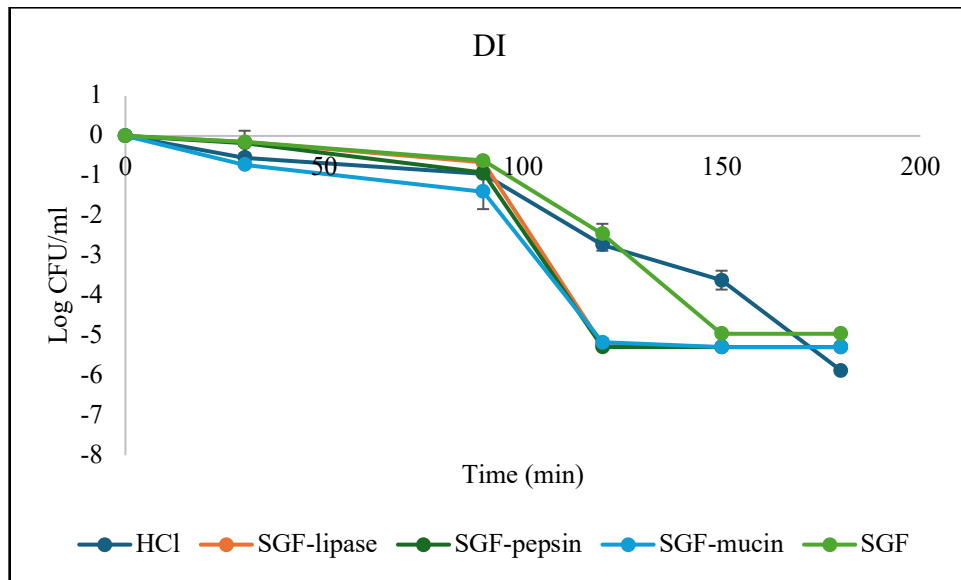
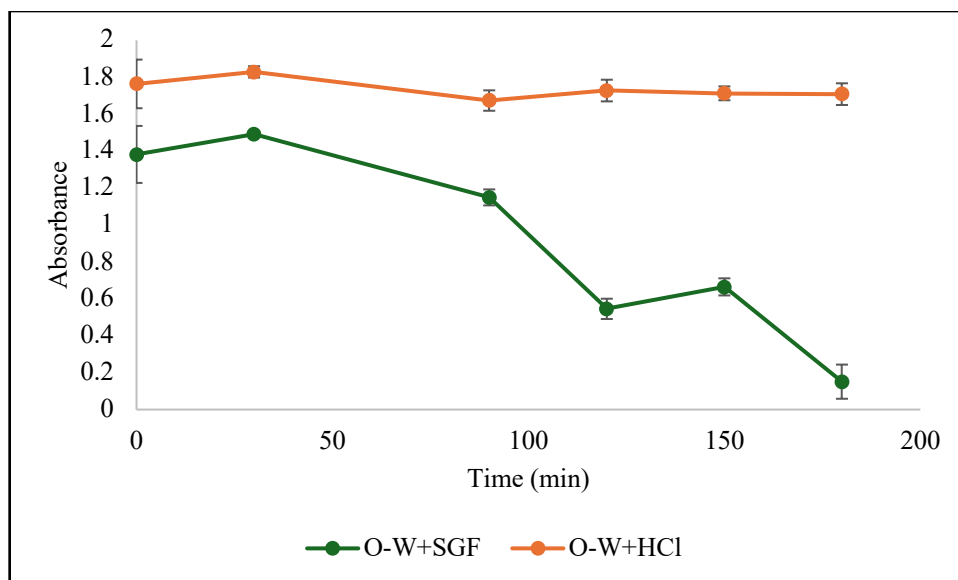


Table 3-1. Summary of the impact of individual digestive enzyme on *Salmonella* survival during simulated gastric digestion.

	Lipase	Pepsin	Mucin
In W-O	Enhance inactivation	Enhance inactivation	No change
In O-W	Reduce inactivation	Reduce inactivation	No change
In DI	Reduce inactivation	Reduce inactivation	Reduce inactivation

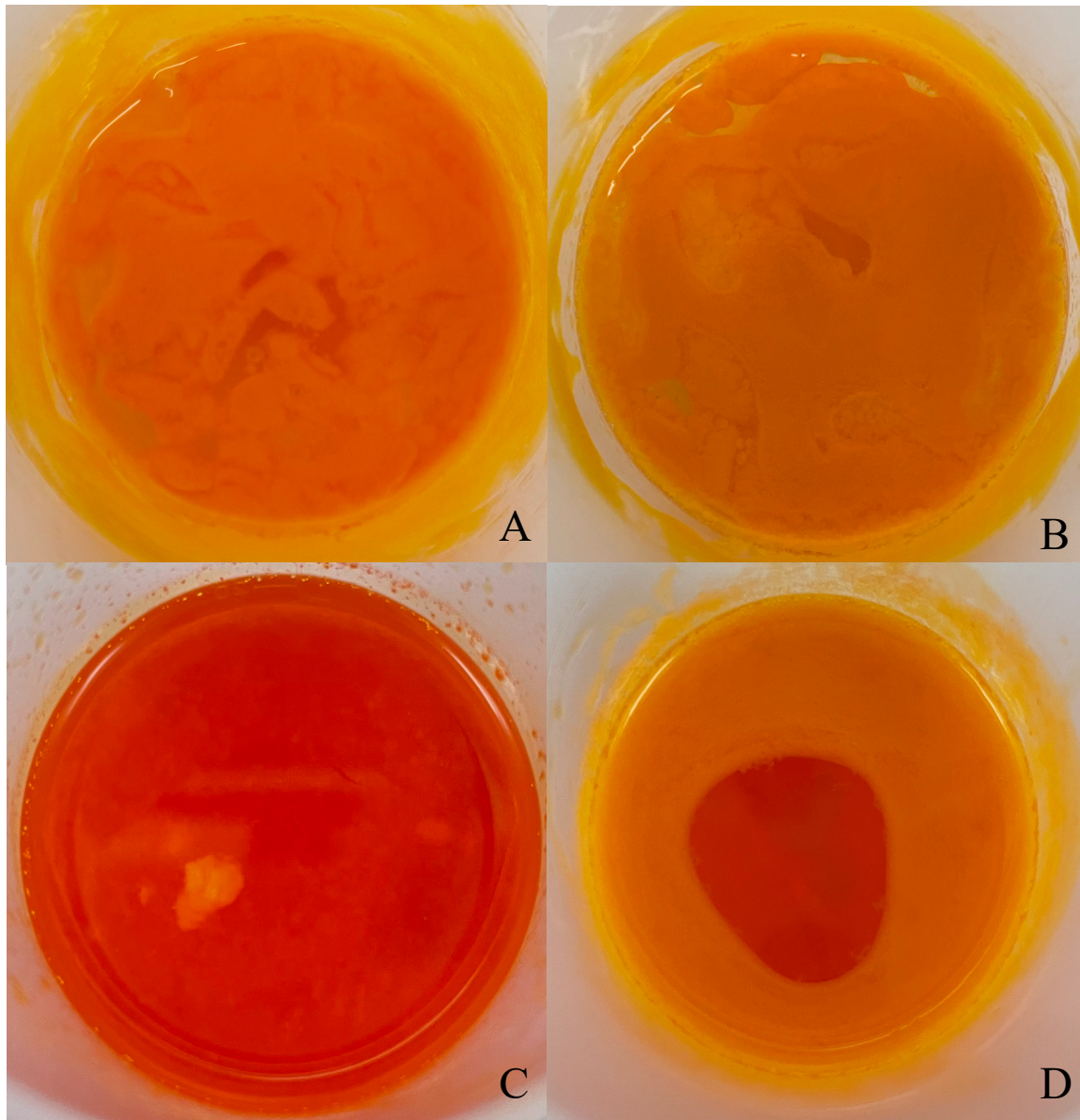
A further investigation was conducted to understand the impact of digestive enzymes on the stability of the emulsion structure and its disruption during gastric digestion under dynamic pH environment. To study this, we dyed the oil phase with highly oil soluble color, Oil Red O. This dye provides contrast, and its concentration can be measured by spectrophotometric measurement at 492 nm. The oil separation from the homogeneous O-W emulsion in the event of its disruption would result in the loss of absorbance from the bottom layer in the homogeneous digesta and can be used as an indicator of emulsion disruption. Thus, the stability of O-W emulsion structure was estimated by measuring the intensity of red color from the bottom of sampling cup at 492 nm (Figure 3-4). When the O-W was digested with HCl solution, the intensity of red color remained at the same level over the 180-min digestion treatment. On the other hand, with the addition of full profile of digestive enzymes, there was a drastic decrease in red color intensity as the pH dropped from 4.0 to 1.5 during the 180-min digestion treatment, indicating a progressive phase separation of oil from the emulsion phase.

Figure 3-4. Spectrophotometric measurement of absorbance at 492 nm for dyed O-W emulsion digested with SGF and HCl. Each data point represents mean $\pm$ standard deviation (SD).



As for W-O emulsion, the original emulsion displayed a creamy orange color at the beginning of digestion for both SGF (Figure 3-5A) and HCl (Figure 3-5B) groups. SGF led to a significant disruption of emulsion structure at 180 min as evident in Figure 3-5C where large amount of freely floating oil in bright red-orange color can be observed, whereas HCL solution group still had large amount of intact emulsion in creamy orange color at 180 min in Figure 3-5D.

Figure 3-5. Pictures of dyed W-O emulsion digested with SGF and HCl. A: T₀ W-O digested with SGF. B: T₀ W-O digested with HCl. C: T₁₈₀ W-O digested with SGF. D: T₁₈₀ W-O digested with HCl.



In addition to digestive enzyme impact, other factors of simulated digestion were also investigated. Compared to the previous study (Gao & Tikekar, 2023), the current study a) was conducted at a dynamic pH environment instead of a static pH level, b) replaced the mechanical

mixing of gastric digestion from stomacher with orbital shaker, and c) had an improved SGF enzyme formula including lipase, pepsin, and mucin. In Figure 3-6A and 3-6B, the experiments were conducted at a fixed pH without adjustment. Figure 3-6A first investigated the extent of pH impact under the current experiment design with complete digestive enzyme profile of SGF including lipase, pepsin and mucin. *Salmonella* was inoculated in 1:9 emulsion matrices and DI water, following the same manner of simulated gastric digestion but without any pH adjust throughout the treatment. The chyme pH was maintained at 2.18 ± 0.12 over the full 180 min. With the complete digestive enzyme profile, W-O emulsion still offered significant protection compared to O-W emulsion and DI water groups in terms of total inactivation for 180 min ($p < 0.05$). In this case, O-W emulsion even enhanced the bacterial inactivation as it achieved a 5.28 log CFU/ml reduction at 120 min, whereas it was 2.41 and 3.52 log CFU/ml for W-O emulsion and DI water, respectively. On the other hand, we also investigated the survivor curves of *Salmonella* inoculated in 1:9 ratio emulsion matrices and DI water, digested with previous SGF formula only containing pepsin (Gao & Tikekar, 2023), but agitated using orbital shaker instead of stomacher (Figure 3-6B). The pH level was maintained at 2.54 ± 0.10 throughout the experiment without adjustment. Under this experiment setup, O-W remarkably showed highest survivor levels throughout the 180-min digestion treatment compared to W-O emulsion and DI water groups ($p < 0.05$). However, the difference between two emulsion matrices were always within 1 log CFU/ml. The survivor curves for all three matrices showed a clear two-phase pattern: a rapid inactivation in the first 90 min followed by a plateau in the next 90 min. Lastly, the impact of orbital shaker (Figure 3-6B) was compared with stomacher mixing using previously obtained results (Figure 3-6C). In Figure 6C, the inoculated matrices were digested with previous SGF formula only containing pepsin (same formula as Figure 3-6B) but mixed in stomacher. While W-

O exhibited 2 to 3 log reduction in both Figures 3-6B and 3-6C, stomacher mixing significantly enhanced the bacterial inactivation in O-W emulsion and DI water groups in 3-6C compared to them in 3-6B ($p<0.05$). Hence, a significant protection was observed in W-O emulsion in Figure 3-6C only when the chyme was vigorously mixed in stomacher.

Figure 3-6A. Survival kinetics of *Salmonella* inoculated in emulsion matrices and DI water during static pH simulated gastric digestion with complete SGF mixed with orbital shaker. Each data point represents mean $\pm$ standard deviation (SD).

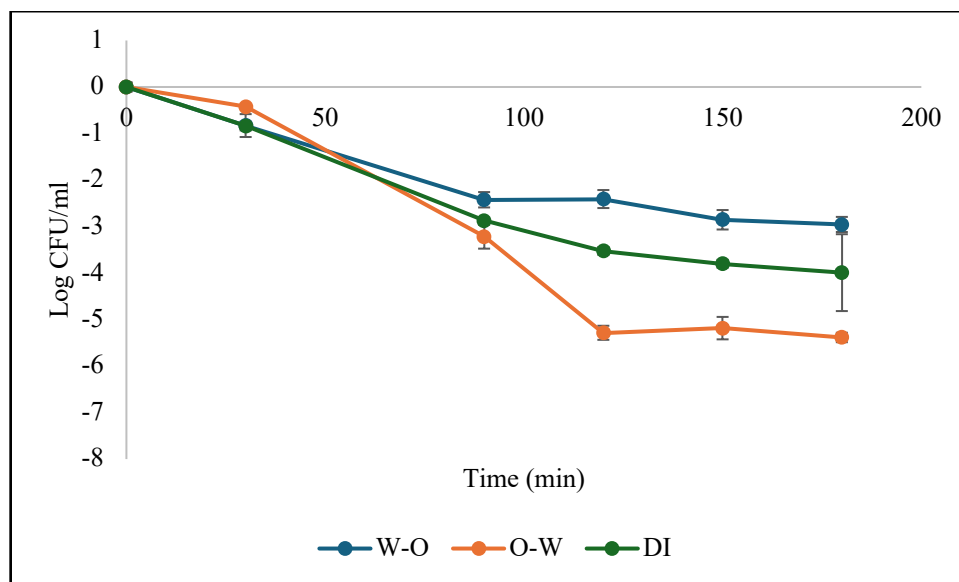


Figure 3-6B. Survival kinetics of *Salmonella* inoculated in emulsion matrices and DI water during static pH simulated gastric digestion with simplified SGF mixed with orbital shaker. Each data point represents mean $\pm$ standard deviation (SD).

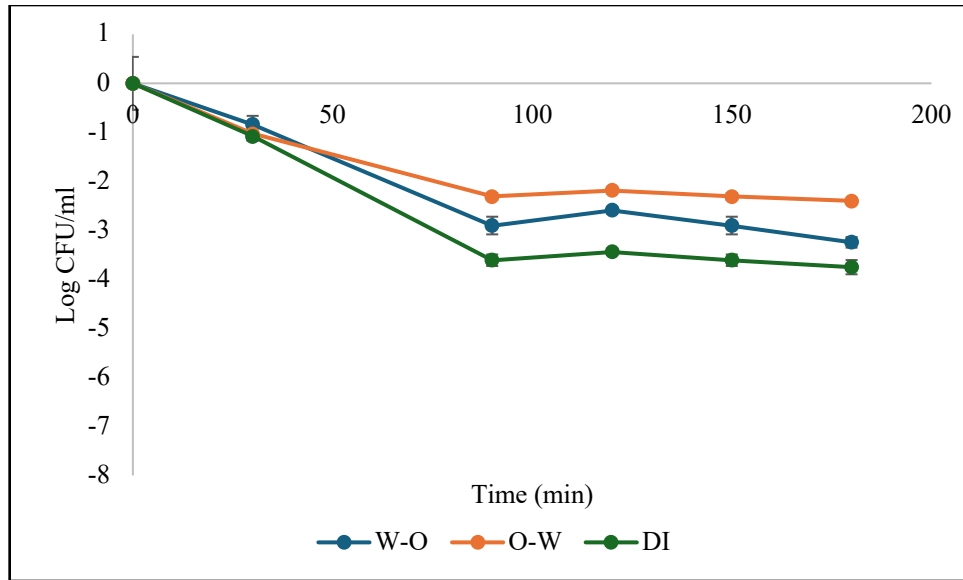
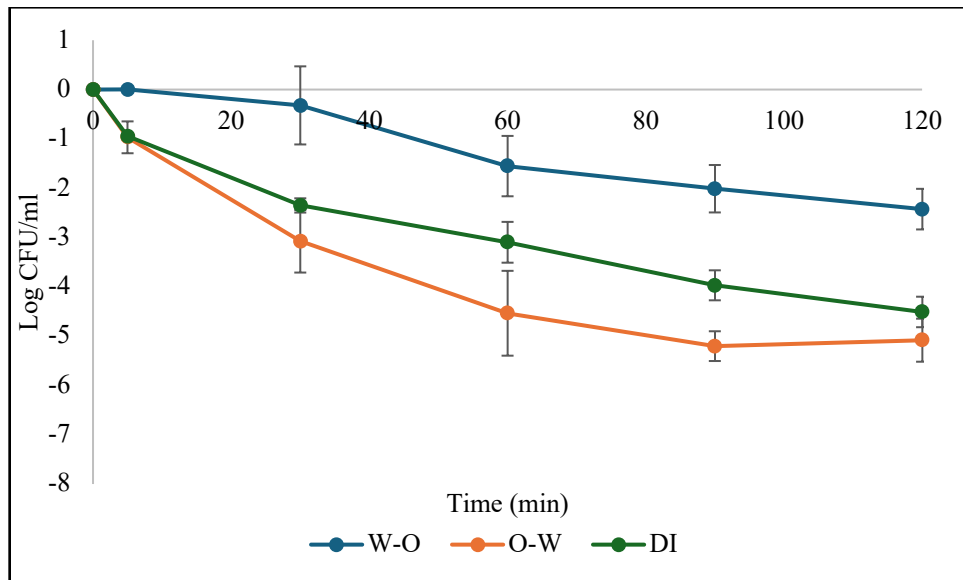


Figure 3-6C. Survival kinetics of *Salmonella* inoculated in emulsion matrices and DI water during static pH simulated gastric digestion with simplified SGF mixed with stomacher. Each data point represents mean $\pm$ standard deviation (SD).



3.4 Discussion

The current study demonstrated the survival kinetics in a specific scenario where *Salmonella* was entrapped in emulsion matrices and digested in a full stomach. The full stomach

situation was created by the dynamic pH which decreased over time. In an ideal semi-dynamic *in vitro* gastric digestion process, it is recommended to have continuous pH adjustment such as using an auto-titrator (Jiménez-Munoz et al., 2021; Mulet-Cabero et al., 2020; Zhou et al., 2023). Under the current experimental setup, pH level potentially altered the enzyme activity from SGF and essentially the emulsion stability. With the elevated enzymatic activities from SGF at certain pH levels, emulsifiers and lipids were rapidly digested that released the entrapped *Salmonella* into gastric acid. This increase in enzymatic activity was sufficiently significant to overcome the differences in emulsion types and the lipid content in the emulsion matrices, thus, we observed a different phenomenon than the previous study (Gao & Tikekar, 2023), where W-O emulsion did not offer protection to *Salmonella* against simulated gastric digestion.

In both W-O and O-W emulsions, lipids are major components for the emulsion structure, both as dispersed/continuous phases and emulsifiers. Therefore, the digestion of lipid during gastric digestion process is critical to understand how it may alter the potential protection to the entrapped *Salmonella*. The general understanding of lipid digestion is that lipid starts to get emulsified in the stomach due to the mechanical mixing and partial lipolysis from gastric lipase. After the initial emulsification and minor hydrolysis in stomach, the main process of lipolysis happens in small intestine facilitated by pancreatic triacylglycerol lipase, bile salts and other cofactors (Bauer et al., 2005). Therefore, the initial stage of lipid digestion in stomach is also critical to the overall process. According to the manufacturer, the amano lipase A used in this study has an optimum pH and temperature at 6.5 and 45 °C. Although the chyme pH was always below 6.5 throughout the whole digestion process, the first stage at pH 4.0 for 30 minutes is expected to partially activate the lipase activity and it may continue to the second stage at pH 3.0 for 60 minutes. Similar to pH, the treatment temperature was maintained at 37 °C, which was below the

optimal temperature, but a partial enzymatic activity at this temperature is plausible. The emulsion structures are anticipated to be digested mainly due to the overall enzymatic activity from lipase in the first 90 minutes of digestion. There was not a drastic decrease in bacterial survivor levels at pH 4.0 and 3.0 because they were not critically lethal to this *Salmonella* strain under such condition. It can be evidenced by the similar inactivation levels observed in *Salmonella* inoculated in DI water groups in both Figures 1 and 2. As the pH continued dropping, the enzymatic activity from lipase was expected to diminish, but pH 2.0 and 1.5 are both severely lethal to *Salmonella* cells, thus, accelerated inactivation was observed in the second half of digestion treatment.

On the other hand, when the digestive enzymes were removed from SGF and only left HCl in Figure 3-2, 1:9 W-O emulsion showed significant protection. The unique difference was observed from 150 to 180 minutes. In the last stage, the chyme pH was at 1.5 where lipase had lost its activity and pepsin was still active, although there was no protein in the emulsion. The higher survivor level at 180 min in 1:9 W-O emulsion may be attributed to a) the absence of enzymes reduced the damage on emulsion structure, and b) the lack of active pepsin reduced the bacterial inactivation. In Figure 3-5, the pictures indicated that the absence of enzymes (Figure 3-5D) significantly reduced breakage of the emulsion structure throughout the digestion process compared to full profile of SGF (Figure 3-5C). As for the antimicrobial properties of pepsin, Schreiber et al. has demonstrated rapid loss of motility of bacteria due to the presence of pepsin: *Helicobacter pylori* lost its motility irreversibly within 1 minute at pH levels of 2.0 and 3.0 (Schreiber et al., 2005). In addition, the presence of pepsin continued to induce structural damage to bacterial cells because of its proteolytic activity (Schreiber et al., 2006). Similar phenomenon was also reported by Zhu et al. with *E. coli* as the target strains (Zhu et al., 2006). By conducting bacterial survival kinetics study in gastric juice with and without pig pepsin A at various pH levels,

they revealed a critical role of pepsin proteolysis activity in bacterial inactivation. In the current study, this phenomenon can be supported by the survival kinetics in W-O emulsion (Figure 3-3A), where removing lipase or pepsin significantly reduced the overall *Salmonella* inactivation resulted in higher survivor levels throughout the study compared to other enzyme controls.

However, the lipase and pepsin activity observed in W-O emulsion (Figure 3-3A) do not explain the results in O-W emulsion (Figure 3-3B) and DI water (Figure 3-3C). In the latter two matrices, the absence of lipase or pepsin indeed accelerated the bacterial inactivation when the chyme pH dropped to lethal pH (below 3.0). Considering the water-like status of O-W and DI water matrices, HCl is expected to directly get in contact with *Salmonella* cells from the beginning of experiments since the bacterial culture was initially inoculated in water phase. Previous study had shown the bacterial cells had a potential to cross the barrier and enter the oil phase (Gao & Tikekar, 2023). With the full profile of SGF, O-W emulsion also experienced disruption over time (Figure 3-4). Thus, the presence of lipase and pepsin is anticipated to reduce the potential habitat for *Salmonella* and increase the total inactivation compared to partial profile of SGF missing either of the enzyme; however, we observed contradictory results. Future research is needed to understand the possible reasons of this phenomenon.

Besides the digestive enzyme profile of SGF, the digestion experiment setup also altered the bacterial survival kinetics. At a fixed pH level of 2.18 (Figure 6A), lipase is not active as it is not at its optimum pH. In the well-established standardized static *in vitro* digestion model, the gastric chyme after 1:1 mixing with SGF should be adjusted to pH 3.0 and agitated for 2 hours (Brodkorb et al., 2019; Minekus et al., 2014). However, with the potential resource limitation, some research projects may not be able to include gastric lipase from animal source and use other types of lipases instead. The limited enzymatic activity at such a low pH level should be considered

during analysis of results. Agitation is also considered as a standard mixing approach in static model (Brodkorb et al., 2019; Minekus et al., 2014). Compared to orbital shaker (Figure 3-6B), stomacher mixing (Figure 3-6C) induced a substantially higher overall bacterial inactivation in O-W emulsion. It may be attributed to the severe mechanical force created by stomacher mixing. In human stomach, stomach contents are mixed and disrupted by peristaltic waves (Boland, 2016; Bornhorst, 2017), which is considerably less vigorous than stomacher mixing and closer to agitation. Although the mechanical mixing such as stomacher is not a lethal factor or sublethal stress to bacteria, the comprehensive experiment setup may essentially change the final results.

In summary, the current study demonstrated the critical roles of lipase and pepsin as well as the pH levels in inactivation of *Salmonella* entrapped in W-O and emulsions as well as in DI water during gastric digestion. The presence of digestive enzymes effectively disrupts the W-O emulsion structure and facilitates the inactivation of bacterial cells. Under the full stomach situation where gastric pH drops over time, when *Salmonella* was inoculated W-O emulsion, O-W emulsion and DI water, there was no significant differences in their survival rates among all three matrices.

With the understanding of the differences between two emulsions and two digestion models, we would like to expand the food matrix impact investigation to other common foods with complex ingredients. In addition, we would like to also change the focus from a single pathogen to complex microbiome community that naturally presented on food. In the next chapter, a complex microbiome community was used to study its inactivation kinetics in various common food matrices during dynamic pH simulated gastric digestion.

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Chapter 4: Investigation on survival of natural microbiome in emulsion and other food matrices during simulated gastric digestion

Abstract

Previous studies have demonstrated the impact of food emulsion matrices on bacterial survival during certain gastric digestion scenarios. However, the studies were conducted only with a single pathogenic strain *Salmonella enterica* subsp. *enterica* serovar Typhimurium. In the current study, the food matrix impact on bacterial survival has been expanded and extensively studied in various food matrices using a tailored natural microbiome community. Using fresh apples as a natural microbiome source, this study investigated the potential of enriching the natural microbiome and understanding their survivability during simulated gastric digestion. Fresh Golden Delicious and Empire apples were cut into wedges with peel on and transferred into Tryptic Soy Broth (TSB) with pre-adjusted pH at 7 or 5. The samples were incubated at 30 °C for 1 to 3 day(s), and the microbiome was collected by centrifugation at 5750 ref for 10 minutes. The microbiome was then transferred into different matrices including deionized water, apple sauce, sweet potato puree, chicken puree, or a water-in-oil emulsion. The augmented food matrix samples with enriched apple microbiome were 1:1 mixed with simulated gastric fluid (HCl, NaCl, gastric mucin, pepsin, and amano lipase A) and kept on an orbital shaker in a 37 °C incubator for up to 180 min. The pH of the digesta was adjusted to pH 4 at 0 min, pH 3 at 30 min, pH 2 at 90 min, and pH 1.5 at 150 min. Samples were taken every 30 min for bacteria enumeration. Based on the 16S rRNA sequencing results, no human pathogen was detected in the native and enriched apple microbiome, and only commensal bacteria were enriched. During gastric digestion, the enriched apple microbiomes reduced from 8.44 ± 0.39 log CFU/ml to 7.55 ± 0.38 log CFU/ml in the first 90

minutes in deionized water for both apple cultivars from both enrichment condition. As pH decreased beyond the initial stage, microbiome inactivation was observed to be matrix dependent. That is, the inclusion of fat and protein in the augmented food matrices (chicken puree) significantly prolonged survivability of the apple microbiomes during the pH 3 digestion stage by 3 log CFU/ml compared to sweet potato puree ($p < 0.05$), and the water-in-oil emulsion appeared to significantly protect the microbiome, as the bacterial concentrations remained above 4 log CFU/ml throughout the entire digestion process. There was no significant difference in inactivation rates observed between the two apple cultivars or two enrichment pH levels within the same food matrix ($p > 0.05$). The results demonstrated the feasibility and possible benefits of enriching natural microbiome from apples and the potential of reintroducing the natural microbiome back into other food products. This work will be extended to understand the ecological dynamics of the apple microbiome and implications for food quality and consumer health.

4.1 Introduction

It has been demonstrated in previous studies that W-O emulsions can offer protective effect to *Salmonella enterica* subsp. *enterica* serovar Typhimurium during gastric digestion in some specific conditions (Gao & Tikekar, 2023). While the previous study was restricted to a specific gram-negative human pathogen, it is worth studying the same matrix impact with a complex live microbiome community that better represents the bacterial population in actual food. To obtain a mixed bacteria culture with various bacterial genera and species, one of the common methods is to mix a few bacteria cultures and form a cocktail with known composition under specific criteria. However, real food such as fresh fruits and vegetables usually carry large and extremely diverse microbial population (Leff & Fierer, 2013). It is exceptionally challenging to mimic a natural microbiome profile by manually combining individual strains. Therefore, utilizing naturally

presented microbiome community from a food source was considered the optimum choice to understand how food microbiome survive during gastric digestion.

Among common fruits and vegetables, apples are the most consumed fruits worldwide (Wassermann et al., 2019). In addition, people consume apples in various forms including fresh apple fruits, apple juice, apple sauce, and varieties of apple snacks. In commercial practice, harvested apples are precooled, followed by washing and sanitizing, and waxing. These treatments enable the low temperature (1-2 °C) storage of apples for up to 6-12 months before they are released into market (Abdelfattah et al., 2020). Studies have shown the bacterial community isolated from fresh apples carry a relative high operational taxonomic unit (OTU) richness level, which refers to high level of diversity, and the bacterial diversity is considerably dependent on storage time and postharvest treatments (Abdelfattah et al., 2020; Leff & Fierer, 2013; Wassermann et al., 2019). \ Besides the treatments preserving the fruit quality, it would be beneficial to explore the potential of preserving the fresh natural microbiome.

Such knowledge is also critical for successfully delivering probiotic bacteria into the gastrointestinal tract (Luo et al., 2022). Food structure, composition and processing all play a role in transport and release nutrients in human gastrointestinal tract (Guo et al., 2020). In human stomach, the bolus experiences antral mixing, acid hydrolysis, dilution, and enzymatic digestion (Bornhorst, 2017; Guo et al., 2020). Different food matrices may offer distinct digestion and release rates during gastric digestion. Thus, five liquid/semi-liquid food matrices were selected for comparison in this study, including deionized water (DI), apple sauce (AS), sweet potato puree (SP), chicken puree (CK), and W-O emulsion (W-O). The selection is mainly dependent on their unique food composition. The general objectives of this study are to explore the potential of enriching a natural microbiome profile and understand the food matrix impact on the enriched

microbial community survivability during gastric digestion. Based on the results from previous objectives, we hypothesize that food matrices that contain high lipid contents will offer protection to the natural microbiome during gastric digestion.

4.2 Materials and methods

Apples. Golden Delicious (GD) and Empire (EP) apples were kindly donated by a local farm in Maryland, USA. Approximately 40 apples of each apple cultivar were directly picked from the apple trees on the same day without cross-contact between the two cultivars. All apples were transferred into zipper bags and stored at 4 °C throughout the study. After equilibrated at 4 °C for more than 48 h, the study started on the third day from the apple harvesting.

Food matrices. Shelf-stable apple sauce (AS), canned sweet potato puree (SP), and canned chicken (CK) puree were obtained from a local grocery store. The same batch of products were used throughout the study.

Native apple microbiome collection. Ten apples from each cultivar were randomly picked from the stock. Unwashed and unpeeled apples were cut into 8 pieces using an apple cutter, cores were removed, and each sliced apple was transferred into a 227-ml stomacher bag. One hundred ml of phosphate buffer solution (Catalog No. J62036.K7, Thermo Scientific, Waltham, MA) (PBS) was added into the first stomacher bag, the apple was hand massaged for 1 min to dislodge the microbiome. The apple wash water was then transferred to the second apple bag and hand massaged for 1 min. Using the same aliquot of 100 ml PBS, this step was repeated until all 10 apples from each cultivar were washed to concentrate the microbiome from all 10 apples in small amount of PBS. The final apple wash water was transferred into a 50-ml centrifuge tube and then centrifuged at 7000 rcf for 10 min. Supernatant was decanted and the pellet was re-suspended in

45 ml of PBS. The re-suspended culture was then served as the inoculum for subsequent food matrix preparation.

Apple microbiome enrichment. Three apples from each cultivar were randomly picked from the stock. Unwashed and unpeeled apples were cut into 8 pieces using apple cutter, cores were removed, and each apple slice was transferred into a 50-ml centrifuge tube containing 30 ml of Tryptic Soy Broth (TSB) (Catalog No. BD211825, BD Bacto™, San Jose, CA) with 50 mg/L of cycloheximide (Catalog No. J66901, Thermo Scientific, Scientific, Waltham, MA) that was pre-adjusted to pH 5 or 7 using 6N HCl. Cycloheximide was added to inhibit the growth of molds. The enrichment broth tubes were then transferred to a 30 °C incubator. The incubation periods for pH 7 and pH 5 broth were 1 day and 3 days, respectively. After the incubation, all broth from the same apple cultivar and same pH level was combined into a 50-ml centrifuge tube and then centrifuged at 7000 rcf for 10 min. Supernatant was decanted and the pellet was re-suspended in 35 ml of PBS. The re-suspended culture was then served as the inoculum for subsequent food matrix preparation. Figure 4-MS outlined the schematic of apple microbiome enrichment process.

Food matrices inoculation. Deionized water (DI) & Apple Sauce (AS): Fifteen ml of prepared inoculum was mixed with 15 ml of sterile DI water or apple sauce. After thoroughly mixing, the simulated digestion experiments were initiated. **Sweet Potato Puree (SP) & Chicken Puree (CK):** The centrifuged pellet from microbiome enrichment was stored in 4 °C refrigerator for 3 days and was then re-suspended only with 5 ml of PBS. One ml of resuspended culture was inoculated into 35 ml of sweet potato puree or chicken puree. After thoroughly mixing, the prepared food matrices were stored in 4 °C refrigerator overnight for equilibrium and digested with simulated gastric digestion treatment on the second day. **W-O:** The centrifuged pellet from microbiome enrichment was stored in 4 °C refrigerator for 7 days and was then re-suspended only

with 5 ml of PBS. Water-in-oil emulsion (W-O) was then prepared exactly following the methodology described in previous study (Gao & Tikekar, 2023). The prepared emulsions were stored in 4 °C refrigerator overnight for equilibrium and digested with simulated gastric digestion treatment on the second day.

Simulated gastric fluid. Simulated gastric fluid (SGF) was prepared following the formula from Swackhamer et al (Swackhamer et al., 2019). The base of simulated gastric fluid was pH 1.8 ± 0.02 HCl solution using sterile DI water and 6N HCl. Then 6.67 mg/ml of pepsin powder (300 U/g, MP Biomedicals, CAS: 9001-75-6), 1.50 mg/ml of gastric mucin (Porcine stomach - Type II, Sigma-Aldrich, CAS: 84082-64-4), 3.33 mg/ml of Amano Lipase A (120,000 U/g, Sigma-Aldrich, CAS: 9001-62-1), and 8.78 mg/ml of NaCl (Fisher Scientific, CAS: 7647-14-5) were suspended in the acid solution to make the final simulated gastric fluid. The prepared SGF was stored in 4 °C refrigerator and used within 48 hours.

Simulated gastric digestion. All the bacterial survival digestion experiments were conducted in triplicate sample cups simultaneously. Before the gastric digestion, the prepared SGF and inoculated food matrices were transferred to a $37 \pm 1^\circ\text{C}$ incubator and preheated for 30 min. The simulated gastric digestion system was prepared by transferring 10 ml of SGF and 10 ml of prepared food matrices to three of 100 ml-sample cups, respectively. The remaining food matrix was utilized to measure the initial bacterial population levels. The pH of the gastric digesta mixture was immediately adjusted to 4.0 ± 0.1 using 1N NaOH and placed onto an orbital shaker in the incubator that was equilibrated in $37 \pm 1^\circ\text{C}$. The orbital shaker was operated at 100 rpm and a separate timer was initiated at the same time. At 30, 90, and 150 min, the pH of the gastric digesta was adjusted using HCl to 3.0, 2.0 and 1.5, respectively. The overall pH adjustment added less than 1.5 ml of total liquid volume in the gastric digesta, which was negligible to affect the enzyme

concentration in gastric digesta. At 30, 90, 150, and 180 min, 0.1 ml of the gastric digesta sample was directly taken from the upper half of each sample cup before the pH adjustment. In some cases, 120 min samples were also taken in the same manner. All samples were serially diluted in 0.1% BPW and plated on duplicated TSA plates with spiral plater (Neutec Group Inc., Nr 10003700/113, Farmingdale, NY) with E50 mode. The plates were dried at room temperature for 10 min, and then inverted and incubated at 37 °C for 20-24 h. Plates were enumerated using an automatic plate counter (Neutec Group Inc., Nr 10006021/182, Farmingdale, NY), and the counts were converted to log₁₀ values. In native apple microbiome experiments, the samples with extremely low bacterial population were measured using a 3-tube MPN method with TSB as the growing media following the U.S. Department of Agriculture Most Probable Number Procedure and Tables (U.S. Department of Agriculture, 2014).

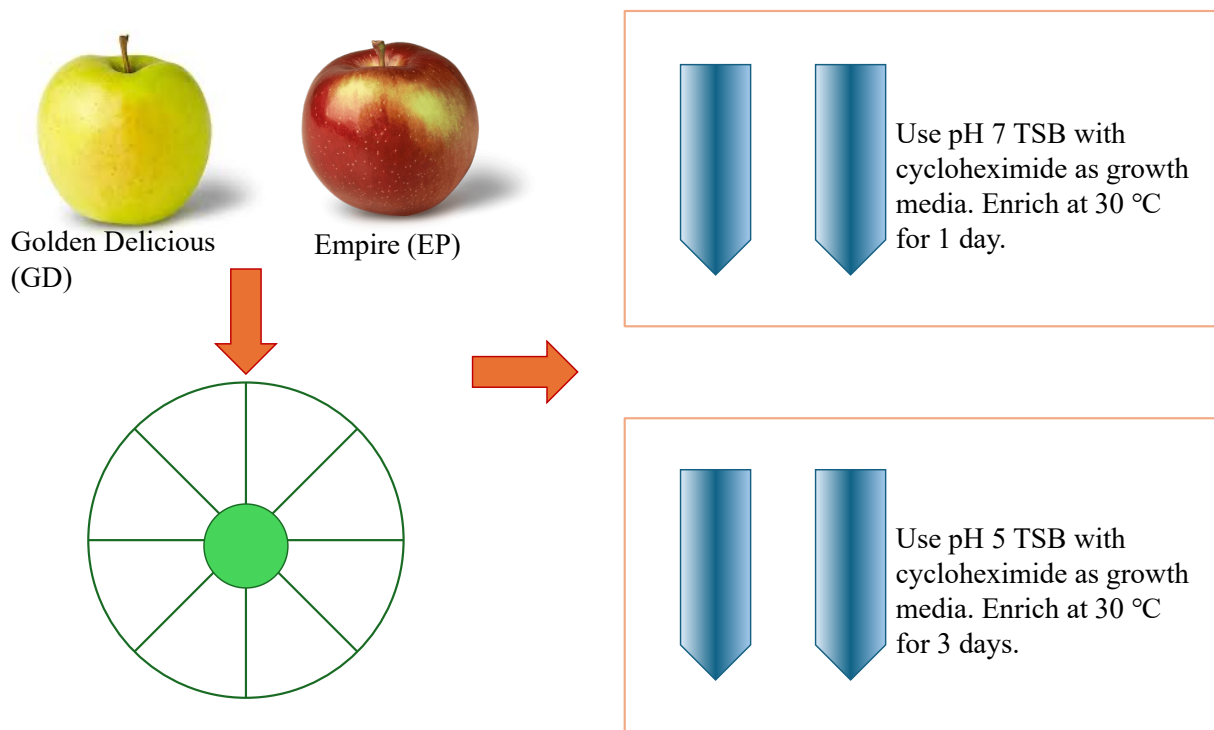
Plate count data analysis. Considering the equal portion mixing at the beginning of digestion, the initial bacterial population levels obtained directly from emulsion sample were divided into half and then used as T₀ bacterial levels for data analysis. The collected bacterial population data were normalized respect to T₀ bacterial population, calculated for the means and standard deviation, and the survivor curves were plotted. All data points below the lower detection limit (LDL) were assigned a value of $0.5 \times 200 = 100 = 2 \log$ and then used for normalizing. The survivor levels at certain time points were compared with a one-way analysis of variance and Tukey honest significant difference test at $p = 0.05$.

DNA extraction and 16S rRNA sequencing. The samples were stored at -20 °C freezer and the DNA extraction was performed within 30 days. The DNeasy Powersoil kit (Catlog No. 47014, Qiagen, Hilden, Germany) was used to extract DNA from samples. DNA concentration and 260/280 were measured by an NanoDrop One C (Thermo Fisher, Waltham, MA) for quality

control purpose. Quick-16S Plus NGS Library Prep Kit (Zymo Research, Irvine, CA) was used to prepare the amplicon libraries for the V3-V4 hypervariable region of 16S rRNA genes using a QuantStudio 3 Real-Time PCR System (Thermo Fisher, Waltham, MA). ZymoBIOMICS qPCR standards and Microbial Community DNA standards were run on the plate to approximate 16S copies per reaction based on Cq values. They also served as controls to validate absence of foodborne pathogens in the apple samples. Monarch PCR Cleanup Kit (NE Biolabs, Ipswich, MA) was then used to clean the pooled PCR products. DNA concentration of the final amplicon library pool was quantified with a Qubit 4 fluorimeter (Invitrogen, Waltham, MA). Paired-end sequencing (2×300 cycles) was performed on an Illumina NextSeq1000 at the Joint Institute for Food Safety and Applied Nutrition (JIFSAN).

16S rRNA sequencing results analysis. QIIME2 v.2023.9 (Bolyen et al., 2019) was used to analyze the sequenced reads. The paired-end sequences were denoised with the DADA2 workflow to generate amplicon sequence variants (ASVs) and taxonomy was assigned with the silva-132-97-nb-classifier (Quast et al., 2012). The online visualization module (<https://view.qiime2.org>) was used for tabulated read counts for ASVs at taxonomic levels spanning phylum to genus. Downstream analysis was performed in R v.4.3.1. SCRuB (Austin et al., 2023). Negative sequencing controls including blanks for DNA extraction and PCR library prep was used for removing potential ASV contaminants in analysis. To further filter reads related to eukaryotic DNA, counts assigned to Chloroplast and Mitochondria that were uncharacterized past the order- and family-level, respectively, were removed. The resulting microbiota profile counts tables were converted to relative abundance values. Vegan (Oksanen et al., 2022) was used to compute alpha-diversity (Shannon index) and beta-diversity (Bray-Curtis dissimilarity) metrics.

Figure 4-MS: The schematic of apple microbiome enrichment process.



4.3 Results

Before understanding the survival kinetics of natural and enriched apple microbiome, 16S rRNA sequencing was conducted to understand the composition of the native and enriched apple microbiome. In total, 71 bacterial genera were detected in the native microbiome collected at the beginning of the study. After the enrichment at pH 5 or pH 7, 28 of total genera were detected in both apple cultivars (Table 4-1). As the TSB broth enrichment with inclusion of cycloheximide was supposed to only support the growth culturable bacteria, the alpha diversity that measured the differences in total taxa was expected to reduce during the enrichment (the Shannon indexes in Figure 4-1). The digesta samples collected at the end of digestion treatment were also analyzed using the same approach. There was an exceptionally high increase in their Shannon indexes in Figure 4-7 with a high variation. It may be attributed to the residual DNA from the food matrices

due to various ingredients and manufacture processing. The beta diversity measurement in Figure 4-8 also confirmed the distinct compositional profiles among native, enrichment and digesta microbiome.

Table 4-1. Bacterial genera composition in enriched apple microbiome (combined results from GD and EP).

Genus/Taxa	Percent (%)
<i>Pantoea</i>	36.009
<i>Gluconobacter</i>	16.091
<i>Bacillus</i>	13.667
<i>Enterobacteriaceae</i> unclassified (a)	10.345
<i>Kosakonia</i>	9.762
<i>Rahnella</i>	3.484
<i>Pseudomonas</i>	3.194
<i>Microbacteriaceae</i> sp.	1.612
<i>Enterobacteriaceae</i> uncl. (b)	0.992
<i>Lactococcus</i>	0.873
<i>Streptococcus</i>	0.557
<i>Erwinia</i>	0.540
<i>Lysinibacillus</i>	0.518
<i>Sarcina</i>	0.491
<i>Curtobacterium</i>	0.334
<i>Xanthobacteraceae</i> uncl.	0.318
<i>Clostridium sensu stricto 1</i>	0.302
<i>Gaiella</i>	0.141
<i>Acidobacteria</i> Subgroup 6	0.138
<i>Solirubrobacter</i>	0.130
<i>Sphingomonas</i>	0.117
<i>Micrococcaceae</i> uncl.	0.088
<i>KD4-96</i>	0.088
<i>IMCC26256</i>	0.050
<i>Gaiellales</i> sp.	0.042
<i>Lactobacillus</i>	0.042
<i>Burkholderiaceae</i> uncl.	0.038
<i>Leuconostoc</i>	0.035

Figure 4-1A. Shannon Indexes of apple microbiome composition from different groups of samples (combined results from GD and EP).

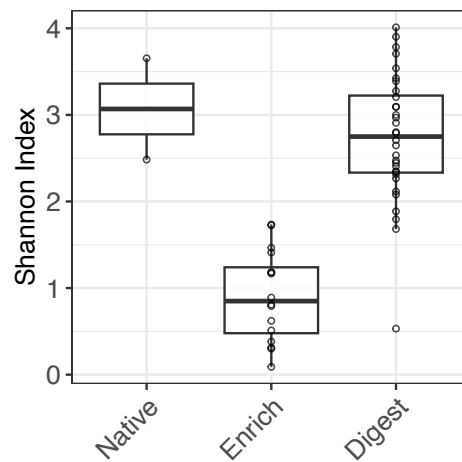
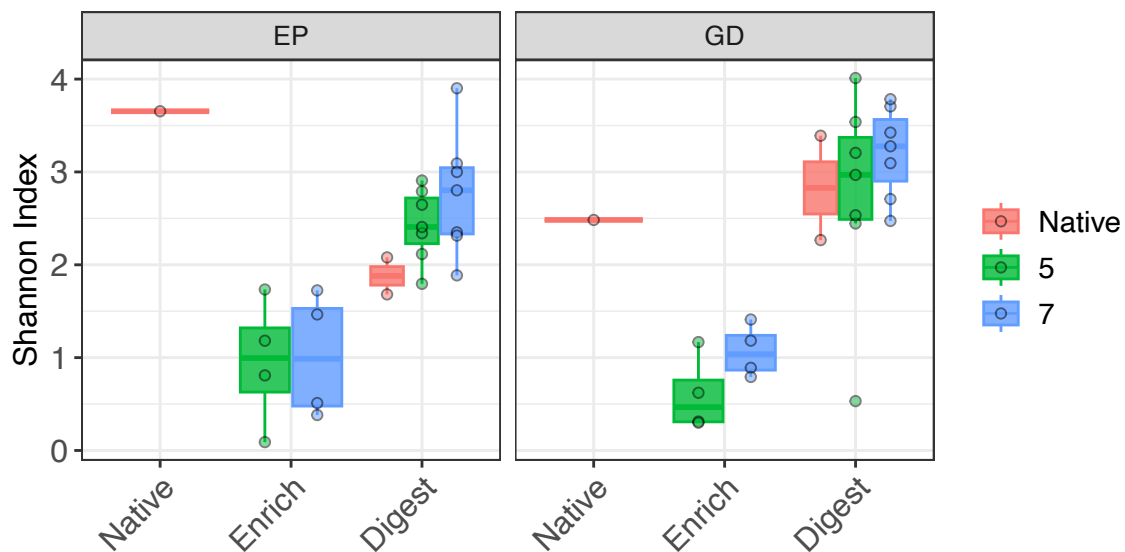


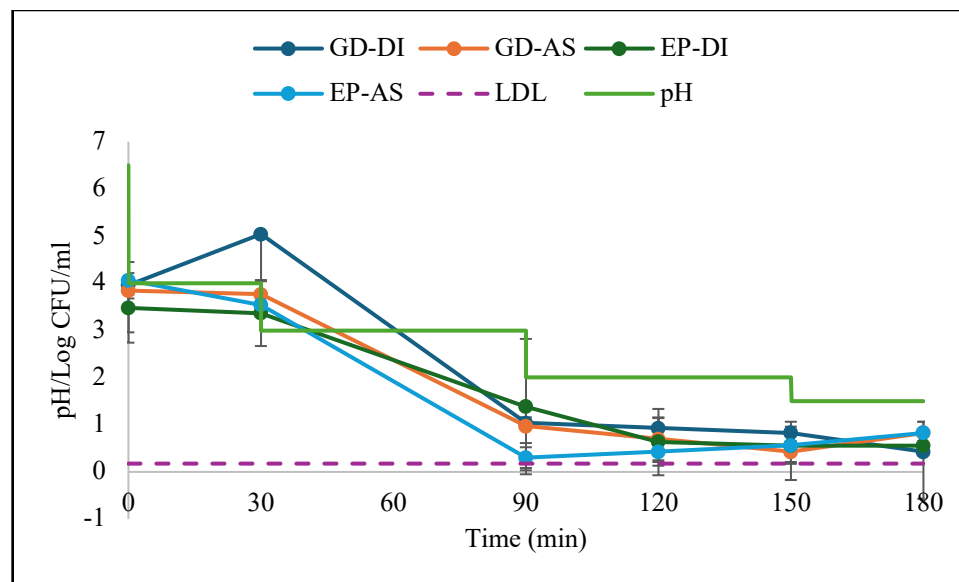
Figure 4-1B. Shannon Indexes of apple microbiome composition from different groups of samples (separate analysis from GD and EP in respect of different enrichment condition).



The survival kinetics study started from understanding the behavior of native microbiome from apples during gastric digestion (Figure 4-2). The initial microbial load collected from 10 apples from each cultivar was estimated to be approximately 4 log CFU/ml. To better simulate the digestion of real apple, the collected microbiome was only inoculated in sterile DI water and shelf-

stable apple sauce for the simulated gastric digestion treatment. In general, two apple cultivars GD and AS showed similar inactivation kinetics in both DI and AS throughout the 3-hour gastric digestion. At pH 4.0 for the first 30 minutes, the microbial loads remained unchanged and then started to decrease substantially from 30 minutes when the pH dropped to 3.0. The total microbial counts remained detectable throughout the treatment as MPN method was applied for microbial enumeration. In fact, the data obtained from all groups starting from 90 min were generally below 1 log CFU/ml, which was less practical to be considered as live microbiome.

Figure 4-2. Survival kinetics of native apple microbiome inoculated in different food matrices during simulated gastric digestion. Each data point represents mean $\pm$ standard deviation (SD). GD: Golden Delicious apple. EP: Empire apple. DI: Deionized water. AS: Apple sauce.



The study was then focused on the enriched apple microbiome and the comparison between two apple cultivars, the enrichment conditions, and the impact from various food matrices. The fresh pH 7 enrichment bacteria from GD and EP were inoculated in both DI and AS (Figure 4-3A). After the 1-day enrichment, we obtained more than 8 log CFU/ml of bacteria to initiate the gastric digestion treatment. The bacterial loads in the first 90 min remained above 7 log CFU/ml

for both cultivars and both in DI and AS. In other words, pH 4.0 and pH 3.0 conditions only induced less than 1 log CFU/ml of inactivation in the fresh pH 7 enriched microbiome. As the pH reduced to 2.0 at 90 min, the inactivation happened rapidly as all groups declined to around lower limit of detection within the next 30 min. Some groups, EP in DI and AS as examples, remained detectable in the last one-hour stage of digestion treatment, but the actual bacterial counts were particularly low for practical consideration. The refrigerated apple enrichment culture was then inoculated into CK and SP (Figure 4-3B). After the refrigeration, the initial bacterial levels from the two cultivars decreased to 7.39 to 7.64 log CFU/ml. In addition, their acid resistance substantially reduced compared to fresh culture. pH 3 condition induced significant reduction from 30 to 90 min in both cultivars and in both CK and SP. The lowest bacterial reduction at 90 min was observed in EP inoculated in CK, which was 2.19 log CFU/ml on average. The rest of groups decreased below lowest detection limit at 90 min.

Figure 4-3A. Survival kinetics of freshly harvested pH 7 enriched apple microbiome inoculated in different food matrices during simulated gastric digestion. Each data point represents mean $\pm$ standard deviation (SD). GD: Golden Delicious apple. EP: Empire apple. DI: Deionized water. AS: Apple sauce.

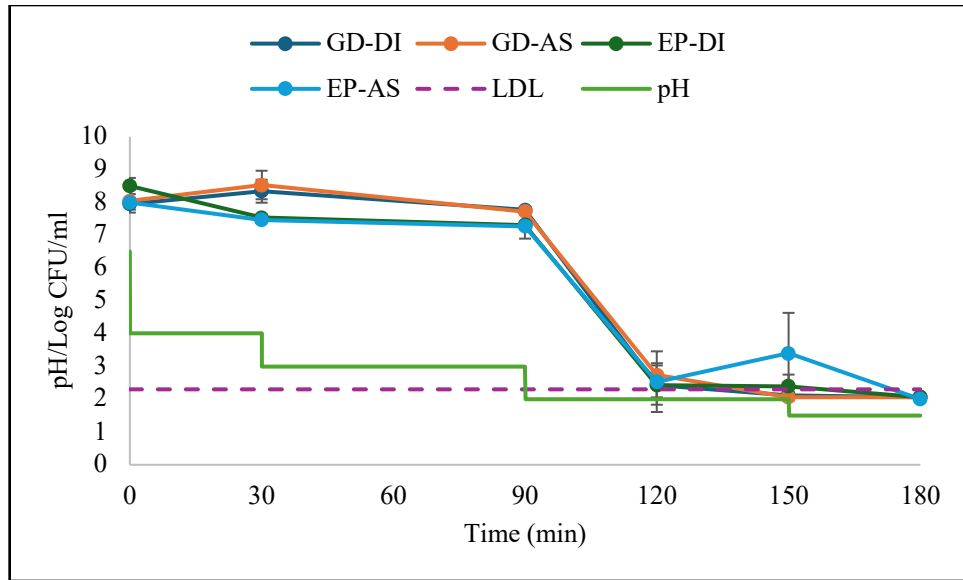
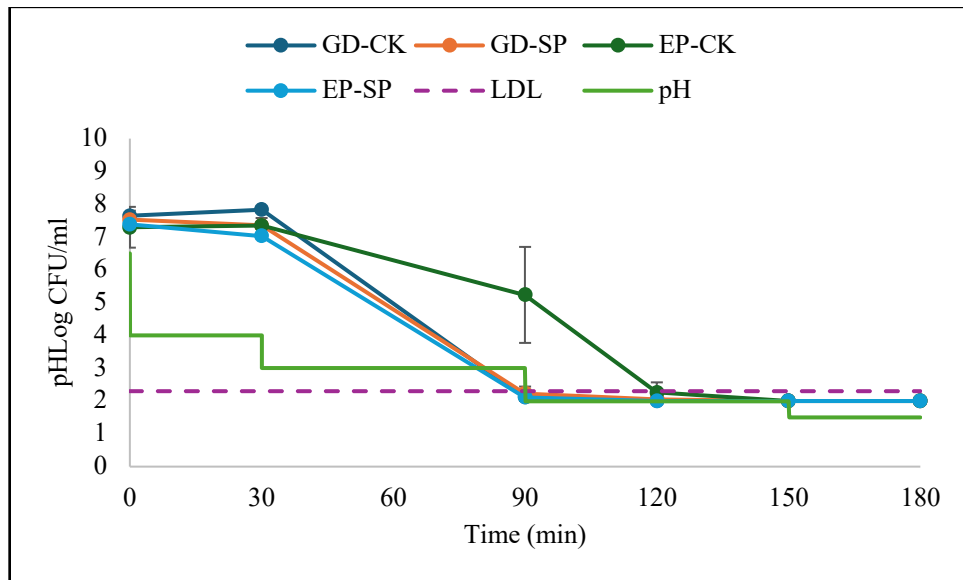


Figure 4-3B. Survival kinetics of refrigerated pH 7 enriched apple microbiome inoculated in different food matrices during simulated gastric digestion. Each data point represents mean $\pm$ standard deviation (SD). GD: Golden Delicious apple. EP: Empire apple. CK: Chicken puree. SP: Sweet potato puree.



Similarly, the same food matrices and apple cultivar comparison were conducted on pH 5 enrichment (Figures 4-4A & 4-4B). Enrichment at pH 5 also successfully increased the bacterial loads to 8 to 9 log CFU/ml to initiate the digestion experiments (Figure 4-4A). At 90 min, both

cultivars in both DI and AS showed substantially high survivor levels with less than 2 log CFU/ml inactivation. The lowest bacterial survivor level for fresh culture was observed in EP inoculated in AS at 6.21 ± 0.85 log CFU/ml. When the gastric digesta pH levels reduced to 2.0, the survivor levels in all groups approached to the lowest detection limit within 30 min of treatment at 120 min. As for the food matrix comparison between AS and CK, the experiments were also conducted using refrigerated apple enrichment culture (Figure 4-4B). Again, the initial bacterial loads decreased to 7 to 8 log CFU/ml due to the refrigeration storage, and their acid resistance significantly reduced as the survivor levels at 90 min considerably declined compared to the initial levels. Besides, CK offered significant protection to the enriched bacteria in both cultivars compared to SP at 90 min ($p < 0.05$).

Figure 4-4A. Survival kinetics of freshly harvested pH 5 enriched apple microbiome inoculated in different food matrices during simulated gastric digestion. Each data point represents mean $\pm$ standard deviation (SD). GD: Golden Delicious apple. EP: Empire apple. DI: Deionized water. AS: Apple sauce.

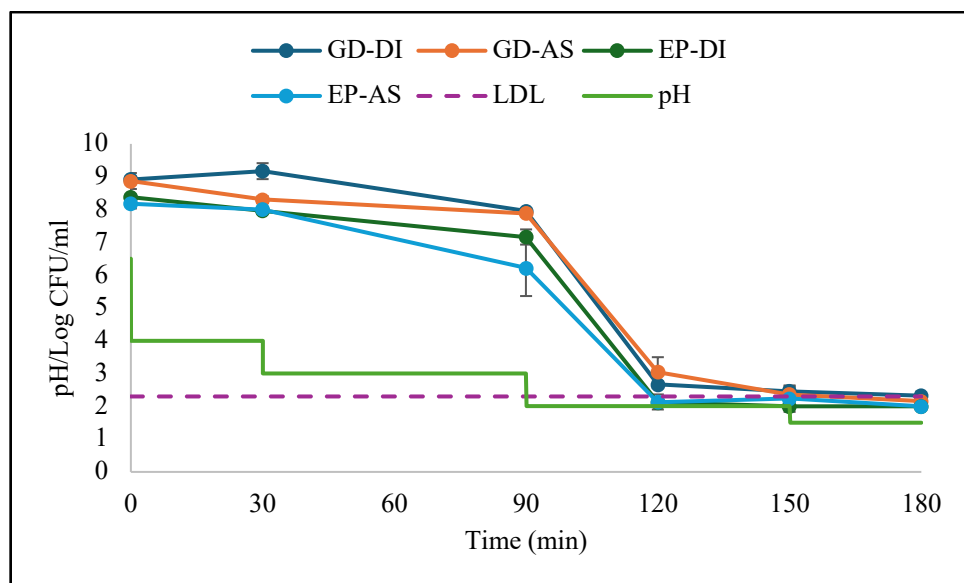
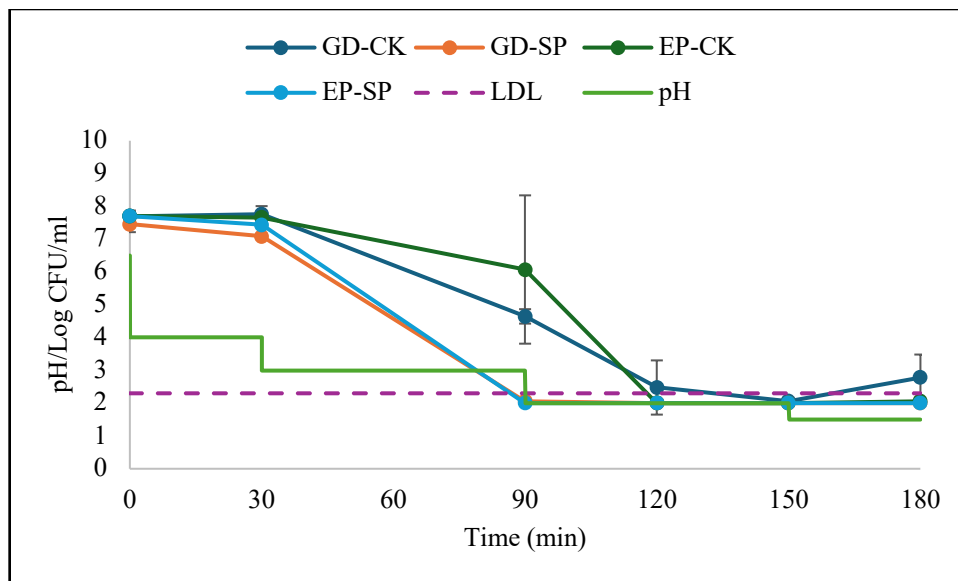


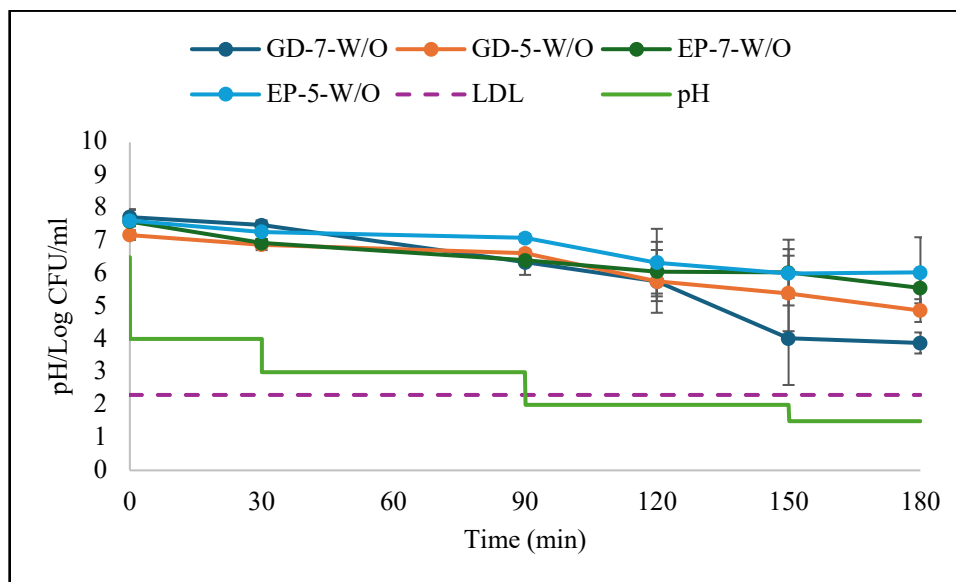
Figure 4-4B. Survival kinetics of refrigerated pH 5 enriched apple microbiome inoculated in different food matrices during simulated gastric digestion. Each data point represents mean $\pm$ standard deviation (SD). GD: Golden Delicious apple. EP: Empire apple. CK: Chicken puree. SP: Sweet potato puree.



The last survival kinetics study was conducted on W-O emulsion using the 7-day refrigerated bacterial culture (Figure 4-5). The initial bacterial levels for both cultivars from both enrichment conditions fell into the range of 7 to 8 log CFU/ml. Unlike the substantial inactivation observed in DI, AS, SP, or CK, W-O emulsion offered significant protection throughout the full 3-hour of digestion treatment. In Figures 4-2 to 4-4, the bacterial inactivation rates were mostly dependent on the pH condition over the specific treatment period and marginally affected by the food matrix type. In Figure 4-5, when the enriched bacteria were inoculated in W-O emulsions, the inactivation curves generally followed first order kinetics over treatment time and less affected by the actual pH conditions. At the end of digestion treatment, the highest survivor level was

observed in EP pH 5 enriched bacteria at 6.03 ± 1.08 log CFU/ml, whereas the lowest survivor level occurred in GD pH 7 enrichment at 3.88 ± 0.32 log CFU/ml.

Figure 4-5. Survival kinetics of refrigerated pH 7&5 enriched apple microbiome inoculated in different emulsions during simulated gastric digestion. Each data point represents mean $\pm$ standard deviation (SD). GD: Golden Delicious apple. EP: Empire apple.



4.4 Discussion

A complex tailored apple microbiome profile was used to study the impact of food matrices on the microbial survivability against simulated gastric digestion. The primary purpose of using a tailored apple microbiome community is to investigate the food matrices impact that previously discovered with a single pathogenic bacterial strain and expand the food matrix study into other commercially available products. This study also explored the possibility of enriching natural microbiome community to obtain a complex profile of commensal and potential probiotic bacteria.

In the current study, 16S rRNA results confirmed the absence of known human pathogens, and the rest of taxa are mainly commensal bacteria. The alpha diversity results also revealed that it was a tailored apple microbiome profile after enrichment. The top five bacterial genus that were more than 4% of total composition in the enriched microbiome were *Pantoea*, *Gluconobacter*, *Bacillus*, *Enterobacteriaceae*, and *Kosakonia*. Some strains of *Pantoea* was reported to be beneficial to rich growth by promoting plant growth, protecting from abiotic stress, and preventing from diseases and pathogen infection (Lv et al., 2022). *Gluconobacter* is classified as acetic acid bacteria and also reported to increase the biomass and height of plants (Dwivedi, 2020). Some strains of *Bacillus* are considered as human pathogen but there are also probiotic strains that can hydrolyze food compounds and produce beneficial bioactive compounds (WoldemariamYohannes et al., 2020). Besides *Bacillus*, *Enterobacteriaceae* is another large group of gut microbiome but it is often linked with inflammatory bowel diseases (Baldelli et al., 2021). Lastly, *Kosakonia* was also reported as a growth-promoting bacteria to plants (Jan-Roblero et al., 2020). Among these commensal bacteria taxa, some of them may also exhibit beneficial functions to human. It would be helpful to conduct future studies on functional predictions by analyzing functional genes and using multi-omics approaches (O'Donnell et al., 2020; Wang et al., 2021).

Two apple cultivars in this study were selected based on the seasonal availability, and the major purpose was to investigate the potential difference in their natural microbiome during enrichment and gastric digestion. Both apple cultivars in this study were collected from the same farm on the same day. The apples were all grown under conventional management, and the two types of apple trees were physically close to each other. As a result, there was no major difference in their compositional profile from their native microbiome at the beginning of the study. The enrichment broth in this study is tryptic soy broth which only provides nutrients to culturable

microorganisms, and the supplementation of cycloheximide minimized the growth of yeasts and molds. Therefore, only culturable bacteria from apples were expected to be enriched. Even though it is still debatable on the exact proportions of culturable bacteria across major biomes, a large number of bacteria are still considered to be unculturable (depending on the definition of the diversity unit) (Martiny, 2019). Under the current understanding of microbiome diversity, the enrichment of apple microbiome in this study is a selective enrichment of culturable bacteria and results in a tailored apple microbiome profile. In addition, as the enrichment will always utilize manually prepared culturing media, it is almost impossible to fully enrich the complete profile of apple microbiome as of natural environment. Considering the spoilage potential and over competition from yeasts and molds, they were removed from the current study. It would be worthwhile to explore the probiotics yeast enrichment and their survival kinetics during gastric digestion in the future studies.

The enrichment method reported in the current study utilized cut apple slices as the seeding material, and each of the apple slice contains unwashed peel and flesh. Wassermann et al reported that native bacteria were presented on both apple peel and flesh (Wassermann et al., 2019). Thus, this enrichment method is expected to provide nutrients to all bacteria that presented on the edible part of apples. Another enrichment trial was conducted using TSB blended with apple peels and blended with apple flesh separately (data not shown). The trial was designated to understand the difference between peel and flesh microbiome and to better utilize the natural apple nutrients during the enrichment. However, the enriched microbiome did not show high survivability as slice enrichment, or the collected bacteria population was not sufficient to conduct digestion study. Moreover, the blended apple pieces created difficulties in collecting enriched bacteria at the end

of enrichment. Thus, an improved enrichment and collection methodology is needed to study the effect of peel vs. flesh as the enrichment seeding material.

The two critical parameters in the current enrichment condition are temperature and pH levels. 30 °C was selected since it is a mildly increased temperature compared to the natural apple growing environment. The slight elevation in temperature is considered to enhance and accelerate the growth of bacteria (Yang et al., 2018). On the other hand, it is preferred to enhance the bacterial acid tolerance during the enrichment as it can help bacterial cells overcome the gastric digestion treatment. Low-pH adaptation is one of the common approaches. It has been well demonstrated that pre-exposure and adaptation to low pH environment enhances the acid tolerance in various bacteria species (Sánchez et al., 2007; Trček et al., 2015; Wu et al., 2014). Therefore, a pH 5 enrichment condition was added in parallel with pH 7. However, there was no significant difference in their enriched bacteria survivability during gastric digestion. There might be a few reasons behind this unexpected phenomenon: a) pH 5 condition is not acidic enough to trigger strong bacterial response to acidity. The pH of apple fell in a range of 2.71 to 4.70 depending on the cultivar and maturity (Xu et al., 2012). Thus, the microbiome presented in the flesh may require a lower pH to trigger their acid response. b) In figures 1-5, the drastic drop in bacterial population was generally observed at pH 2 stage. With the enzymatic activity from gastric fluid, pH 2 is extremely lethal to majority of bacteria even they have enhanced acid tolerance. Therefore, the bacterial population harvested from both pH enrichment conditions showed dramatic decrease as the activated acid tolerance cannot overcome the extremely low pH. c) The potential acid tolerance induced by low-pH adaptation during enrichment was essentially a rapid stress response mechanism. In the current study, harvested bacteria were inoculated in various food matrices, with or without overnight equilibrium, and then subjected to 3-hour gastric digestion treatment. It is

possible that the bacteria had switched off the acid responses when the actual gastric digestion took place. It would be worthwhile to explore other approaches to enhance the bacterial acid tolerance during enrichment and verify it through gastric digestion.

The other major objective of this study is to understand the impact of different food matrices on bacterial survival during gastric digestion. DI water was selected as a baseline control; apple sauce was selected to mimic the original food source of the apple microbiome; shelf-stable sweet potato puree was selected to represent a food matrix high in carbohydrates (starch and dietary fiber); shelf-stable chicken puree was selected as a food matrix high in protein and fat; W-O emulsion was specifically selected as the previous study indicated protective effect on bacterial survival (Gao & Tikekar, 2023). While the bacteria in DI water and apple sauce showed high survivor levels at pH 3 stage during digestion, the starter cultures were fresh and unsusceptible, which may be the major reason of high bacterial levels rather than the food matrix impact. Among all the food matrices, W-O emulsion showed substantial protection to the enriched apple bacteria community. The second protective food matrix was observed to be chicken puree compared to sweet potato puree under the same conditions. The high lipid content in both W-O emulsion and chicken puree is estimated to mainly contribute to this protective effect (Aviles et al., 2013). In addition, the sweet potato puree and chicken puree were canned products, so the inoculation was simply mixing the bacterial PBS suspension into the puree. It would be beneficial to inoculate the bacterial community while manually making the purees. The homogenization process may create coarse emulsion structure or micelles that may offer additional protective effect to bacteria.

In conclusion, this study demonstrated the possibility of partially enriching a natural microbiome profile presented on/in fresh apples and evaluated its survivability during gastric digestion. The alpha diversity of the enriched microbiome significantly decreased whereas the

bacterial population reached to substantially high levels (above 8 log CFU/ml). Among the five matrices studied in the project (DI, AS, SP, CK, and W-O), W-O emulsion and chicken puree (CK) offered significant protection to bacteria against simulated gastric digestion treatment. In general, there was no difference observed in bacterial survivability between two apple cultivars (Golden Delicious and Empire) or the enrichment pH (5 or 7).

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Chapter 5: Overall conclusions and future work

5.1 Overall conclusions

Various emulsions as well as other food matrices showed distinct impact on bacterial survival during gastric digestion. In static pH simulated gastric digestion, where the digestive enzyme activity was expected to be limited due to the low pH levels, W-O emulsion showed significant protection to *Salmonella* survival compared to O-W emulsion. There was a remarkably difference in viscosity between W-O and O-W emulsion at a 1:9 dispersed to continuous phase ratio. However, increase in viscosity of O-W emulsion did not improve the *Salmonella* survivability during gastric digestion. In addition, inoculation in water phase vs. oil phase was also compared in Chapter 2. Because of the spontaneous bacterial migration from oil phase to water phase, majority of inoculated bacterial cells were expected to locate in water phase when the gastric digestion was initiated.

When the *Salmonella* cells were challenged with a dynamic pH gastric digestion treatment, the dynamic pH levels enabled partial digestive enzyme activity during the treatment. The activated digestive enzymes particularly enhanced the emulsion structure disruption and hindered the protective effect from W-O emulsions. In dynamic pH gastric digestion, there was no significant difference between the emulsion types (W-O vs. O-W) and the ratios of dispersed to continuous phase (1:9 vs. 4:6). The results in Chapter 3 also revealed the impact of gastric digestion parameters on the final bacterial survival kinetics: pH, SGF composition, and mechanical mixing approaches all played a role in digestion outcomes. Removing lipase or pepsin from SGF both

induced lower total bacterial inactivation in W-O emulsion, but higher bacterial inactivation in O-W emulsion and DI water.

Enriched apple microbiome showed slightly different survival patterns during dynamic pH gastric digestion. The alpha diversity was reduced during enrichment since only the culturable bacteria was able to utilize the enrichment broth and growth competition may occur. The final enriched microbiome community was sensitive to cold temperature storage but was robust during dynamic pH gastric digestion when entrapped with W-O emulsion. Chicken puree with high fat and protein contents also showed protective effect on bacterial survival during digestion. Other food matrices including DI, AS, and SP did not enhance the survivability of the bacterial cells. Enrichment at pH 5 and pH 7 induced similar acid resistance to the enriched microbiome as their survivor levels were generally similar under the same condition throughout the digestion treatment.

5.2 Future work

The current study mainly focused on the survivability of one pathogenic bacterial strain *Salmonella enterica* subsp. *enterica* serovar Typhimurium during gastric digestion. However, it would be beneficial to also include the pathogenicity analysis along with survival kinetics during gastric digestion. When bacterial cells were exposed to lethal and sub-lethal stresses, it would trigger their stress responses and potentially alter its pathogenicity expression. The pathogenicity analysis throughout the digestion treatment may provide additional information to comprehensively understand the interaction between bacterial cells, digestion conditions and food matrices. The second way to expand the knowledge is to include other foodborne pathogens. With different pathogenicity and stress response pathways, their survivability and gene expression are expected to show differences during gastric digestion. Moreover, the current project started to

explore the potential of manually enrich and utilize a complex food microbiome community as the inoculum for kinetics study. It would be valuable to discover other potential natural food microbiome community for this type of study. Utilizing a natural complex microbiome community may provide better insights on how commensal bacteria survive during gastric digestion in a real food consumption scenario. In addition, this enrichment may support the growth of some natural bacteria that have probiotics effect. Functional genome analysis is expected to comprehensive categorize and classify the potential health benefits from these enriched apple microbiomes.

On the other hand, the simulated gastric digestion treatment can also be improved. Due to the accessibility issue, lipase from *Aspergillus* was used in this study and its optimal pH is higher than the gastric digestion condition. It would be valuable to validate the results in a digestion treatment setup utilizing lipase from animal sources. With accelerated enzymatic activity from lipase, bacteria inoculated in W-O emulsion and other high-fat food matrices may show different survival rates. In addition, the study was only conducted on simulated gastric digestion without following intestinal digestion. Lipid contents from food is largely digested during intestinal digestion, where complex enzymatic reactions continuing disrupt the food structure from gastric chyme, but the pH is neutral and not lethal to bacteria. It is a reasonable step to have follow-up digestion phase and understand the interaction of chyme, bacteria, and intestinal digestion. Gastric emptying effect can also be studied if the chyme is then proceeded with simulated intestinal digestion. Oral digestion was not included in this project considering the liquid nature of emulsion, and the absence of oral digestion is consistent throughout the whole study. The future work should consider the inclusion of oral digestion if expand to other complex food matrices and extend to intestinal digestion.

Chapter 6: Limitation and Take-home Message

6.1 Limitations of the project

This project specifically focused on the food matrix impact on bacterial survival during simulated gastric digestion, and some experimental parameters were simplified along the project. The potential limitations and their impact on the results are discussed separately in this section.

In chapter 2, W-O and O-W emulsion impact was evaluated using static pH simulated gastric digestion approach. Lipase was not included in the simulated gastric fluid and the pH of digesta was maintained at 2.54 ± 0.10 . Although the experimental lipase may not be active at this low pH level, the absence of this enzyme may slightly reduce the extent of emulsion structure disruption and bacterial cell damage. Considering both W-O and O-W emulsion were evaluated using the same SGF, the inactivation results were comparable. Moreover, the presence of excessive oil composition in W-O emulsion may also create large barrier to lipase activity even if the lipase was included. The second limitation in this chapter occurred in bacterial inoculum preparation. The *Salmonella* cells were inoculated in both water and oil separately. The inoculation method in water phase was achieved by simply adding culture suspension into DI water, while the inoculation method in oil included a drying step before resuspended in peanut oil. The desiccation stress is expected to trigger bacterial stress responses and induce cross-protection to other stressors including acid resistance (Aviles et al., 2013; Gruzdev et al., 2011). In the current study, the drying process lasted within three hours and the oil suspension was then used to prepare emulsion matrices. Tables 2-1A and 2-2A had revealed that majority of bacterial cells migrated into water phase during the overnight storage. Thus, the bacterial stress response to desiccation was expected to be restored, but it would be fair to expose the water inoculation bacterial cells to the same

conditions for paired comparison. In addition, most of figures were converted into the form of showing log reduction over time. There were two major reasons: 1) the oil phase inoculation had approximately 2-log lower bacterial levels compared to water phase inoculation; 2) the W-O emulsion had substantially higher viscosity than O-W emulsion, which created difficulties to take a representative sample. Thus, the W-O emulsion data was normalized in respect to 5-min data point where the digesta was fully mixed with SGF, whereas the O-W emulsion was normalized in respect to 0-min data where the digesta was homogenously mixed at the beginning. The normalization eased the results demonstration but also limited the space of showing original data levels.

In chapter 3, W-O and O-W emulsions were challenged with dynamic pH simulated gastric digestion treatment. The impact of mixing approach during simulated gastric digestion and the effects from individual digestive enzyme were also separately studied. Both pepsin and lipase are protein, which have their buffering capacities to pH changes (Heda et al., 2023; Miled et al., 2000). When the digestive enzyme was individually removed from SGF, the pH of the digestion treatment was controlled but the potential protein buffering capacity was not considered. Therefore, it would be beneficial to supplement equivalent amount of protein in these treatments (Figures 3-3A, B & C). In addition, a separate control of HCl solution with equivalent amount of protein should be included besides the pure HCl solution control group. In addition, peanut oil was used to prepare emulsions both in chapters 2 and 3. Researchers had demonstrated fungal lipases were able to hydrolyze edible oils including peanut oil and produce polyunsaturated fatty acids and short-chain fatty acids that exhibited antimicrobial properties (Kotogán et al., 2022). The amano lipase A used in chapter 3 and 4 was isolated from *Aspergillus*, so it was possible that hydrolyzed fatty acids also contributed to bacterial inactivation.

In chapter 4, the food matrix impact study was expanded from emulsions to other common food products, and the focus changed from single strain of *Salmonella* to complex microbial communities. Even though the study only focused on the matrix impact during gastric digestion, the nature of different food matrix may impact the digestion efficacy or directly affect bacterial behaviors. For example, the chicken puree used in this project had significantly higher viscosity compared to the rest of food matrices, this may alter the rate of digestion treatment. Apple sauce tends to have an acidic pH level, and its acidity may trigger bacterial stress responses prior to the digestion treatment. While the study was intended to utilize and evaluate the food matrices' natural properties, it would be beneficial to specify their major differences with additional experiments or with information from literatures and manufacturers. Tailored complex microbial communities were used as the target for inactivation in this chapter. However, only 16S rRNA sequencing was used to analyze the microbial composition before and after the enrichment (before the simulated gastric digestion treatment). We conducted 16S rRNA sequencing to the final digesta after the digestion treatment, but the noise from food matrices created disorder in data analysis resulting in meaningless results. If time and resources permit, the best approach to study the survivor bacteria would be through metagenomics approach. With metagenomics analysis, we would also be able to incorporate *Salmonella* in the complex microbial community to investigate the potential impact from other natural microflora on pathogen survival during gastric digestion.

Throughout the study, the statistical analysis was usually conducted on the survivor or inactivation levels at specific sampling points. Inactivation rates, often obtained through linear regression analysis, are commonly used in bacterial inactivation kinetics studies. However, in chapters 3 and 4, the pH of digesta was changing over time. The lethal stressors for bacterial cells

were not constant throughout the treatment, thus, it would be unrepresentative to have linear regression for the entire 3-hour treatment or with the limited data points at each pH level.

6.2 Take Home Messages

This study revealed the food matrix impact on bacterial survival during simulated gastric digestion. Beyond the abstractive experiments and analysis, there are two major aspects to apply the findings from this project.

1. From food manufacturing perspective, the results indicated that complex food systems such as emulsions may pose higher foodborne pathogen risks to consumers. The more reliable manufacturing approach may be processing the ingredients separately before making them into a complex food matrix in a safe way.
2. From a consumer and a public health standpoint, the same pathogenic bacteria may exhibit different level of risks via different food sources. W-O emulsion and foods rich in fat and protein can create protection for pathogenic bacteria to help them survive through gastric digestion and may lead to foodborne diseases. On the other hand, we can also utilize these food matrix characteristics to deliver beneficial microorganisms to consumers.

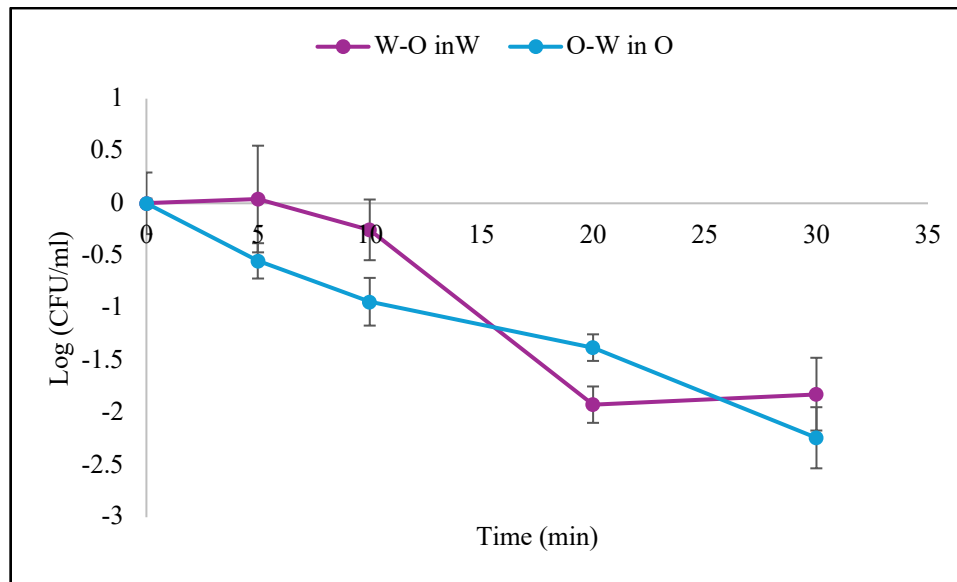
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Appendix A: Supplemental Results for Chapter 2

Figure SA-1: Survival kinetics of *Salmonella* inoculated in the dispersed phases in emulsion models during simulated gastric digestion.



Summary: Separate experiments were conducted to further analyze *Salmonella* inactivation kinetics in W-O and O-W emulsions during the first 30 minutes of simulated gastric digestion. The results were not consistent with the 2-hour digestion kinetics results. It may be attributed to the frequent sampling which interfered the digestion temperature and stomacher mixing.

Table SA-1: *Salmonella* migration between water phase and oil phase without supplementation of lecithins with initial inoculation in water. (Lower detection limit: 2.30 log (CFU/ml).)

	T _{0h}	T _{24h}
DI	6.87 ± 0.13	7.05 ± 0.81
Peanut oil	5.32 ± 0.32	3.87 ± 0.22

Table SA-2: *Salmonella* migration between water phase and oil phase without supplementation of lecithins with initial inoculation in oil. (Lower detection limit: 2.30 log (CFU/ml).)

	T _{0h}	T _{24h}
DI	3.55 ± 0.50	2.60 ± 0.00
Peanut oil	2.20 ± 0.35	3.98 ± 0.64

Summary: Separate experiments were conducted to understand the natural potential of bacterial cells migrating between water and oil phase. Lecithin was not included, and water/oil phase was separately inoculated in the same manner outlined in Chapter 2. The prepared water and oil were then 1:1 (v/v) mixed and homogenized at 24,000 rcf for 90 seconds. The results indicated the natural migration of *Salmonella* cells between water and oil phases with the absence of emulsifiers.

Appendix B: Supplemental Studies for Chapter 4

The inactivation kinetics results reported in Chapter 4 all utilized the enrichment from the first round of enrichment. There were additional two rounds of enrichment process conducted for other purposes. In the second round of enrichment, the microbiome was enriched in the same approach as outlined in Chapter 4. After the enrichment cycle finished, all of the broth from two apple cultivars and two pH levels was collected, respectively. After centrifugation, the pellets were resuspended with 5 ml of PBS, and 1 ml of resuspended culture was transferred into 10 g of dextrin. After vigorously shaking, the inoculated dextrin was freeze dried, and the final products were stored at room temperature.

Table SB-1: Bacterial level in the freeze-dried dextrin powder (obtained by TSA plate counts).

Unit: log CFU/g.

GD pH 7	GD pH 5	EP pH 7	EP pH 5
4.48 ± 1.10	2.71 ± 0.20	3.81 ± 1.19	4.52 ± 0.26

The third round of enrichment was conducted to explore other enrichment approaches that might increase the bacterial survivability during gastric digestion as well as increase the alpha diversity of the enriched microbiome community. Three apples from each cultivar were randomly selected and peeled with sterile apple slicer. The peel and flesh were collected separately and blended with TSB (pH 5 & 7) on a 1:1 ratio (w/v). The blended apple peel and flesh were transferred into 50-ml centrifuge tubes and incubated at 30 °C (3 days for pH 7 and 4 days for pH 5). The enriched apple crumbs were then centrifuged at 7000 rcf for 10 min to collect the enriched apple microbiome and apple crumbs. They were then resuspended with PBS on an approximate

1:1 (v/v) ratio, and the final products were challenged with dynamic pH simulated gastric digestion as outlined in Chapter 4. Figure SB-1 showed the survival kinetics of apple microbiome pH 7 enrichment using peel and flesh. The survivability from both apple cultivars and both peel and flesh were reduced compared to the results obtained from the first round of enrichment in Chapter 4. As for pH 5 enrichment (Figure SB-2), there was no bacteria detected on TSA plates from EP groups throughout the digestion treatment including T_0 counts. The T_{90} bacterial levels in GD groups at pH 5 were higher than them in Figure SB-1 at pH 7, but the overall survivability was not significantly enhanced compared to the first round of enrichment. It may be attributed to the decrease in alpha diversity in the natural microbiome on apples during storage, and it was evident in 16S rRNA results in Appendix C Figure SC-3 where the enriched microbiome from different rounds of enrichment were significantly different.

Figure SB-1: Survival kinetics of apple microbiome enriched at pH 7 using blended apple peel and flesh during simulated gastric digestion. Each data point represents mean $\pm$ standard deviation (SD). P: blended apple peel; F: blended apple flesh.

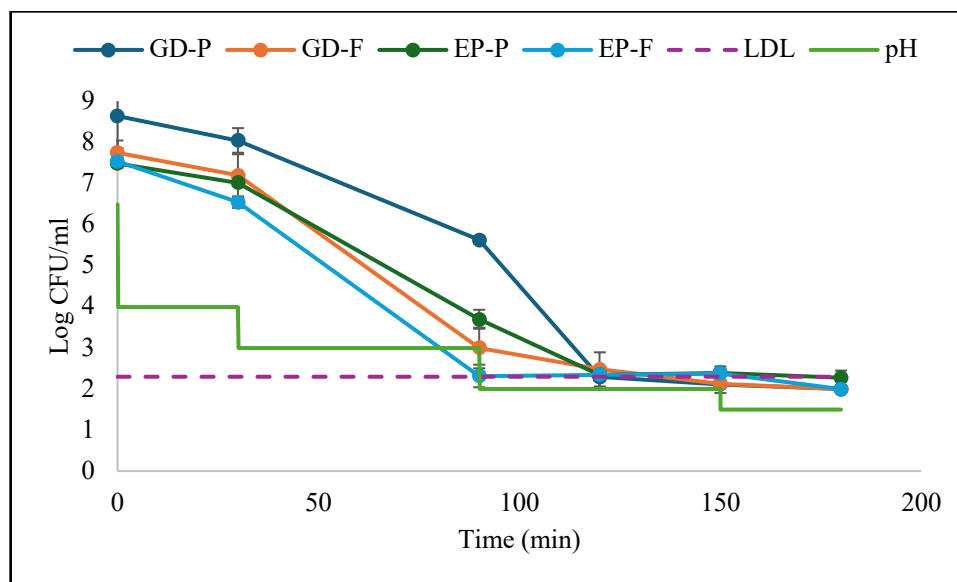
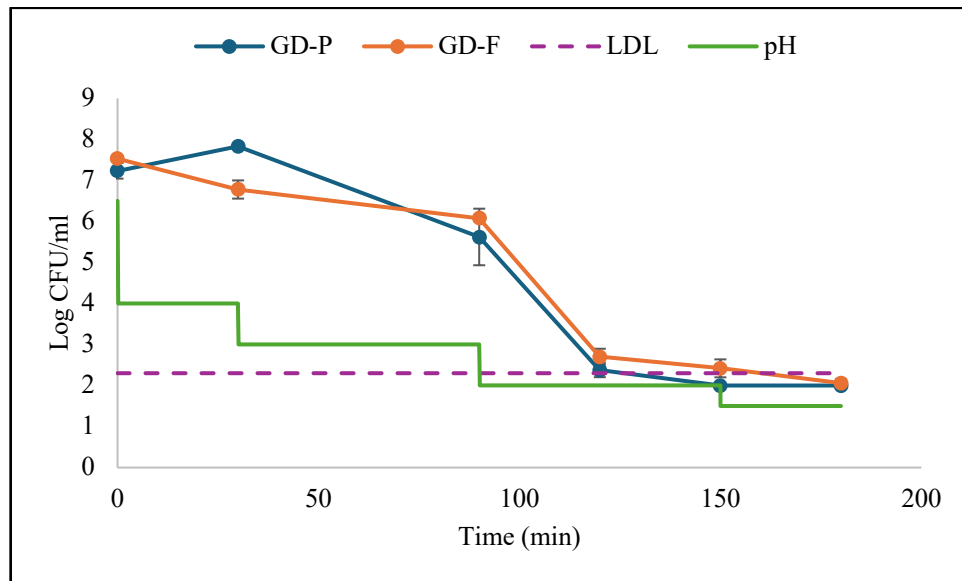


Figure SB-2: Survival kinetics of apple microbiome enriched at pH 5 using blended apple peel and flesh during simulated gastric digestion. Each data point represents mean $\pm$ standard deviation (SD). P: blended apple peel; F: blended apple flesh.



Appendix C: Supplemental 16S rRNA Analysis for Chapter 4

Figure SC-1: Bacterial phyla detected in original native apple microbiome. Native: microbiome collected in initial native apple wash water; Digest: microbiome collected in final digest samples after 3-hour gastric digestion treatment. 1: GD native microbiome; 2: EP native microbiome; 21: GD native microbiome inoculated in DI at T₁₈₀ gastric chyme; 22: GD native microbiome inoculated in AS at T₁₈₀ gastric chyme; 23: EP native microbiome inoculated in DI at T₁₈₀ gastric chyme; 24: EP native microbiome inoculated in AS at T₁₈₀ gastric chyme.

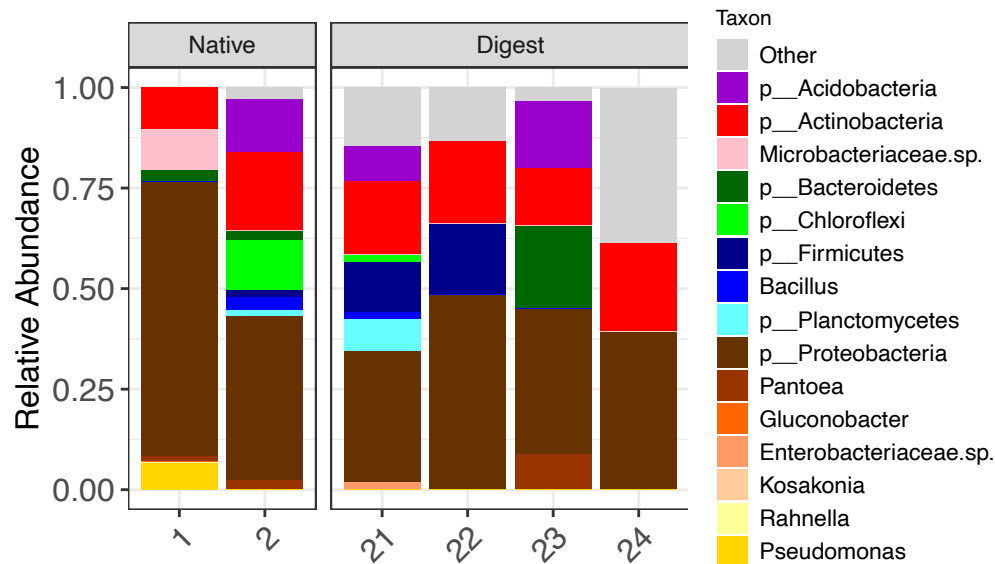


Figure SC-2: Bacterial phyla detected in different enrichment pH conditions and different rounds of enrichment.

Top left: 35: GD at pH 7, 1st round; 37: EP at pH 7, 1st round; 39: GD at pH 7, 2nd round; 41: EP at pH 7, 2nd round; 43: GD peel at pH 7, 3rd round; 44: GD flesh at pH 7, 3rd round; 45: EP peel at pH 7, 3rd round; 46: EP flesh at pH 7, 3rd round.

Top right: 10: Digesta of GD at pH 7, 1st round in CK; 11: Digesta of GD at pH 7, 1st round in SP; 12: Digesta of EP at pH 7, 1st round in CK; 13: Digesta of EP at pH 7, 1st round in SP; 52: Digesta of GD at pH 7, 1st round in W-O; 19: Digesta of EP at pH 7, 1st round in W-O; 25: Digesta of GD peel at pH 7, 3rd round; 26: Digesta of GD flesh at pH 7, 3rd round; 27: Digesta of EP peel at pH 7, 3rd round; 28: Digesta of EP flesh at pH 7, 3rd round; 51: Digesta of GD at pH 7, 1st round in DI; 3: Digesta of GD at pH 7, 1st round in AS; 4: Digesta of EP at pH 7, 1st round in DI; 5: Digesta of AS at pH 7, 1st round in AS.

Bottom left: 36: GD at pH 5, 1st round; 38: EP at pH 5, 1st round; 40: GD at pH 5, 2nd round; 42: EP at pH 5, 2nd round; 47: GD peel at pH 5, 3rd round; 48: GD flesh at pH 5, 3rd round; 49: EP peel at pH 5, 3rd round; 50: EP flesh at pH 5, 3rd round.

Bottom right: 14: Digesta of GD at pH 5, 1st round in CK; 15: Digesta of GD at pH 5, 1st round in SP; 16: Digesta of EP at pH 5, 1st round in CK; 17: Digesta of EP at pH 5, 1st round in SP; 18: Digesta of GD at pH 5, 1st round in W-O; 20: Digesta of EP at pH 5, 1st round in W-O; 29: Digesta of GD peel at pH 5, 3rd round; 30: Digesta of GD flesh at pH 5, 3rd round; 31: Digesta of EP peel at pH 5, 3rd round; 32: Digesta of EP flesh at pH 5, 3rd round; 6: Digesta of GD at pH 5, 1st round in DI; 7: Digesta of GD at pH 5, 1st round in AS; 8: Digesta of EP at pH 5, 1st round in DI; 9: Digesta of AS at pH 5, 1st round in AS.

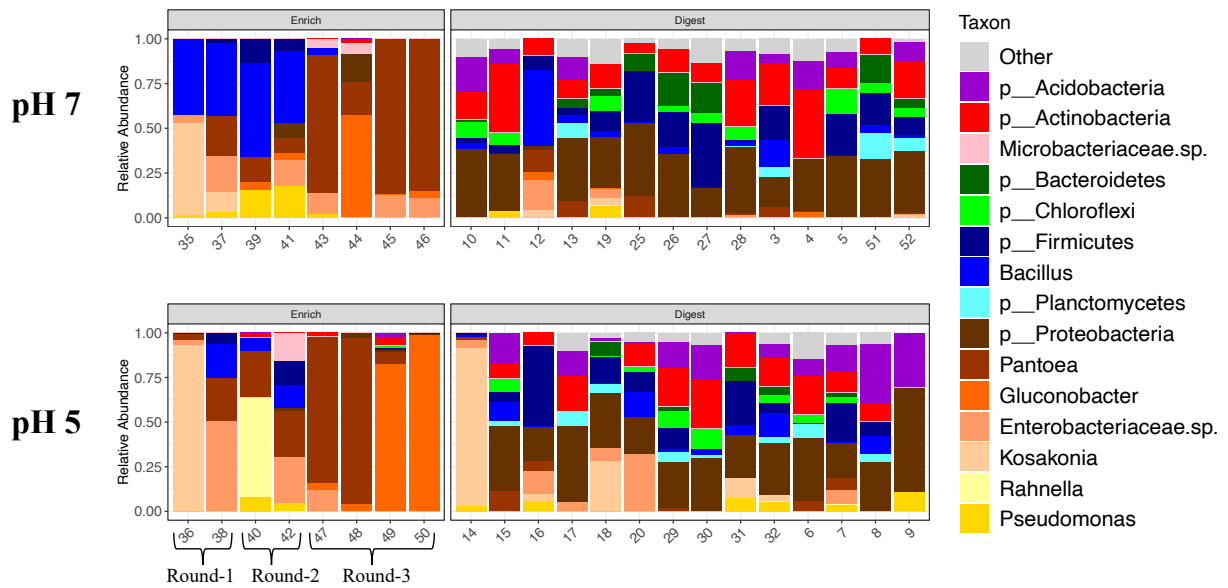


Figure SC-3: PCoA representing genus-level beta-diversity across the different types of samples consist native microbiome and from two enrichment pH levels.

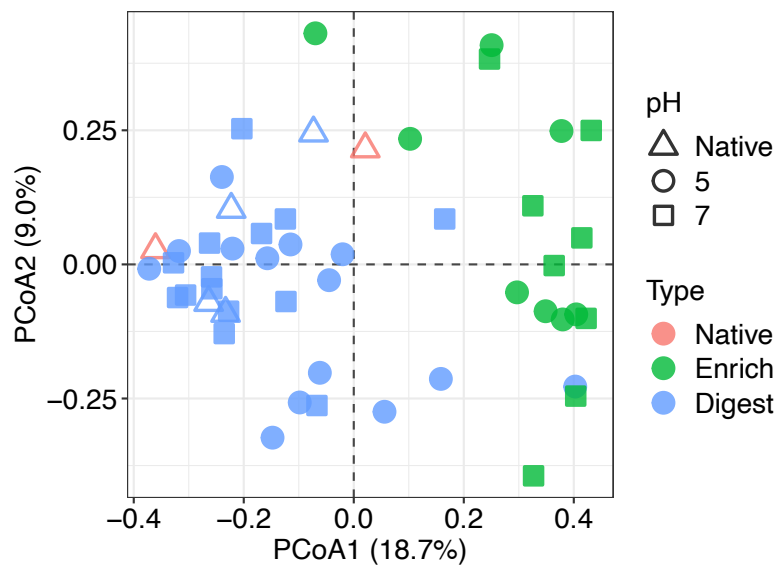


Figure SC-4: PCoA representing genus-level beta-diversity across the different rounds of enrichment from two enrichment pH levels.

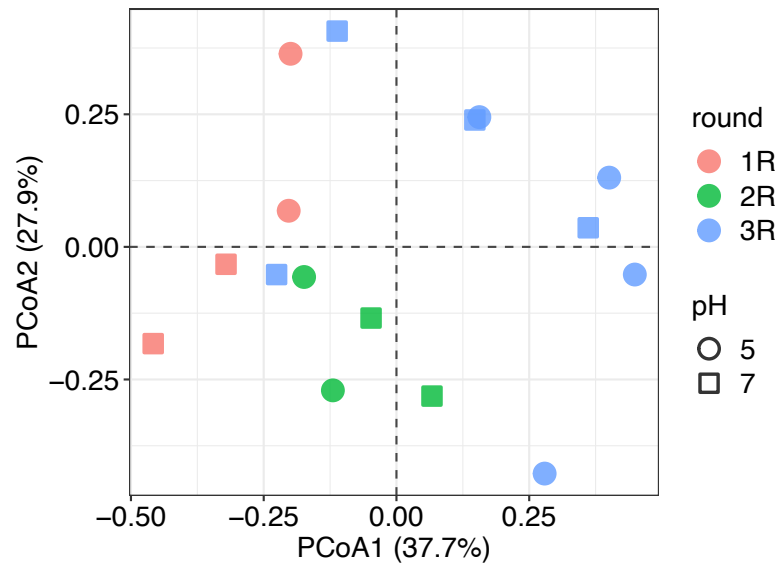
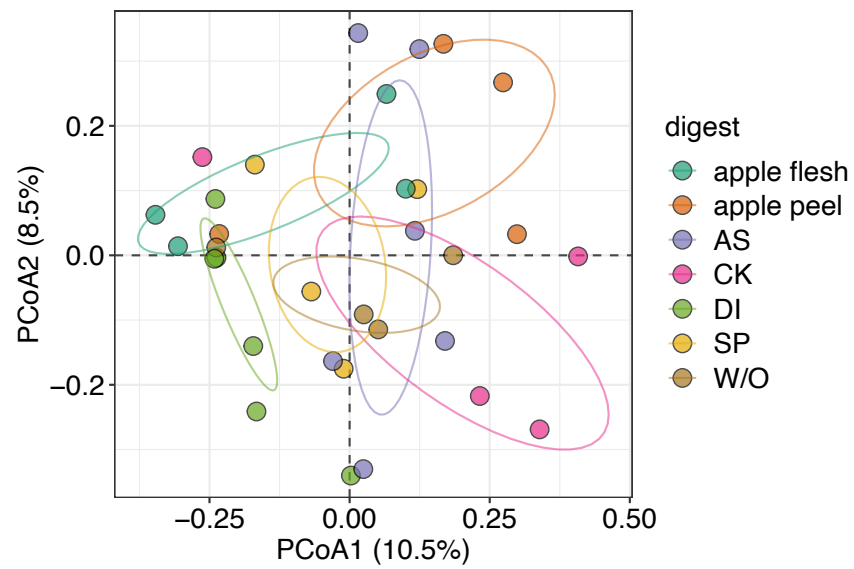


Figure SC-5: PCoA representing genus-level beta-diversity across digested samples in different food matrix.



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