ABSTRACT

Title of Dissertation: ENHANCEMENT OF THERMAL PROCESSING WITH FOOD-GRADE ANTIMICROBIAL COMPOUNDS IN LOW-MOISTURE FOOD MATRIX TO IMPROVE FOOD SAFETY

Qiao Ding, Doctor of Philosophy, 2022

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Heat resistant foodborne pathogens have been a concern in low-moisture foods and ingredients (LMFs). Increased thermal resistance of pathogens such as *Escherichia coli* O157:H7 and *Salmonella* Typhimurium at low water activity (a_w) reduces the efficiency of thermal treatment in LMFs. Alternative methods are therefore needed to augment thermal processing and reduce food safety risk. This study investigated the enhancement of thermal treatment efficiency against pathogenic bacteria in LMF matrices at different a_w by inclusion of food-grade antimicrobial compounds. Based on their similar target sites in cells, it is hypothesized that antimicrobial compounds may work synergistically with heat treatment in LMF matrices. The treatment effect may be dependent on both a_w and matrix compositions. Physiological and transcriptional changes may take place within cells adapted to different environments and contribute to the varied bacterial resistance. A combination of Butylparaben (BP), a known

antimicrobial and thermal treatment was tested to enhance inactivation rates of *S.* Typhimurium and *E. coli* O157:H7 in meat and bone meal (MBM) equilibrated to water activity (a_w) of 0.4 and 0.7. Presence of BP significantly accelerated thermal inactivation of *E. coli* O157:H7 in MBM at both 0.4 and 0.7 a_w. However, inactivation rate of *S.* Typhimurium was not affected by the combined treatment at either 0.4 or 0.7 a_w. BP accelerated the inactivation of the thermal-resistant subpopulation in *E. coli* O157:H7 but not *S.* Typhimurium. Therefore, further studies were conducted to identify alternative antimicrobial compounds to enhance thermal treatment against *S.* Typhimurium in LMF matrices and investigate the resistance mechanisms. Trans-cinnamaldehyde (CA) and eugenol (EG) were selected from a screening study to assist thermal treatment against *S.* Typhimurium adapted to different a_w in whey protein (WP), corn starch (CS) or peanut oil (PO). Although addition of CA or EG significantly accelerated thermal inactivation of *S.* Typhimurium in water and LMF components at 0.9 a_w, similar effect was not observed in bacteria adapted to lower (0.4) a_w in any of those matrices. The matrix effect on bacterial thermal resistance was observed at 0.9 a_w and were ranked as WP > PO > CS. The combined heat treatments reduced bacterial metabolic activity, which was partially dependent on the food components as well. *S.* Typhimurium had lower membrane fluidity because of membrane fatty acid composition change when adapted to a lower a_w, which contributed to resistance against the combined heat treatments. Based on the a_w- and matrix- dependent bacterial resistance and physiological changes observed in the two previous studies, transcriptional analysis was conducted to further understand the molecular mechanism behind the bacterial resistance. Expression of nine stress-related genes in *S.* Typhimurium adapted to different a_w in LMF components were analyzed with or without the CA-assisted heat treatment. The upregulation of *rpoH* and *dnaK* in *S.* Typhimurium was induced by the stress during bacterial adaptation to the

low a_w environments as well as the combined heat treatment, which contributed to the bacterial resistance to both desiccation and the combined treatment. Although its link to the desiccation response in bacteria is not fully understood, the downregulation of *ompC* during the combined treatment also partially contributed to the treatment resistance. The upregulation of *rpoE*, *otsB*, *proV* and *fadA* in *S*. Typhimurium was induced by the desiccation stress during incubation at the low a_w environment but was not a major contributor to the resistance during the combined treatment. The observed upregulation of *fabA* and downregulation of *ibpA* could not be directly linked to either bacterial resistance to desiccation or the combined heat treatment. Differential expressions were also observed among different a_w levels or in different matrices at the same a_w. The inconsistencies between the expression profiles of *dnaK* and *ompC* and bacterial resistance during the combined treatment suggested the presence of additional stress-response pathways.

In conclusion, the results have demonstrated the potential of using food-grade antimicrobial compounds to complement thermal treatment in LMF during processes that start with a relatively high a_w (such as dehydration). However, their effectiveness may subside at low a_w environments. Future works should focus on optimizing the parameters of the CA/ EG-assisted heat treatment for higher efficiency, studying the molecular mechanism behind the bacterial resistance at global scale, and exploring alternative non-thermal processing technologies to enhance thermal treatment efficiency in LMFs without raising thermal treatment intensity.

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IMPROVE FOOD SAFETY

by

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Dedication

I would like to dedicate my dissertation work to my parents, Zunian Ding and Haiying Xiao, who raised me up with all their love and caringness. It is their guidance, supports and encouragements that shaped me during the past twenty-eight years and made me who I am today. None of this would be possible without the love and supports from my mom and dad.

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Acknowledgements

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List of Abbreviations

LMFs: Low-moisture foods and food ingredients

A_w: Water activity

DNA: Deoxyribonucleic acid

RNA: Ribonucleic acid

BP: Butylparaben

EO: Essential oil

MBM: Meat and Bone Meal

CFU: Colony forming unit

HPLC: High Performance Liquid Chromatography

CA: trans-Cinnamaldehyde

EG: Eugenol

PO: Peanut oil

WP: Whey protein

CS: Corn starch

GC: Gas chromatography

UFA: Unsaturated fatty acids

SFA: Saturated fatty acids

qPCR: Quantitative polymerase chain reaction

Hsps: Heat shock proteins

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1 Chapter 1: Literature review and statement of problem

1.1 *Low-moisture Foods and Food Ingredients (LMFs) and related food safety concerns*

Low-moisture foods and food ingredients (LMFs) are products with a water activity (a_w)

< 0.85, which is below the minimum water activity level required for the growth of many

spoilage or pathogenic microorganisms and is thus considered less susceptible to microbial

spoilage and the growth of foodborne pathogens (Maltini et al., 2003; Sánchez-Maldonado et al.,

2018). They are either naturally low in moisture or that have been deliberately dried or

incorporated with ingredients to reduce their a_w (Beuchat et al., 2013; Podolak & Black, 2017).

Processes such as dehydration, curing, salting, and addition of sugar can all be used to reduce the

a_w of food products, which have been mainstream food preservation method for centuries

(Podolak & Black, 2017). Some of the most common LMFs include flour, cereals, chocolate,

spices, powdered infant formula, and animal feeds (such as meat and bone meal (MBM)).

Although the low a_w condition of LMFs does not support microbial growth, microorganisms

already present within the products may survive for a long time. Their growth may recover if

LMFs are to be rehydrated in subsequent steps (such as contaminated flour being used to make

raw cookie dough (Neil et al., 2012)) or if a_w exceeded 0.85 due to improper processing or

storage conditions. This can lead to potential food safety concerns.

In recent years, LMFs have been frequently identified as vehicles for foodborne

pathogens. The contamination of LMFs have been linked to an increasing number of outbreaks,

with 41 foodborne outbreaks and 7,315 illness cases linked with the consumption of LMFs

worldwide that resulted in 536 hospitalizations and 63 deaths between 2007 and 2012 (Table 1-

1). In addition, 5,141 LMFs products were recalled in the United States from 2007 to 2012 due to

possible contamination with foodborne pathogens (Table 1-2) (Santillana Farakos & Frank, 2014).

Many pathogenic microorganisms, such as *Salmonella* Typhimurium, *E. coli* O157:H7, and *Cronobacter Sakazaki*, are known to be able to survive in a desiccated environment (with a_w < 0.85) and remain viable for several months or even years (Beuchat et al., 2013). Those pathogenic bacteria already presented in LMFs are capable of causing foodborne diseases by themselves after direct consumption. Once the a_w of the food rises above the required minimum level for growth due to improper storage or handling, the surviving cells can grow back and increase the risk for causing foodborne diseases. Other common foodborne pathogens, such as *Bacillus cereus*, *Clostridium botulinum*, and *Listeria monocytogenes* have also been isolated from LMFs in the past and have been linked to cases of foodborne outbreaks (Beuchat et al., 2013; Podolak & Black, 2017). It has been determined that improper practices within processing facilities, such as sanitation (including contamination on floor, equipment and workers (T. Morita et al., 2006)), design and maintenance of facility and equipment (such as leaking pipes and over-crowded equipment (GMA, 2010)), ingredient control (including sourcing, delivery, storage and handling of raw ingredients (Koch et al., 2005)), and other operational practices (such as violation of good manufacturing practices (GMPs)), can all contribute to outbreaks associated with LMFs (Podolak et al., 2010). Therefore, it is critical to develop measures to reduce the pathogen load in LMFs in order to control the food safety risk. Understanding of the most prevalent pathogens in LMFs is needed before an efficient strategy can be developed. Next, I will discuss *Salmonella* Typhimurium and *Escherichia coli* O157:H7, two of the pathogens that are most commonly associated with LMFs.

Table 1-1. LMFs related foodborne-disease outbreaks from 2010 to 2021 (Sánchez-Maldonado et al., 2018; Santillana Farakos & Frank, 2014; U.S. Food and Drug Administration, 2022a)

Food Type	Pathogen	Affected Cases	Country	Year	Notes
Cashew brie	*S.* Duisburg *S.* Urbana	7	USA	2021	Cashews identified as the likely contamination source
Cake mix	*E. coli* O121	23	USA	2021	
All-purpose flour	*E. coli* O26	21	USA	2021	Patients reported eating, licking, or tasting raw, homemade dough or batter
Dried wood ear mushroom (fungus)	*Salmonella* spp.	43	USA	2020	
Tahini	*S.* Concord	6	USA	2019	
All-purpose flour	*E. coli* O26	21	USA	2019	
Cereal	*S.* Mbandaka	100	USA	2018	
Dried coconut	*S.* Typhimurium	14	USA	2018	
Nut butter	*E. coli* O157:H7	32	USA	2017	
Flour	*E. coli* (STEC O121 and STEC O26)	63	USA	2016	Pathogens were isolated from flour from patients' homes; likely caused by consumption of raw dough
Raw pistachios	*S.* Montevideo and *S.* Senftenberg	11	USA	2016	
Raw sprouted nut butter spreads	*S.* Paratyphi	13	USA	2015	
Dried vegetable spice mixes	*S.* Enteritidis phage type 13a	174	Sweden	2014-2015	Pathogens isolated from an unopened jar of dried vegetable spice mix in a

					restaurant with links to 108 cases.
Sprouted chia seed powder	*S*. Newport, *S*. Hartford, *S*. Oranienburg, and *S*. Saintpaul	63	Canada	2014	
	S. Newport, *S*. Hartford, *S*. Oranienburg,	31	USA	2014	
Almond and peanut butters	*S*. Braenderup	6	USA	2014	Pathogen was only found in the processing facilities instead of the products.
Roasted pistachios	*S*. Senftenberg	8	USA	2013	Pathogen was isolated from patients, pistachios, and environmental samples of the processing facilities
Dry dog food	*S*. Infantis	49	USA (20 states)	2012	Pathogen isolated in the finished product
Dry milk	*S*. Oranienburg	15	Russia	2012	Pathogen isolated in the finished product
Peanut butter	*S*. Bredeney	42	USA	2012	61% were children < 10 yrs old
Raw shelled walnuts	*E. coli* O157:H7	11	Canada	2011	
In-shell hazelnuts	*E. coli* O157:H7	8	USA (3 states)	2011	
Black and red pepper in salami products	*S*. Montevideo	272	USA	2009-2010	Pathogen was detected in both the final product and the container for the black/ red pepper
Raw cookie dough	*E. coli* O157:H7	77	USA (30 states)	2009	
Infant formula	*S*. Kedougou	42	Spain	2008	Pathogen isolated in the finished product
Rice	*S*. Enteritidis	82	USA	2008	Pathogen isolated in the finished product

Sweets and chocolate	*Salmonella* spp.	4	EU	2008	
Peanut butter	*S.* Typhimurium	714	USA (46 states)	2008	Pathogen isolated in the finished product; resulted in hash sentences for the corporation due to intentional cover-ups
Rice cereals	*S.* Agona	28	USA (15 states)	2008	Pathogen isolated in the finished product
Dry pet food	*S.* Schwarzengrund	62	USA (18 states)	2007	Pathogen isolated in the finished product
Peanut butter	*S.* Tennessee	425	USA (44 states)	2007	Pathogen isolated in the finished product
Sweets and chocolate	*Salmonella* spp.	242	EU	2007	Pathogen isolated in the finished product

Table 1-2. LMFs recalled for foodborne bacterial pathogen contamination in the U.S. from 2010 to 2014 (Gurtler et al., 2014; U.S. Food and Drug Administration, 2022b)

Food	Pathogen	Year
Powdered infant formula, pet food,	*Salmonella*	2022
Powdered infant formula	*Cronobacter sakazaki*	2022
Tahini, potato chips, ground coriander, flour, dry cat formula, dog and cat food, pet food, sesame oil, ground sesame paste	*Salmonella*	2021
Milk formula	*Cronobacter sakazaki*	2021
Apple chips, dried fungus, dog food, raw macadamia nuts, curry powder, coconut flour, tahini, crackers, pet food, cake mixes, roasted pistachios, cereal, ground sesame paste	*Salmonella*	2020
Raw nuts (raw macadamia nuts)	*E. coli*	2020
Tahini, taco seasoning, flour	*Salmonella*	2019
All-purpose flour, cookie and brownie mixes	*E. coli*	2019
Dried pet food, dog chews/ treats, chocolate coated candy, potato chips	*Salmonella*	2018
Flour, nut butter	*E. coli*	2018
Ginger powder	*Salmonella*	2017
Ground Malabar pepper, sprouted chia seed powder products, chia and flaxseed powder, dried dog food, dog treats, crushed chili powder, chili powder, ground annatto, sweet basil, organic black peppercorns, cat food, herbal supplement, organic basil	*Salmonella*	2014
Walnuts, shelled walnuts, dog and cat food, peanut butter	*Listeria monocytogenes*	2014
Salted fish	*Clostridium botulinum*	2014
Bird food/ treats, fish food, pet food/ treats, pistachios, dried oregano, dried sweetened breakfast cereal, baking mixes, protein bars	*Salmonella*	2013
Sunflower seeds and snack mixes	*Listeria monocytogenes*	2013
Bird food, tahini, chocolate, chocolate powder, pet food / treats, peanut butter candy, protein/energy/nutritional snack bars, peanuts and peanut butter, multiple peanut- containing	*Salmonella*	2012

foods/candies/desserts/snacks, almond butter, Thai red pepper, ground hot pepper, crackers, brownie and cookies, nutritional prebiotic formula, powdered milk replacer, children's nutritional powdered dietary supplement, dietary supplement capsules, starter yeast, cacao nibs, tempeh, rat and hamster feed, fish food, salt-free seasoning blend		
Popcorn	*Listeria monocytogenes*	2012
Organic celery seed, peanut butter, pine nuts, soybean flour and soy meal, pet treats, cat food, powdered whey protein mix/supplement, whey protein isolate, herbal tea, multiple snack products with chile from one company (wheat snacks, potato chips and corn churritos)	*Salmonella*	2011
Hazelnuts and mixed nut products	*E. coli* O157:H7	2011
Cookies	*Bacillus cereus*	2011
Black bean tortillas	*Clostridium botulinum*	2011
Walnuts, nutmeg, dark chocolate candy, dog treats, cat food, pistachios, black pepper and black peppercorns, garlic powder, sesame seeds, corn chips, potato chips, potato crisps, nuts, granola bars, soy grits and flour, crushed red pepper, multiple food products containing either black pepper, red pepper, or hydrolyzed vegetable protein (e.g., dried soup and sauce mixes, dip mixes, spice packs, snack mixes, bouillon, spice rubs, pretzels, potato chips, corn chips, stuffing, snack mix)	*Salmonella*	2010
Dried fish, dried seafood products from one company	*Clostridium botulinum*	2010
Gingerbread house	*Staphylococcus aureus*	2010

Escherichia coli is a gram-negative, facultative anaerobic bacterium. Most (~ 90%) of the *E. coli* strains live predominantly in animal intestines and have a mutually beneficial relationship with the hosts (Ramos et al., 2020). However, several *E. coli* strains have developed an ability to cause diseases in human gastrointestinal, urinary, or central nervous systems, making them pathogenic. Among them, *Escherichia coli* O157:H7 is an unusually virulent foodborne pathogen with low infectious dose, severe consequences of infection, and apparent resistance to low-a_w environment (Podolak & Black, 2017). It was a member of a group of *E. coli* strains that shared the similar pathogenic potential, known as the enterohemorrhagic (EHEC) *E. coli* (Griffin & Tauxe, 1991). After ingestion from contaminated food or water, the bacterial cells will colonize the colon and produce Shiga toxins, including Shiga toxin 1 (Stx1) and/or Shiga toxin 2 (Stx2), in the intestinal lumen (Buchanan & Doyle, 1997). Members of the Shiga toxin family contain one Stx A subunit with RNA-glycohydrolase activity and five Stx B subunits for toxin binding (Hurley et al., 1999).

Stx B binds to globotriaosylceramides (Gb3s) on the paneth cells in the human intestinal mucosa or kidney epithelial cells, which promotes the internalization of the Stx A subunit. Stx A will then be activated in the host cell Golgi via cleavage and causes cell apoptosis. Infection of *E. coli* O157:H7 may lead to the development of hemorrhagic colitis (HC), which is bloody diarrhea caused by the damage on intestinal tissue, and hemolytic uremic syndrome (HUS), where acute renal failure, thrombocytopenia, and hemolytic anemia can be caused by the toxin damage to the kidney (Croxen & Finlay, 2010; Hurley et al., 1999; Kaper et al., 2004). The death rate of HC alone is 2% in patients > 60 years old but lower and rare for the rest age groups (Mody & Griffin, 2015). Most infected individuals will develop watery/ bloody diarrhea with

crampy abdominal pain or low-grade fever but usually recover within eight days without the need for medical attention (Mody & Griffin, 2015; Petras & Frankel, 2009). However, 6% of the patients (15% in children with < 5 years age) will develop potentially life-threatening systemic complications such as the HUS, which has a fatality rate of 3% in children aged 5 years or younger and 33% in persons aged > 60 years (Mody & Griffin, 2015).*Salmonella* is another gram-negative, facultative anaerobic bacterium commonly found in animal intestinal tract. The original *Salmonella* strain shared a common ancestor with *E. coli* until its acquisitions of *Salmonella* pathogenicity island 1 (SPI-1) at about 100-160 million years ago (Gal-Mor, 2018). A subsequent acquisition of SPI-2 separated *Salmonella* into *S. enterica* and *S. bongori*. The two species of *Salmonella* can be further subtyped by the Kauffmann-White scheme according to three major antigenic determinants expressed on the membrane lipopolysaccharide (O), flagella (H), and capsular polysaccharide (K) (Eng et al., 2015). Among the 2,659 and 22 different serovars of *S. enterica* and *S. bongori*, *S. enterica* subsp. I are responsible for the most (99%) of human *Salmonella* infections (Gal-Mor, 2018; Su et al., 2004). Under *S. enterica* subsp. I, non-typhoidal serovars of *S. enterica* (NTS) are among the leading causes of foodborne illnesses worldwide, and have resulted in more hospitalizations and deaths compared to other foodborne pathogens in the United States (Anderson & Kendall, 2017; Scallan et al., 2011). Within the NTS strains, *S. enterica* serovar Typhimurium (*S.* Typhimurium) is one of the most commonly isolated strains from *Salmonella*-infected patients in the world (Galanis et al., 2006). Infection caused by *S.* Typhimurium typically induces self-limiting gastroenteritis diseases ("stomach flu") such as non-bloody diarrhea, vomiting and abdominal cramps (Anderson & Kendall, 2017; Eng et al., 2015; Gal-Mor, 2018). *S.* Typhimurium ingested in food can survive through the gastric acid barrier and invade the mucosa of the small and large intestine. It has an outer membrane

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protein RcK that will stimulate the host cell to engulf the bacterial cell via endocytosis (Gal-Mor, 2018; Koczerka et al., 2021). By utilizing the SPI-2 encoded type III secretion system (T3SS-2), *S*. Typhimurium can survive and replicate within these phagocytes until it crosses the epithelial barrier (Anderson & Kendall, 2017; Ochman et al., 1996). It can also directly inject effector proteins into host cells using a type III secretion system (encoded by SPI-1), which stimulates gut inflammation, causes diarrhea and may lead to destruction of the mucosa (Eng et al., 2015; Gal-Mor, 2018). Additional systemic diseases may be developed in immunocompromised patients, where the *S*. Typhimurium cells invade into the plasma and cannot be eliminated by immune cells after phagocytosis, leading to the transportation of the bacteria via the lymph system to the liver and lymph nodes and causes more severe symptoms such as chronic sequelae (Gal-Mor, 2018; Keithlin et al., 2015).

As enteric pathogens, *E. coli* O157:H7 and *S.* Typhimurium may enter the LMFs production chain by contaminating raw agricultural commodities through vehicles such as infected animals, raw or improperly composted manure and contaminated agriculture water (Santillana Farakos & Frank, 2014). Pathogens can also be transferred to products via un-sanitized contact surface and human hands (Beuchat et al., 2013). Once attached to the matrix, both *E. coli* O157:H7 and *S.* Typhimurium can survive in LMFs for an extended period of time during storage. Kimber et al. reported that the bacterial population declined by 0.15/ 0.35 log CFU/g/month for *S. enterica* and *E. coli* O157:H7 inoculated on pistachios with 0.4 a_w at 24 °C for 6 months, while < 0.09 log CFU/g/month reduction rate was observed for both bacteria when stored at < 4 °C for one year (Kimber et al., 2012). Kilonzo-Nthenge also reported that *S. enterica* and *E. coli* O157:H7 inoculated in store-bought peanut butter can be detected after 8 or 15 weeks of storage at 4 or 25 °C (Kilonzo-Nthenge et al., 2009). As a result, both of them have

been linked to multiple foodborne outbreaks of LMFs that took place around the world (Table 1-1 and 1-2). It is worth noticing that the involvement of *E. coli* O157:H7 in outbreaks and recalls of LMFs is limited when compared to *S*. Typhimurium, which is likely because of their lower survival rate than *S*. Typhimurium at low-a_w (Kimber et al., 2012; Santillana Farakos & Frank, 2014). However, that does not exclude it from pathogens of concern in LMFs as *E. coli* O157:H7 has low infectious dose and severe consequences, and has been identified as the causative agent in LMFs in several cases of foodborne illness (Podolak & Black, 2017). Both *E. coli* O157:H7 (Ahmed et al., 1995) and *S*. Typhimurium (Beuchat et al., 2013; Podolak & Black, 2017) can also develop increased thermal resistance at low a_w, making it worth to use them as target microorganisms during thermal treatment studies in LMFs. Thus, both *E. coli* O157:H7 and *S*. Typhimurium are among the major pathogens of interests in studies of food safety problems in LMFs. Their ability to survive in LMFs reveals the importance of understanding different factors in LMFs that can affect survival of these pathogens in LMFs. Next, I will discuss how two prominent factors, water activity and composition of LMFs, affect survival of these pathogens and highlight research gaps.

1.1.2 *Effect of water activity on bacterial resistance*

Water activity (a_w) is a parameter that reflects the intensity of interaction between water and solutes. The concept is commonly used in food industry to quantitatively describe the "free" moisture in the system that is available to microorganisms (Caurie, 2011). Since only the free water in food is available to interact with other molecules and participate in reactions such as growth of spoilage or pathogenic microorganisms, the concept of a_w is commonly used by the food industry to predict food spoilage and food safety risk. Growth of microorganisms can be negatively affected or prevented entirely by the reduction in free water content of foods, which

restricts bacteria's access to nutrients necessary for metabolic activity (Beuchat et al., 2013; Podolak & Black, 2017). Therefore, low a_w condition is seen as a growth barrier in foods for many vegetative pathogens. However, it has been observed that many virulent foodborne pathogens, including *Salmonella* spp. and Shiga toxin-producing *E. coli* (STEC) can survive for an extended time at low-a_w conditions (Table 1-3), some of which can even develop higher resistance towards antimicrobial treatments such as heating (Table 1-4) (Sánchez-Maldonado et al., 2018; Syamaladevi, Tang, et al., 2016). The effect of a_w on the survival, growth and resistance of pathogenic bacteria in various food matrices has made it an important factor to be considered for the preservation and inactivation processes during food production.

Table 1-3. Survival of selected foodborne pathogens in LMFs

Pathogen	Food matrix	Survival	Reference
Salmonella Tennessee	Peanut butter	Matrix (initial populations of 6–7 log CFU/g, a_w 0.17 – 0.25) population decreased by 0.15–0.65 and 0.34–1.29 log CFU/g after storage at 4 and 22 °C for 14 days, respectively	(E.-J. Park et al., 2008)
S. enteritidis	Halva	Matrix (initial population 3.87 log CFU/g, a_w 0.18) population decreased to 2.20–2.76 log CFU/g at 6 °C and 2.15–2.70 log CFU/g at 18–20 °C after storage for 8 months	(Kotzekidou, 1998)
S. Typhimurium	Egg powder	1.6–2.8 log reduction in matrix (a_w 0.29–0.37) after storing at 13 °C for 8 weeks	(Jung & Beuchat, 1999)
S. newport	Powdered cake mix/ soup mix, powdered skim milk	Reduction was minimal after storage at 25 °C for 25 days at $a_w < 0.22$, regardless of matrices 4–5-log reduction in matrices with a_w of 0.53 after 25 days storage; 2-log reduction were observed in cake mix (a_w 0.32), skim milk (a_w 0.22), and soup mix (a_w 0.14)	(Beuchat et al., 2013)

		after storing for 10, 9, and 27 weeks, respectively	
S. Anatum, *S.* Cubana, *S.* Infantis, *S.* Stanley, *S.* Montevideo	Alfalfa seeds	Matrix (initial population 4.8 log CFU/g; a$_w$ 0.21 - 0.6) population after storing at 5 °C for 52 weeks was reduced by 0.3 log; Matrix (a$_w$ 0.21, 0.4 and 0.6) stored at 25 °C for 52 weeks were reduced by 1.2, 1.2, and 4.5 log, respectively	(L.R. Beuchat & Scouten, 2002)
S. enteritidis Anatum, Tennessee, Montevideo	Pistachios (in shell)	Matrix (initial population 4.8 log CFU/g, a$_w$ 0.4 - 0.5) population did not reduce at -19 or 4 °C for 12 months; survivals were detectable after storing at 24 °C for 12 months	(Kimber et al., 2012)
Escherichia coli O157:H7	Infant rice cereal	Survived in matrix (a$_w$ 0.35–0.75) at 5, 25, and 35 °C for at least 24, 16, and 9 weeks, respectively	(Y. Deng et al., 1998)
	Biscuit (cookie) cream	Survivals in inoculated matrix (a$_w$ 0.75; initial population 2 log CFU/g) was detected after 2 days at 38 °C, 42 days at 22 °C, and 58 days at 10 °C	(Baylis et al., 2004)

	Potato starch	Matrix (a_w 0.24–0.78) stored at 4 °C; Reduction of 2.5–3.8 log after 33 weeks; More rapid reductions with increased storage temperature (4, 20, and 37 °C)	(C.-M. Park & Beuchat, 2000)
	Alfalfa seeds	Matrix (initial population 2.9–3.2 log CFU/g; a_w 0.15 - 0.54) stored at 5 °C for 52 weeks was reduced by 0.6–0.9 log; Matrix (a_w 0.15, 0.36, and 0.54) stored at 25 °C for 25 weeks were reduced by 2.1, 3.0, and 2.9 log, respectively	(L.R. Beuchat & Scouten, 2002)
Cronobacter sakazaki	Powdered infant formula	Inoculated matrix (a_w 0.14–0.27; initial population, 6 log CFU/g) stored at 20–22 °C; 2.4 log reduction in the first 5 months, 1 log reduction during subsequent 19 months; Survival detected after 687 days.	(Edelson-Mammel et al., 2005)
	Powdered infant formula	Inoculated matrix (a_w 0.21–0.31) stored at 25 °C; < 1 log reduction after 1 month and 4 log	(Osaili et al., 2008)

		reduction after 12 months at 25 °C	
	Infant cereal	Survival in infant cereals (a_w 0.30–0.83) was detected after 24 weeks at 4, 21, and 30 °C	(Gurtler & Beuchat, 2007)

Table 1-4. Summary of selected studies on the thermal resistance of *Salmonella* and *E. coli*

O157:H7 in LMFs (dash indicates data not available)

Target strain	Treatment medium	a_w	Temperature (°C)	D-value (min)	Z-value (°C)	Reference
S. Typhimurium	Peanut butter	0.2	90	3.71	-	(He et al., 2013)
		0.4	90	2.43	-	
		0.6	90	2.07	-	
		0.8	90	1.95	-	
	Milk chocolate	-	70	816	19	(Goepfert & Biggie, 1968)
	Salt solution	0.42	90	32.3	30.3	(Podolak & Black, 2017)
		0.31	90	20	40	
	Sucrose solution	0.83	65.5	40.2	7.6	(Summer et al., 1991)
	Glycerol solution	0.90	57.2	2.6	-	(Goepfert et al., 1970)
		0.75	57.2	8.8	-	
S. Tennessee	Peanut butter	0.2	90	7.05	-	(He et al., 2013)
		0.4	90	2.64	-	
		0.6	90	3	-	
		0.8	90	1.91	-	
	Glycerol solution	0.90	57.2	1.7	-	(Goepfert et al., 1970)
		0.75	57.2	6.0	-	
S. Senftenberg 775W	Milk chocolate	-	70	440	18	(Goepfert & Biggie, 1968)
	Glycerol solution	0.90	57.2	31.5	-	(Goepfert et al., 1970)
		0.75	57.2	41.5	-	
	Animal feed	(<15% moisture)	71.1	10	10.4	(Liu et al., 1969)
		(10% moisture)	71.1	115.2	11.0	
		0.5-0.6	69-71	80	30.3	

S. Weltevreden	Wheat flour	0.46-0.5	69-71	55	53.9	(Archer et al., 1998)
		041-0.45	69-71	55	19.6	
		0.36-0.4	72-74	75	15.2	
		0.31-0.35	69-71	345	29.2	
		0.25-0.3	69-71	165	34.7	
S. Anatum	Milk chocolate	-	90	11	24.2	(Barrile & Cone, 1970)
	Glycerol solution	0.90	57.2	2.3	-	(Goepfert et al., 1970)
		0.75	57.2	7.0	-	
S. Enteridis	Almonds	-	70	1	29	(Podolak & Black, 2017)
	Peanut butter	0.2	90	7.05	-	(He et al., 2013)
		0.4	90	2.64	-	
		0.6	90	3	-	
		0.8	90	1.91	-	
	Almond kernel flour	0.60	70	15.15	10.4	(Villa-Rojas et al., 2013)
		0.72	71	2.06	8.4	
		0.89	68	0.96	6.6	
		0.95	68	0.42	7.2	
E. coli	Glycerol solution	0.90	57.2	8.1	-	(Goepfert et al., 1970)
		0.75	57.2	13.0	-	
E. coli O157:H7	Apple	0.99	54.5	19.3	7.4	(Rana et al., 2021)
		0.90	54.5	23.1	10.9	
	Tofu	0.99	54.5	24.1	6.9	
		0.90	54.5	55.4	7.6	
	Kale	0.99	54.5	19.6	7.5	
		0.90	54.5	22.2	8.7	

1.1.2.1 Water activity in foods

Water is an important component in food systems with critical impact on the properties of foods. Water molecules are comprised of one oxygen atom connecting with two hydrogen atoms through covalent bonds, which forms a bent tetrahydro structure (Reid & Fennema, 2007a). The strong electronegativity of oxygen pulls the electrons from the hydrogen atoms closer to itself, leaving the two hydrogen atoms a partial positive charge, which forms a dipole and makes the molecule polar (Reid & Fennema, 2007a). It also allows the formation of hydrogen bonds with other water molecules or hydrophilic compounds, where the partial positive charge of the hydrogen atom is attracted to an atom of a nearby molecule with partial negative charge (de Jesus & Yin, 2017).

Inside food systems, water can be roughly divided into bound water and free water. Bound water refers to water molecules with reduced freedom of movement due to interaction with solute or to other non-water components in food and may not be available for physicochemical reactions (Caurie, 2011). The mobility of water reduces when the strength of molecular interaction between water molecules and its binding sites suppresses the water-water hydrogen bonds, causing water molecules to pack more densely and become less "mobile" (Reid & Fennema, 2007a). For example, the strong electric fields of some small/ multivalent ions (e.g. Na^+, Li^+ and Mg^{2+}) can interact with its surrounding water molecules via ion-dipole interaction to form a stronger bond and reduce their mobility; hydrogen bonding between water and various groups on hydrophilic compounds (e.g. hydroxyl, carbonyl and amine groups) can also reduce the mobility of adjacent water molecules (Reid & Fennema, 2007a). Due to these changes in interactions, the affected water molecules (known as "bound water") can no longer serve as solvents for essential biological compounds, and therefore become unavailable for the vital

biological reactions that takes place inside microorganisms such as the synthesis of cellular

materials, removal of waste byproducts, and transportation of nutrients (Podolak & Black, 2017).

Free water refers to the water molecules with higher freedom of movement and is only loosely

attached to solid components of foods via capillary forces. and (Caurie, 2011). Only the free

water in food system can support the growth of microorganisms and thus may affect food safety.

The concept of water activity (a_w) was first introduced by G. N. Lewis based on thermodynamic

principles for more than one hundred years ago (Lewis, 1907; Syamaladevi, Tang, et al., 2016).

It is a thermodynamic property that describes the escaping tendency ("fugacity") of water

molecules from food matrices. Water activity is defined as the ratio between the fugacity (i.e.

escaping tendency of the molecules) of water in the mixed matrix and the fugacity of pure water.

Since water vapor is commonly considered as an ideal gas in most food processing conditions, a_w

is more commonly approximated as the vapor pressure of water in a food matrix (P_v) divided by

the saturation water vapor pressure (P_{vs}) (i.e. the vapor pressure of pure water) at the same

temperature (Eq 1) (Podolak & Black, 2017; Syamaladevi, Tang, et al., 2016).

$$a_w = \frac{P_v}{P_{vs}} \text{ (Eq 1)}$$

After reaching an equilibrium with the surrounding air media (i.e. thermodynamic

equilibrium), the vapor pressure of water in the air of a closed system containing the food is

equivalent to the vapor pressure of water in that food matrix once the air reaches thermodynamic

equilibrium with the food matrix (Syamaladevi, Tang, et al., 2016; U.S. Food and Drug

Administration, 1984). Therefore, a_w can be easily determined by measuring the relative vapor

pressure or relative humidity of a small sample in a closed chamber after achieving equilibrium

with a manometer or an electric hygrometer water activity meter (Reid & Fennema, 2007b).

During the storage and processing of food products, a_w can be used to quantitively estimate the

amount free water that is thermodynamically available for various physicochemical or biological reactions, making it a more efficient parameter than moisture content to understand the effect of water on microorganisms in foods.

1.1.2.2 *Bacterial resistance at low water activity: physiological response*

Due to the lack of "free water" to transfer nutrient to the cells and the presence of osmotic pressure across cytoplasmic membranes, most bacteria, including those capable of causing human diseases, require a minimum a_w of 0.87 for growth (except for *Staphylococcus aureus*, which can grow at a_w between 0.85 - 0.87) (Sánchez-Maldonado et al., 2018). However, while a low a_w may serve as growth barrier for many foodborne pathogens, it still does not assure the safety of the food due to the bacterial adaptation at low-a_w for enhanced survivability and resistance.

To overcome the stress and survive at low a_w environments, bacterial cells in a desiccated state can 1) reduce their intracellular a_w through production of osmoprotectants such as glutamic acid or proline to reduce the damage caused by osmotic shock (Sperber, 1983); 2) modify the composition of its outer membrane through the expression of porins (Finn, Condell, et al., 2013); and 3) reduce their metabolic activity or even enter metabolically dormant state to conserve energy and nutrients for long term survival (Beuchat et al., 2013). The priority of bacterial survival mechanism at low a_w is to avoid loss of water by maintaining their turgor pressure. Bacteria usually combat the osmotic stress by first inducing influx of potassium ions through activation of the KdP transporter protein and the synthesis of its counterion glutamate in the cells (Finn, Condell, et al., 2013). Concurrently, bacteria can also increase the intracellular concentration of compatible solutes, also known as osmoprotectants, via biosynthesis and cross-membrane transportation (Balaji et al., 2005). Accumulation of these small electrically neutral

molecules, such as trehalose or proline can help maintain a balanced osmotic pressure between the cytoplasm and the surrounding environment and thus prevent the damage caused by osmotic shock (Sperber, 1983). The expression of outer membrane porins, including OmpF and OmpC, has also been observed in both *E. coli* and *Salmonella* to assist the passive transportation of osmoprotectants at low a_w (Kempf & Bremer, 1998). Additionally, while the supply of nutrients from extracellular environment is severely limited at low a_w, glucose is required for both the transportation and biosynthesis process of many osmoprotectants (e.g. trehalose). It has been reported that bacteria may switch to alternative energy source (such as fatty acid catabolism) as an alternative to generate ATP for essential cellular processes (Finn, Condell, et al., 2013). However, with prolonged adaptation to low-a_w environments, bacteria eventually reduce their metabolic activity or even enter metabolically dormant state ("viable but nonculturable" state) to conserve energy and nutrients for long-term survival (Beuchat et al., 2013).

Many pathogenic bacteria also exhibit a higher tolerance towards heat and other antimicrobial treatments in low a_w environments. The relation between a_w and bacterial heat resistance was first discovered more than 30 years ago on *Salmonella* in dehydrated milk powders (McDonough & Hargrove, 1968). It has been suggested that the molecular mobility of bacterial cells is reduced at low a_w environments, which helps to protect ribosomal units (such as 30S and 50S ribosomal subunits) against irreversible damage caused by thermal energy (Syamaladevi, Tang, et al., 2016). Liu et al. also hypothesized that water loss from bacterial cells at low a_w environment can reduce protein mobilization, and therefore strengthen the structure of heat sensitive proteins and increase bacterial heat resistance (Liu et al., 2018). Cross-resistance may also be induced at low a_w, where the desiccation stress response will lead to the expression of protease and chaperons, which can provide protection against damage from heat and other

antimicrobial treatments by refolding or eliminating denatured proteins and enzymes (Abee, 1999; Lou & Yousef, 1997). The increased synthesis of saturated fatty acids has also been observed in bacteria at low a_w, which can enhance bacterial membrane rigidity and protected the bacteria from heat-induced cellular component leakage and improve the barrier function against incorporation of extracellular antimicrobial compounds (Di Pasqua et al., 2006; Dubois-Brissonnet et al., 2011; Mejía et al., 1995; Scherber et al., 2009). The presence of water molecules alone also contributes to thermal inactivation via physicochemical interactions. Heat-induced destabilization or denaturation of cellular components (such as enzymes) will be less efficient without the presence of free water around the targets (Cebrián et al., 2017; Finn, Condell, et al., 2013). The lack of free water also limits the access of antimicrobial compounds to their targets in bacteria (Helander et al., 1998).

1.1.2.3 *Bacterial resistance at low water activity: genetic response*

The desiccation stress from a low a_w environment can also induce upregulation of stress response genes. Alternative sigma factor *RpoS* (σ^{38}), which serves as the master regulator to induce a general stress response upon exposure to environmental stresses, was activated in bacteria at low a_w (Chen & Jiang, 2017; Rangel, 2011). Similar activation has also been observed on *RpoE* (σ^{24}), the alternative sigma factor that regulates dehydration tolerance in *Salmonella* (Gruzdev et al., 2012). It has also been reported that *RpoH*, the heat shock response regulator (σ^{32}) in many gram-negative bacteria, was expressed in *E. coli* during carbon starvation (Jenkins et al., 1991). The activation of these alternative sigma factors lead to the upregulation/ downregulation of different down-stream stress response genes, which in turns contribute to increased survival and resistance of bacteria at low a_w. A transcriptomic analysis has identified 90 relatively upregulated and 7 downregulated genes in *Salmonella* at 0.4 a_w, which includes

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genes involved in protein biosynthesis, metabolism, energy production, and membrane synthesis (Gruzdev et al., 2012). Through the activation of the *rpoS*, genes related to biosynthesis and cross-membrane transportation (such as *proU, proP*, and *osmU*) are expressed to increase the intracellular concentration of osmoprotectants (Balaji et al., 2005) Chen and Jiang have observed the upregulation of the *dnaK* and *grpE* genes when exposing *S*. Typhimurium to aged broiler litter (0.81 a_w), which can increase bacterial thermal resistance via the expression of heat-shock proteins such as chaperone protein (Hsp70) and GrpE protein (Chen & Jiang, 2017). As discussed above, the heat shock proteins can help stabilize other proteins by repairing misfolded proteins and preventing protein accumulation under the stressed conditions (Roncarati & Scarlato, 2017). The expression of *fadA* gene, which encodes for 3-ketoacyl-CoA thiolase that can catabolize long-chain fatty acids into acetyl-CoA for producing ATP in the tricarboxylic acid cycle, was observed in *S. enterica* after a 6-day desiccation in peanut oil (0.52 a_w) and aged broiled litter (0.81 a_w) (Chen & Jiang, 2017; Fong & Wang, 2016). The downregulation of *fadA* gene was also identified in *Salmonella* incubated at 0.5 a_w (W Chen et al., 2014). It encodes the β-hydroxydecanoyl ACP dehydrase, which can reduce cellular membrane fluidity by producing longer chain fatty acid and thus increase bacterial thermal resistance (Magnuson et al., 1993). Both genes may lead to the changes of membrane fatty acids profiles and eventually affect bacterial membrane rigidity, which would increase bacterial resistance to both heat-induced leakage and permeabilization of extracellular antimicrobial compounds (Di Pasqua et al., 2006; Dubois-Brissonnet et al., 2011; Kollanoor Johny et al., 2017). A summary of selected genes of *Salmonella* Typhimurium that may be differentially expressed at low a_w conditions and may contribute to bacterial resistance is listed in Table 1-5. Our knowledge of the bacterial resistance at low a_w can partially explains the prevalence of both *E. coli* O157:H7 and *S*. Typhimurium in

LMFs and the need to develop enhanced thermal treatment against these pathogens at low-a_w conditions.

Table 1-5. Summary of selected differentially expressed genes that may contribute to *Salmonella* resistance at low a_w

Gene	Function description	Potential contribution to bacterial resistance	Reference
rpoS	Alternate sigma factor	General stress response	(Finn, Condell, et al., 2013; Gruzdev et al., 2012; H. Li et al., 2012)
rpoE	Alternate sigma factor	Heat and desiccation stress response	(Gruzdev et al., 2012; H. Li et al., 2012; Li, Overall, et al., 2015)
otsB	Trehalose biosynthesis	Encodes trehalose-6-phosphate phosphatase, an enzyme for trehalose (an osmoproteatant) biosynthesis.	(W Chen et al., 2014; Finn, Condell, et al., 2013)
proV	Osmoproteatant transporter	Regulates ProU transport system for glycine betaine (an osmoproteatant).	(W Chen et al., 2014; Finn, Condell, et al., 2013)
fadA	Fatty acid metabolism	Encodes 3-ketoacyl-CoA thiolase, which can catabolize long-chain fatty acids into acetyl-CoA for producing ATP in the tricarboxylic acid cycle. Thus it may reduce long chain fatty acid composition in membrane.	(W Chen et al., 2014; Finn, Condell, et al., 2013; Fong & Wang, 2016)
fabA	Fatty acid metabolism	Encodes β-hydroxydecanoyl ACP dehydrase, which introduces the double bond to fatty acid chain during its elongation synthesis,	(W Chen et al., 2014)

		therefore derives unsaturated fatty acid synthesis from saturated fatty acid synthesis.	
dnaK	Chaperon protein	Encodes a chaperone protein (Hsp70) that helps stabilize/ refold proteins during heat shock	(W Chen et al., 2014; Chen & Jiang, 2017; Fong & Wang, 2016)
grpE	Heat shock protein	Encodes the GrpE protein, which can interact and modify Hsp70 chaperone protein and enhance the protection efficiency	(W Chen et al., 2014; Chen & Jiang, 2017; Fong & Wang, 2016)
AcrA/B	Efflux pump	Encodes for the AcrA/B efflux pump, which has a broad specificity and can export a wide class of compounds, including toxic plant essential oils (such as basil EO and linalool).	(Birhanu et al., 2021; Eaves et al., 2004)
ompC	Outer membrane protein (porin)	Encodes ompC porin protein, which is an outer membrane aqueous channel that regulates the diffusion of various compounds across the membrane. Therefore it may provide antibacterial drug resistance.	(Kollanoor Johny et al., 2017)
ibpA	Small heat shock protein	Encodes for a small heat shock protein, which can bind to denatured proteins, prevent their aggregation and prepare them for further refolding by other chaperones (such as the ClpB-DnaK bichaperone system).	(Carroll et al., 2016; Tomoyasu et al., 2003)

1.1.3   *The effect of food matrix composition on bacterial resistance*

The physical structural and chemical composition of many LMFs can be complex, which may affect the treatment effectiveness. When different LMF matrices were used for inoculation, studies have shown that the D-values of *Salmonella* were dependent on matrix composition profiles when the sample a_w (0.3-0.9) and treatment temperatures were the same (Jin et al., 2018; Syamaladevi, Tadapaneni, et al., 2016). It was also found that due to the different sorption isotherm profiles between LMF matrices, the actual a_w in the matrix would develop component-dependent changes as the temperature increased during thermal treatment, causing bacteria inoculated in different food matrices with the same initial a_w level at room temperature to be exposed to different desiccation stress during heat treatments, thus affecting their thermal resistance (Syamaladevi, Tadapaneni, et al., 2016). At the same moisture content, the a_w of carbohydrate or protein-rich foods increases with the temperature, as the hydrogen bonds between water molecules and biomolecules are weakened at higher temperature, leading to the release of the free water from binding sites (Palmer & Fernández-Prini, Roberto Harvey, 2004). The a_w of lipid-rich matrices is partially dependent on the solubility of lipid molecules in water, which increases as the temperature increases (Khuwijitjaru et al., 2002). Therefore, the a_w in lipid-rich matrices may decrease as the temperature increases (Syamaladevi, Tadapaneni, et al., 2016). In addition, Difference in physical structure such as particle size (e.g. beef puree vs. ground beef vs. whole beef) can also lead to significant variations on the resistance of pathogens in LMFs to treatments (Mogollón et al., 2009; Syamaladevi, Tang, et al., 2016). Many LMFs systems also have complex chemical composition that may affect the adaptation of bacteria or the effectiveness of treatments.

High concentration of soluble salts such as sodium chloride or soluble carbohydrate such as sucrose may bind to the free water in the food matrix and reduce the a_w of the food matrix, which has been linked with the increase in thermal resistance of bacteria including *Salmonella* spp. (Gibson, 1973; Hansen & Riemann, 1963; Moats et al., 1971; Stackhouse et al., 2012; Syamaladevi, Tang, et al., 2016). Ca^{2+}, Mg^{2+}, or other divalent cations can increase protein stability, which may also contribute to the increased heat resistance of bacteria when there is high concentration of the corresponding salts (Hansen & Riemann, 1963).

The presence of lipids in LMFs has been shown to have protective effect on pathogenic bacteria against thermal treatments. For example, in ground beef, pork sausage and poultry, *E. coli* O157:H7 had the lowest D-values when treated in media with the lowest fat content (3-7%) compared to media with higher fat content (11-30%) (Ahmed et al., 1995). Similar protective effect from fat has also been observed in other LMFs including peanut butter that was inoculated with *Salmonella* Tennessee (Jin et al., 2018; Ma et al., 2009; Syamaladevi, Tang, et al., 2016). The thermal protective effect from fat may be attributed to the lower a_w within the fat part of the food matrix caused by a) localized absence of moisture (Hansen & Riemann, 1963) or b) increased solubility of fat in water at higher temperature that leads to reduced a_w during the heat treatment (Senhaji, 2007). It is also possible that the increased fat content may alter the heat transfer rate within the food matrix and causes uneven temperature distribution (Hansen & Riemann, 1963; Syamaladevi, Tang, et al., 2016). In addition, due to its nonpolar characteristic, lipids do not interact or form hydrogen bonds with water molecules. In fact, thermodynamically it is more favorable for water molecules to rearrange to form a more orderly structure while the surrounding fat molecules may aggregate so that their interfacial surface area is minimized. This can reduce the inter-phase migration of bacteria (Syamaladevi, Tang, et al., 2016) and result in

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the development of high thermal resistance for the subpopulation partitioning into the oil phase of LMFs with high lipid content.

The presence of lipid materials in food matrix may also affect the efficiency of treatment with antimicrobial compounds. Chen et al. reported that antimicrobial efficiency of cinnamaldehyde against S. Tennessee in the glycerol-sucrose model decreased at reduced a_w (Wei Chen et al., 2015). However, when peanut paste was used as a LMF model, the antimicrobial efficiency of the compounds increased at reduced a_w (Wei Chen et al., 2015). It was hypothesized that the reduced concentrations of protein and carbohydrate in the peanut flour with low a_w might have contributed to the increased antimicrobial activity (Wei Chen et al., 2015). The study also found that the antimicrobial efficiency of the compounds was negatively affected by the fat content in the system (Wei Chen et al., 2015). The authors hypothesized that the added lipids might solubilize and bind hydrophobic antimicrobial compounds, which would create a protective fat layer on the surface of bacterial cells that prevented the antimicrobial compounds from reaching the cell (Wei Chen et al., 2015; Farbood et al., 1976). Similar inhibitory effect from fat content has also been observed in cheese, where the antimicrobial effect of plant essential oils against both S. enteritidis and *Listeria monocytogenes* was reduced in cheese with higher fat content (Smith-Palmer et al., 2001).

The existence of proteins in food matrix can also protect some microorganisms against thermal treatment. It has been observed that bacteria treated in skim milk had much higher thermal resistance than in Ringer's solution (a general-purpose saline solution), as the D-value of *Streptococcus faecalis* in skim milk (3.33 - 10 min) was 2-9 times higher than in saline solution (0.83 - 1.02 min) at the same temperature (60 ℃) (Hansen & Riemann, 1963; White, 1952). Although its protection mechanism is not well understood, protein may increase the

stability of enzymes and other protein structures on cell surface by forming a heat resistant complex via electrostatic attraction (Hansen & Riemann, 1963). Previous studies has also reported that the hydrophobic functional groups on protein molecules have high binding capacity for many phenolic/ terpenoid-like compounds, which may lead to inhibitory effect against the antimicrobial compounds (Baranauskienė et al., 2006; Pol et al., 2001).

It has been reported that in aqueous system, lower (< 5) or higher (> 7.5) pH conditions reduced the thermal resistance of *S. enteritidis* PT4 in modified tryptone soya broth (TSB) (Blackburn et al., 1997; Doyle & Mazzotta, 2000). A decrease of pH from 7 to 3 reduced D_{50} value of *S*. Typhimurium from 28.7 to 0.4 min, while an increase of pH from 6 to 7.7 reduced the D_{56} value of *S*. Typhimurium by three fold (Casadei et al., 2001; Mañas et al., 2003). Disruption to the electron transfer chain, solubilization of membrane protein, or saponification of membrane lipids on bacterial cells in acidic/ alkaline environment may all contribute to the reduced heat resistance (Bender & Marquis, 1985; Mañas et al., 2003; Teo et al., 1996).

Different matrix components may also alter bacterial transcriptional responses at low a_w. In one study, the expression level of the *kdp* genes, which encodes a potassium transporter, were expressed > 170-fold higher in *Salmonella* exposed to 0.3 M NaCl than bacteria in 0.6 M sucrose. However, few other studies have looked at the effect of different LMF components on bacterial transcription. Hence, besides a_w, it is also important to investigate the effect of different matrix components on bacterial resistance while developing antimicrobial treatments in LMFs.

1.2 Thermal Processing

1.2.1   The status of current thermal processing technologies

To improve the safety of LMFs, pathogen levels can be controlled by specific processing steps designed to 1) effectively inactivate microorganisms ("kill step"), 2) maintain a sanitary environment, and 3) avoid cross-contamination during the manufacturing process (Podolak & Black, 2017). Among them, thermal processing is the mainstream technology used as the kill step during LMFs production, which utilizes heat to inactivate microorganisms and enzymes in foods and enhance both the safety and stability of the products (Grasso et al., 2014). In addition, energy supplied by heat treatment can also promote beneficial reactions, including Millard reaction, protein denaturation and starch gelatinization, to improve the sensory and digestibility of food while reducing antinutritional compounds such as legumes (Fellows, 2017). However, heat processing may also compromise the nutritional and sensory quality of foods. The higher temperature may induce deleterious effects on heat-sensitive nutrients such as vitamins, amino acids or phytochemicals, promote lipid oxidation, and reduce favorable odorous compounds (Fellows, 2017).

During thermal processing, the processed food matrix is heated to the pre-determined target temperature and held at this condition with enough time to achieve the desired levels of inactivation, sensory and nutrition improvement. In terms of food safety, the choice of thermal processing parameters (temperature – time combination) is usually determined based on the choice of target microorganism, which is usually the most thermal-resistant microorganism in the food product (Kubo et al., 2021).

Based on their target objectives and treatment intensities, thermal processing can be divided into pasteurization and sterilization treatments (May & Campden, 2001; M. van Boekel

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et al., 2010). Pasteurization processing is designed to inactivate vegetative pathogenic bacteria and some spoilage enzymes in foods. Inactivation of vegetative pathogens such as *Salmonella* spp. can be achieved by applying mild heat treatment (< 95 °C) for a specified time (based on food matrices and target inactivation levels), while the quality of foods can be also be maintained (Silva & Gibbs, 2012). It is also used to extend the shelf life of foods that are stored at refrigeration conditions. Sterilization aims to inactivate not only vegetative cells, but also microbial spores as well as many toxins existed in foods. This process generally requires a treatment temperature at > 100 °C in order to ensure the stability of the foods at room temperature (M. van Boekel et al., 2010). While being effective against pathogens and their spores, the intensive treatment condition may negatively affect the organoleptic and nutritional properties of the products (Cebrián et al., 2017).

Traditional thermal process, such as the retort technology, usually involves the generation of heat outside the product (or containers holding the product) being processed, which is transferred to the products via the conduction and convection methods (May & Campden, 2001). Heat transfer may occur through either direct (e.g. air, steam, water) or indirect (e.g. hot surface heated by a heating medium) contact with a heating medium (May & Campden, 2001; Vicente & Machado, 2011). Due to the limitations associated with traditional heating such as energy loss on the heat transfer surface and limited heat transfer efficiency, novel thermal processing technologies, such as ohmic heating, dielectric heating, inductive heating and infrared heating, have been developed as an alternative to be used during food manufacturing (Leong & Oey, 2022; Vicente & Machado, 2011).

In ohmic heating, heat is generated by the passing alternating electrical current (AC) through the food matrix via the electrodes installed at both ends of the product (Richardson,

2001). The food system serves as an electrical resistance in the circuit where heat is being generated. Inactivation during ohmic heating is mainly caused by the heat generated, with additional damage caused by electroporation, where electrical charges may cause damage to bacterial membrane. Treatment effect is determined by the electric field strength applied by the electrodes and the electrical conductivity of the food system. Dielectric heating includes both microwave processing and radio frequency processing. In microwave heating, water molecules in foods vibrate under a high frequency electromagnetic field (915 – 2450 GHz), which, together with the oscillatory movement of ionic charges, induce volumetric heating of the food (Vicente & Machado, 2011). In radio frequency processing, a high frequency AC electric field (13.56 – 40.68 MHz) is applied between the plates of a capacitor (radio frequency applicator) (Richardson, 2001). Heat is also generated by the movement of polar dielectric molecules (such as water) within foods after the molecules absorb energy in the electric field. The effect of microwave processing is mainly dependent on the dielectric properties and temperature of food matrix, while the radio frequency processing effect is also dependent on the electrical conductivity of the foods (Richardson, 2001). In infrared heating, treatment is applied by shining infrared light (0.7 µm – 1 mm) directly onto food surface. Energy from the wave is transmitted to food via interaction with molecules and penetrating through the food matrix by conduction (Vicente & Machado, 2011).

For most of the alternative thermal processing technologies, heat is generated from the interaction between electric field or electromagnetic waves with molecules inside the food (Leong & Oey, 2022; Vicente & Machado, 2011). This may allow food to be heated more evenly and efficiently, which may lead to less energy consumption and improvement in nutritional quality of food (Vicente & Machado, 2011). However, disadvantages such as high equipment

and operating costs, difficult process validation, varied efficiency for different products, and lack of understanding for certain treatment mechanisms still present challenges for the adoption of the novel thermal processing technologies (Vicente & Machado, 2011). Overall, the effect of current thermal processing technologies is highly dependent on the complexity and inherent properties of the food matrices, including thermal conductivity, water activity, moisture content, presence of particulates, rheological properties, food porosity, and electrical resistance, and may pose potential risk to food safety when its efficiency is reduced.

1.2.2 *Inactivation mechanisms of thermal treatment*

Generally, inactivation of vegetative bacteria takes place at 50 °C with increasing inactivation rate at higher temperatures, while bacterial spores can be more thermally resistant and require a significantly higher temperature (> 100 °C) to reduce their viability (Russell, 2003). The thermal treatment efficiency can be described with the inactivation kinetics, represented by the Log_{10} value of the number of survivors versus treatment time (Cebrián et al., 2017). When the inactivation kinetics follows a first-order kinetics, i.e. the number of surviving population is an exponential function of the thermal treatment time, the thermal treatment can be described with the log-linear model (Bevilacqua et al., 2015; Jay et al., 2005) (Eq 2).

$$\log N_t = log N_0 - \frac{t}{D} \quad \text{(Eq 2)}$$

At a given time t, the logarithm of the initial microbial population and the survivors is described as log N and log N_0. D is the decimal reduction time determining the required treatment time to reach a 1-log reduction within the microbial population at a specific temperature. After acquiring data from treatments at different temperatures, the increase of temperature resulting in 90% reduction of the D value, known as the Z value, can be computed

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by plotting the *D* values versus treatment temperatures, which describes the effect of temperature on thermal inactivation (Jay et al., 2005).

The log-linear model assumes that the percentage of inactivated microorganisms is constant after equal time interval during the heat treatment at a constant temperature,. In other words, the treatment is an isothermal process and all cells in the target population are equally resistant to the treatment (Cebrián et al., 2017; Kubo et al., 2021). However, fitting the log-linear model to thermal inactivation kinetics in LMFs can sometimes be challenging. Firstly, due to lack of convection, the uniform distribution of heat in the matrix can often be jeopardized by components with different thermal conductivity. Secondly, as discussed previously, bacteria such as *Salmonella* can develop a_w-dependent thermal resistance in LMFs, which may hinder the effect of heat treatment. Both reasons could lead to the observation of shouldering and tailing (lag phase in the beginning or end of an inactivation curve) in thermal inactivation kinetics of LMFs. The shouldering part of the inactivation curve represents a treatment period where stresses have accumulated within the target sites without causing instant inactivation, while the tailing part suggests that a subpopulation of the microorganisms are either intrinsically more resistant than others or have developed higher resistance during the treatment by factors such as a_w change or deactivation of antimicrobial agents (Xiong et al., 1999). As a result, non-linear kinetics models such as the Weibull model (Eq 3), have been used to describe the thermal inactivation kinetics in LMFs (Dhaliwal et al., 2021).

$$log\left(\frac{N_t}{N_0}\right) = -\frac{1}{2.303}\left(\frac{t}{\alpha}\right)^\beta \text{ (Eq 3)}$$

Where α is a scale parameter that describes the characteristic time and β is a shape parameter of the inactivation curve. Based on the Weibull model, the time needed to reach a 1-log reduction (t_{D1}) can be computed using equation (Eq 4)

$$t_{D1} = \alpha(2.303)^{\frac{1}{\beta}} \ \text{(Eq 4)}$$

The Weibull model considers different shapes of survival kinetics as cumulative forms of

Weibull distributions of individual thermal resistance among the microbial cells within the

population and can accurately describe straight, concave upward and downward curves (Kubo et

al., 2021; Peleg & Cole, 1998; M. A. J. S. Van Boekel, 2002). Three additional mathematical

models used to fit the survival or inactivation curves were summarized in Table 1-6.

The inactivation mechanism of heat on microorganisms has been widely studied. It has been

widely accepted that thermal inactivation on bacterial cells is a multi-target phenomenon

(Cebrián et al., 2017; Mackey et al., 1991; Syamaladevi, Tang, et al., 2016; Tattawasart et al.,

2000). The function and structure of many critical cellular components can be affected by

thermal treatment, which includes the cellular membrane, nucleic acids (DNA/ RNA),

ribosomes, proteins and enzymes (Cebrián et al., 2017; Syamaladevi, Tang, et al., 2016).

Thermal treatment can induce loss of membrane components and damage to the integrity of the

cytoplasmic membrane, which affect cellular respiration activity, intracellular osmotic and pH

homeostasis, and the ability to control the entry and exit of solutes/ nutrients (Kramer &

Thielmann, 2016; Leguérinel et al., 2007; Marcén et al., 2017; Teixeira et al., 1997).

Morphological and structural changes, increase of membrane permeability and fluidity, and

release of lipopolysaccharide have been found on heat treated *E. coli* cells (Katsui et al., 1982;

Tsuchido et al., 1985). The loss of intracellular materials (potassium ions, nucleotides, amino

acids) and increased sensitivity to hydrophobic antibiotics after thermal treatment have also been

reported (Cebrián et al., 2017; Mackey, 1983). Although the heat-induced alteration to the

cytoplasmic membrane has induced apparent disruption to cellular activities, a clear correlation

between membrane damage and bacterial inactivation has not been shown (Marcén et al., 2017;

Russell, 2003). However, this damage can be of paramount importance with the simultaneous presence of other preservative factors during the treatment (such as hydrophobic antimicrobial compounds). For Gram-positive bacteria such as *S. aureus*, heat treatment can induce the reduction of D-alanine from the teichoic acids on its peptidoglycan cell wall, causing chelation of the Mg ions and affecting bacterial metabolic activity (Cebrián et al., 2017).

Nucleotides, including both deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) molecules, can be affected by heat treatment. Although it is considered as one of the most thermally resistant cellular components, bacterial DNA may suffer from single or double strand breaks when exposed to sterilization temperatures (Mackey et al., 1991; Russell, 2003). Less-intensive heat treatment can also induce mutations and damages on DNA, which increase its exposure to endonucleases and cause higher degree of denaturation after the thermal treatment (Cebrián et al., 2017). DNA is also a critical component for the cellular damage-repairing activities. Heat-induces damages to other cellular components can only be repaired or replaced if DNA is sufficiently functional to provide the appropriate genetic information (Russell, 2003). RNA and ribosomes are more thermosensitive than DNA. It has been identified that the irreversible destabilization of ribosomes, specifically 30S and 50S ribosomal subunits, contributed to thermal inactivation of *E. coli* and *Lactobacillus plantarum* (Lee & Kaletunç, 2002; Mackey et al., 1991; Syamaladevi, Tang, et al., 2016).

Structural and functional changes (mainly the disruption of the complex hydrogen-bonded structure) may take place on proteins during heat treatment (Russell, 2003). The heat-induced denaturation and/ or aggregation of proteins, including both structural protein and enzymes, can lead to major disruption to cellular function and contribute to the inactivation. For example, damage-repairing enzymes that play an important role in the bacterial repairing and

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recovery process, such as chaperones, proteases, and DNA repair enzymes, are all susceptible to thermal denaturation (Cebrián et al., 2017). RNA polymerase could also be targeted by the heat-induced denaturation, further reducing the repair efficiency of bacteria to resynthesize replacement proteins. Additionally, thermal denaturation of detoxifying enzymes, including catalase and superoxide dismutase, has been reported to have caused the accumulation of reactive oxygen species in heat-treated bacteria, leading to further damages on cellular components including membrane lipids, proteins, and the DNA (Arku et al., 2011; Marcén et al., 2017).

Together, thermal inactivation is contributed by the alteration of at least one cellular targets beyond the critical threshold (Cebrián et al., 2017). Since the damage/ repair of many cellular components are interrelated, the ultimate inactivation is likely to be induced by the parallel alteration of multiple cellular targets.

Table 1-6. Summary of additional mathematical models to describe bacterial inactivation

kinetics

Model	Mathematical formula	Description	Reference
Cerf	$$\frac{N(t)}{N_0} = fe^{-k_1 t} + (1-f)e^{-k_2 t}$$	k_1 and k_2 stand for the two different slopes in the inactivation kinetic. f is the fraction of the initial population (less resistant) characterized by the death rate k_1, while $(1-f)$ stands for the second subpopulation (more resistant) characterized by the death rate $k2$	(Cerf, 1977; Xiong et al., 1999)
Whiting	$$\frac{N(t)}{N_0} = \log\left(\frac{F_1(1+e^{-b_1 t_1})}{1+e^{b_1(t-t_1)}} + \frac{(1-F_1)(1+e^{-b_2 t_1})}{1+e^{b_2(t-t_1)}}\right)$$	b_1 and b_2 stand for the maximum specific death rate of the major population and the initial subpopulation; F_1 stands for the fraction of the initial population in the major population; t_1 stands for the time where the shoulder ends.	(Whiting, 1993)
Cole	$$logN(t) = \alpha + \frac{\omega - \alpha}{1 + e^{4\sigma(\tau - \log t)/(\omega - \alpha)}}$$	Alpha stands for upper asymptote (shoulder); Omega stands for the lower asymptote (tail); Sigma stands for the maximum slope of the curve; Tau stands for the position (on the log t axis) with the maximum slope.	(Cole et al., 1993)

1.3 *Hurdle technology to enhance treatment effect*

Hurdle technology refers to a deliberate combination of preservative technologies to build up a series of preservative factors (known as "hurdle"), where each of the hurdle acts as a barrier that bacteria must "leap over" in order to survive (Leistner & Gorris, 1995). When all the preservative factors are applied together, the combination of hurdles cannot be overcome by target microorganisms presented in foods (Leistner, 2000; Leistner & Gorris, 1995). Sometimes the intensity of a particular hurdle by itself is not strong enough and the target microorganisms can easily overcome the stress; or in some other cases, the side-effect of a particular hurdle is so strong that it may cause detrimental effect towards product quality (Leistner, 2000). However, with hurdle technology, existing and novel treatment methods are intelligently combined and applied with reduced doses and interact synergistically to achieve greater antimicrobial efficiency without sacrificing the quality of the final products (Khan et al., 2017; Singh & Shalini, 2016).

Some pathogens may develop higher resistance to different hurdles via the expression of stress response genes when exposed to stress from a single treatment ("cross protection"). However, during a combined treatment, different hurdles may disrupt the intracellular homeostasis of microorganisms (such as osmotic balance, pH, or oxidative stress), which leads to inhibition of growth or inactivation (Leistner & Gorris, 1995). The mechanisms developed by microorganisms to restore homeostasis, such as the activation of ion pumps or the synthesis of osmoprotectants, can be energy consuming and may lead to metabolic exhaustion (Leistner, 2000; Leistner & Gorris, 1995). Therefore, when stress from different hurdles during a combined treatment are applied simultaneously, bacteria are forced to spend more energy to neutralize the hurdle effect and are likely to suffer from metabolic exhaustion (Leistner, 2000; Singh & Shalini,

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2016). It is plausible that with a rational selection of different hurdles that target same or different cellular components as the heat treatment, the efficiency of existing thermal processing method on LMFs can be enhanced through addition of food-grade antimicrobial compounds with sub-lethal intensity.

Previous studies have demonstrated enhancement of thermal treatment by inclusion of food-grade antimicrobial compounds such as parabens (Gao et al., 2020; Gurtler & Jin, 2012; Ruan & Buchanan, 2016) and essential oil (EO) compounds(Espina et al., 2012, 2014; Sarbu et al., 2019) in aqueous system, which have been summarized in Table 1-7. However, only a few have studied the effect of antimicrobial compounds-assisted heat treatment in LMFs or at low-a_w conditions. In one study, Amalaradjou et al. reported that a combination of 0.5% CA induced > 6 log reduction on *Staphylococcus aureus* inoculated in reconstituted infant formula stored at 23 °C for 10 h (Amalaradjou et al., 2009). Chen et al. has also observed the antimicrobial effect from 0.12% carvacrol, cinnamaldehyde, and lauric arginate at room temperature for 3 days in both the glycerol-sucrose model and peanut paste (a_w 0.3 - 1) (Wei Chen et al., 2015). A study conducted Xu et al reported that heat treatment at 80 °C for 5 min followed by 3-d treatment with cinnamon oil vapor (0.83 µL/mL at 25 °C) has induced > 5 log reductions of *Salmonella* on sesame seed and flaxseed (a_w 0.44) (Y. Xu et al., 2022). However, another study reported that although there was synergistic effect between oregano oil (1%) and heat treatment (56 – 58 °C) in tahini (sesame seed paste) with high a_w (1.0), the effectiveness of the EO to enhance thermal inactivation was absent at lower a_w (0.3) (Y. Xu et al., 2021). The inactivation mechanism of the antimicrobial compounds will be discussed in details in the next section.

Table 1-7. Representative studies on food-grade antimicrobial compounds-assisted thermal processes in different medium (Adapted from "Inactivation of foodborne pathogens by the synergistic combinations of food processing technologies and food-grade compounds" by Zhang, H., Tikekar, R. V., Ding, Q., Gilbert, A. R., & Wimsatt, S. T., 2020, *Comprehensive Reviews in Food Science and Food Safety*, *19*(4), 2110–2138. Copyright 2020 by John Wiley & Sons. Adapted with permission.)

Processing conditions	Food grade compounds	Target pathogens	Food/ medium	Synergistic antimicrobial effects	Reference
47-53 °C	Malic acid 0.5% sodium benzoate 0.1% potassium sorbate 0.1%	*Escherichia coli* O157: H7	Apple cider	The addition of malic acid, sodium benzoate, or potassium sorbate reduced the D-value to 13.87, 6.99, and 13.18 min, respectively. D-value for heat treatment at 50 °C alone was 65.2 min.	(Dock et al., 2000)
52-58 °C	Methyl-, ethyl-, propyl-, butyl-, and hyptyl-paraben	*Cronobacter sakazakii*	BHI broth	Heating in combination with 125 ppm parabens led to 2.7 to > 6.0 log reductions at 58 °C for 900 s. Heating alone inactivated < 2.5 log bacteria, and parabens (≥ 125 ppm) alone did not pose appreciate effects.	(Ruan, 2016)
65 °C 5 min	Enterocin AS-48 20 μg/mL	*Staphylococcus aureus*	Reconstituted skimmed milk	The combined treatment reduced the bacterial population to below the detection limit. Comparatively, thermal treatment inactivated 97.7% but > 6 log CFU/mL bacteria was recovered;	(Muñoz et al., 2007)

				Treating by enterocin alone obtained > 2 log recovery.	
54 °C 10 min pH 4.0	Citrus fruit essential oils 200 µg/L	*Escherichia coli* O157: H7	Citrate phosphate buffers	The combined treatment resulted in > 5 log reduction within 4 min, whereas heating inactivated bacteria by < 1 log CFU/mL, and essential oil alone inactivated bacteria by < 0.5 log CFU/mL.	(Espina et al., 2012)
55 °C	Magainin II amide 5 µg/mL	*Listeria monocytogenes* *Escherichia coli* BM	TPB broth (*Listeria*) Nutrient broth (*E. coli*)	The combined treatment resulted in > 4.8/2.0 log reductions for *L. monocytogenes/E. coli* in 4 min. The thermal process resulted in < 0.5 log reduction for both bacteria in 4 min, and Magainin II amide inactivated 2.9 log *L. monocytogenes* or < 0.5 log *E. coli* in 7 h.	(Ueckert et al., 1998)
65 °C 32 s	Nisin and lysozyme 31.25 AU/cm^2	*Listeria monocytogenes*	RTE low-fat turkey bologna	Sample with the addition of nisin or lysozyme both resulted in 3.8 log reduction and reduced to below the detection limit within 4 weeks. While thermal treatment inactivated *Listeria* by 3.5 logs following by 2 logs of recovery after 12 weeks, and no antimicrobial effect was observed for nisin.	(Mangalassary et al., 2008)
100 °C	Nisin	*Bacillus* sporother	Skim milk	Sample with the addition of 134 UI/mL nisin was inactivated by ~ 5 log	(Aouadhi et al., 2014)

				CFU/mL after heat treatment at 100 °C for 13 min but nisin alone only inactivated by ~ 0.9 logs. D-value for heating alone was 142 min.	
	modurans spores				
45 °C	S-carvone 5 mmol/mL Carvacrol 2 mmol/mL Thymol 2.5 mmol/mL Cinnamaldehyde 2 mmol/mL Decanal 2 mmol/mL	*Listeria monocytogenes* (exponential phase)	HEPES buffer	Sample with the addition of S-carvone, carvacrol, thymol, cinnamaldehyde or decanal was inactivated by ~2/2/1.5/3/2 log CFU/mL after heat treatment at 45 °C for 30 min. No effect was observed for individual treatments.	(Karatzas et al., 2000)
Steaming 100 °C	Liquid smoke 0.2 mL/frank	*Listeria monocytogenes*	Fully cooked franks	Sample with the addition of frank liquid smoke was inactivated by ~ 3 log CFU/cm^2. Control sample with no liquid smoke resulted in ~ 2 log reduction; 0.2 mL/frank liquid smoke alone inactivated by ~ 1 log CFU/cm^2.	(Murphy et al., 2005)
Steaming at 143 kPa (~70 °C after 10 s)	Lactic acid 2%	*Escherichia coli* O157: H7 *Salmonella* Typhimurium	PVC and stainless steel 316 coupons	Combined treatment led to ~ 4.73/4.71/4.94 log reduction. Comparatively, *E. coli* O157: H7/*S. Typhimurium/L. Monocytogenes*, control resulted in ~ 2.05/2.21/2.1 logs reduction; Sample with lactic acid treatment alone	(Ban et al., 2012)

		Listeria monocyogenes (Biofilms)		was inactivated by ~ 0.84/0.44/0.67.	
55-70 °C	*Pinhao* seed coat extract (aqueous extraction) 1%	*Listeria monocytogenes*	Distilled water	D-value for heat treatment at 55 °C alone was 3.56 min; the addition of pinhao coat extract reduced the D-value to 1.73 min with the same heat treatment. Pinhao coat extract did not inactivate bacteria.	(Trojaike et al., 2019)
45 °C 2-8 min	Octanoic acid (OA) 1-3 mM	*Escherichia coli* O157: H7	Wash water grape tomatoes	Control samples in water did not inactivate bacteria; 2 mM OA induced up to 3.4 log reduction in 6 min compared to 2.5 log reduction at 25 °C in 8 min. Bacteria load on tomatoes was inactivated by > 4.5 log CFU/g compared to ~3 log CFU/g for 3 mM OA wash without heat in 2 min.	(H. Zhang et al., 2019)
Microwave (MW) 700W 2450 MHz 50-60 °C	Hydrogen peroxide (H_2O_2) 0.05%-0.1%	*Escherichia coli K12*	Distilled water	MW + H_2O_2 0.075% reached >1 log of additional inactivation compared to individual treatments based on the response surface analysis.	(Koutchma & Ramaswamy, 2000)
Radio frequency (RF) 27 MHz 60/63/65 °C	Nisin 500 IU/mL	*Escherichia coli K12*	Salmon and sturgeon caviars	Combined treatment of RF (equivalent to 4D thermal process) and nisin resulted in a 7.3 log reduction at 65 °C. Individual RF process (equivalent to an 8D	(Al-Holy et al., 2004)

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				thermal process) and nisin led to 2.6 log reduction and no inactivation.	
Ohmic heating (OH) 60 Hz 12.1/11.5 V_{rms}/cm	Carvacrol 1.3 mM Citral 1 mM Eugenol 1 mM Thymol 1 mM Carvone 1 mM	*Escherichia coli O157: H7 Salmonella Typhimurium Listeria monocytogenes* *MS-2 bacteriophage*	Salsa	For *E. coli/Salmonella/Listeria*, OH (12.1 V_{rms}/cm) + carvacrol resulted in >5 log reduction compared to ~0.6 and ~2.1 log reduction caused by carvacrol and ohmic heating alone (50 s); OH (11.5 V_{rms}/cm) + thymol resulted in 4.0/4.3/2.3 log reduction compared to ~1 and ~0.2 log reduction caused by OH and thymol alone. OH + citral resulted in 3.3 log reduction only for *Salmonella* (36 s). For MS-2 bacteriophage, OH + carvacrol resulted in 6.2 log reduction compared to 0.16 log and 4.2 log reduction caused by carvacrol and ohmic heating alone (100 s).	(S.-S. Kim & Kang, 2017a, 2017b)

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1.4 *Treatment with food-grade antimicrobial compounds*

As discussed above, although being an efficient treatment against pathogens in LMFs, heat treatment efficiency is highly dependent on the complexity and inherent properties of the food matrices and may pose potential risk to food safety when its efficiency is reduced. It may also pose negative impact to the nutrient and organoleptic quality of foods. An alternative method that may serve as a hurdle to induce synergistic effect with heat treatment in LMFs is with the use of food-grade antimicrobial compounds. Food grade antimicrobial compound is a substance that is either a food additive, which is subject to premarket review and approval by the FDA; or a Generally Recognized as Safe (GRAS) compound. GRAS compounds are substance that is generally recognized to be safe under the conditions of its intended use, which is established through either scientific procedures by qualified experts or experience based on their common use in food before 1958 (U.S. Food and Drug Administration, 2019). Examples of the food-grade antimicrobial compounds includes benzoic acid derivatives (e.g. parabens) and essential oils and their components, which have been chosen based on their antimicrobial efficiency and regulatory status to be used in foods.

1.4.1 *Paraben*

Parabens, also known as esters of *p*-hydroxybenzoic acid, have a general structure as shown in Figure 1-1. Based on their chemical structure, the solubility for parabens in water is inversely related to the alkyl chain length. Methyl paraben has water solubility of 0.25 g/100 g at room temperature, while the solubility for butyl paraben is at 0.02 g/ 100g (Lück & Jager, 1997). Owing to its hydrophobicity, parabens, especially those with high alkyl chain length, have few application in aqueous system and relatively unfavorable distribution within oil in water emulsions (Lück & Jager, 1997).

In the United States, methyl (21 C.F.R. § 184.1490, 2019) and propyl paraben (21 C.F.R. §

184.1670, 2019) are classified as generally recognized as safe (GRAS) products at maximum

concentration of 0.1% each, while butyl paraben is allowed to be added as a flavoring agent in

food and can be used in accordance to the good manufacture practices (21 C.F.R. § 172.515,

2019).
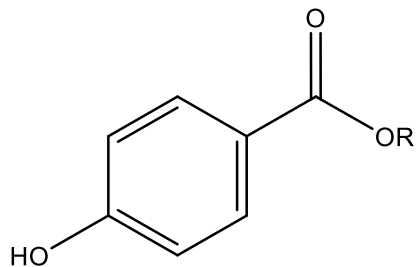
Figure 1-1. General Structure for Parabens

Parabens have effective antimicrobial activity at pH range around 3 to 8, which makes

them more effective than the acidic preservatives in food systems with neutral pH (Aalto et al.,

1953). The antimicrobial action of the parabens is proportional to the length of their alkyl chain

(in the alcohol component) (Davidson et al., 2005). Investigations on the antimicrobial effect of

parabens with different alkyl chain length against *E. coli* revealed that the uptake of parabens

was logarithmically proportional to the chain length (Fukahori et al., 1996).

The inactivation mechanism of parabens has not been well understood, but studies have

already indicated that the destruction of cytoplasmic membrane structure might be one of the

main reasons (Davidson, 2005; Lück & Jager, 1997; Tatsuguchi et al., 1991). Leakage of

intracellular compounds such as ribonucleic acid (RNA) (Furr & Russell, 1972), and inhibition

of amino acids and essential nutrients uptake (Tatsuguchi et al., 1991) have been detected in

different bacterial species including *E. coli* and *B. subtilis*, indicating cytoplasmic membrane

disruption. It has also been postulated that parabens may neutralize chemical and electrical forces that are responsible for establishing a normal membrane gradient (Eklund, 1980).

Recently, a number of these compounds have been found to enhance thermal inactivation of foodborne pathogens, achieving shorter heating times or allowing lower processing temperatures (Ait-Ouazzou et al., 2011; Dock et al., 2000; Espina et al., 2012, 2014; Ruan & Buchanan, 2016). The parabens are among the compounds that have demonstrated significant enhancement of thermal inactivation at mild treatment temperatures (Gao et al., 2020; Gurtler & Jin, 2012; Ruan & Buchanan, 2016). However, these studies were conducted in microbiological media or aqueous foods with high a_w.

Currently, parabens are predominately used as preservatives in cosmetic products (Davidson et al., 2005). It has been reported that the major drawbacks that prevent parabens from being used in food industry include consumer health concern and problems with its "metallic-bitter" taste (Lück & Jager, 1997). These drawbacks have prevented it from being used in food products at a dose with enough efficiency for it to be used alone during processing.
In the past decade, the use of parabens have also become controversial due to concerns over potential toxicological effects at high levels associated with endocrine disruption in specific test animals (Fransway, Fransway, Belsito, & Yiannias, 2019; Routledge et al., 1998). As a result, parabens have been the subject of extensive research over the past 20 years, including being the subject of a National Toxicology Program evaluation (NTP, 2005), extensive regulatory reviews by FDA and EFSA (Angerer et al., 2010; Anton et al., 2004), and cosmetic industry scientific panels. The uptake, metabolism, and excretion of parabens have been studied extensively (Abbas et al., 2010; Elder, 1984; Jones et al., 1956; Moos et al., 2016; Soni et al., 2001), and is considered non-mutagenic in multiple *in vitro* systems and therefore not likely to be carcinogenic

via classical mutation of cell lines (EFSA, 2020). Testing at high doses in *in vitro* systems and laboratory animals have led researchers to hypothesize potential adverse effects such as endocrine disruption (Boberg et al., 2010; Darbre & Harvey, 2008), breast cancer (Byford et al., 2002; Wróbel & Gregoraszczuk, 2014), and teratogenicity/ reproductive toxicity (Boberg et al., 2010; Oishi, 2002); as well as concerns related to childhood exposure risks (Towers et al., 2015)and psychosocial issues (Shiue, 2015). Many of these hypotheses have not been supported by subsequent studies consistent with how parabens are employed. For example, the use of patch tests have found parabens as one of the least allergenic preservatives available for controlling microbiological contamination in pharmaceutical products (Fransway, Fransway, Belsito, Warshaw, et al., 2019). Similarly, recent studies have not supported the hypothesis that maternal and paternal pre-conception exposure to parabens led to adverse behavioral effect in the offspring (Skarha et al., 2020). Therefore, there has not been enough definitive human or animal study data to confirm or disprove the significance from any of the previous findings about the adverse health effect of parabens.

1.4.2   *Plant-derived antimicrobial compounds*

Recently, the food industry has developed a growing interest in researching and developing natural compounds either for direct addition or in synergy with current treatments to achieve greater antimicrobial efficiency. Among the vast range of compounds being studies, many essential oils (EOs) have been reported to possess significant antimicrobial activities in aqueous systems (Table 1-8) (Burt, 2004; Chouhan et al., 2017). They are aromatic oily liquids existed in different parts of plants to protect them against pathogenic microorganisms and insects and can be extracted by steam distillation. The chemical profiles of EOs are complex natural mixtures of 20–60 components at different concentrations, where there are usually 2-3 dominant

components that has higher concentrations (20-70%) and antimicrobial effect (Riley et al., 1983).

These components in EOs are usually terpenes and their oxygenated derivatives such as aromatic

terpenoids and phenolic compounds. Same as EOs, most of the effective antimicrobial

compounds are hydrophobic. It limits their solubility in aqueous system but enables them to

interact and partition inside the phospholipid membrane of bacterial cells, which can damage

bacterial membrane integrity (Bajpai et al., 2013; Chouhan et al., 2017; Lv et al., 2011). Critical

cellular components like DNA/RNA, electrolytes, reducing sugars and ATP may leak into the

extracellular environment due to changes in the membrane permeability, which serves as the

dominant inactivation mechanism of these antimicrobial compounds (Devi et al., 2010). Trans-

cinnamaldehyde (CA) and eugenol (EG) are two of the fast-acting phenylpropene compounds

among the major antimicrobial EO components (Burt, 2004; Chouhan et al., 2017). It has been

reported that 0.3% CA or 0.15% EG can induce 50% inactivation of *E. coli* and *S. enterica* in

apple juice at room temperature for 5 min (Friedman et al., 2004). Both CA (21 C.F.R. § 182.60,

2019) and EG (21 C.F.R. § 184.1257, 2019) are also approved by the FDA as generally

recognized as safe (GRAS) compounds.
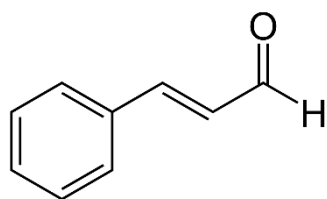
Figure 1-2. Chemical Structure for trans-Cinnamaldehyde (CA)
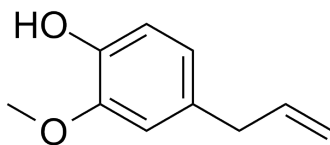
Figure 1-3. Chemical Structure for eugenol (EG)

Trans-cinnamaldehyde (CA), or cinnamic aldehyde, is a phenylpropene aldehyde with a solubility of 0.14 g/ 100 g (Figure 1-2). It is a yellowish oily liquid at room temperature and can be either extracted from cinnamon (*Cinnamonum*) EO or synthesized by condensation of benzaldehyde and acetaldehyde (Friedman, 2017). It has been found that CA can interact with cellular membrane of *E. coli* and reduce energy metabolism by inhibiting the membrane-bound adenosine triphosphatase (ATPase) activity and disrupting proton motive force across the membrane (Gill & Holley, 2006b). Alteration to the membrane composition and morphology were also observed on CA treated *E. coli* cells, which could facilitate the incorporation of other antimicrobial compounds if presented (Di Pasqua et al., 2006). The membrane modification is partially caused by the aggregates formed by CA and membrane lipid molecules (Nowotarska et al., 2014, 2017).

Eugenol (EG) is a clear to pale yellow oily liquid with a solubility of 0.24 g/ 100 g, which can be extracted from EOs of clove, nutmeg, cinnamon, basil and bay leaves or synthesized by bio-engineered microorganisms (Chouhan et al., 2017; Mishra et al., 2013). It is a phenylpropene phenol (Figure 1-3). Like CA, its antimicrobial effect is also contributed by the attachment and accumulation within the cytoplasmic membrane, which causes damage to bacterial membrane

integrity, increases the non-specific permeability (Devi et al., 2010; Filgueiras & Vanetti, 2006; Gill & Holley, 2006a). Leakage of critical cellular components such as such as electrolytes, proteins and nucleic acids have been observed on eugenol-treated *E. coli* and *Listeria monocytogenes*, which can eventually lead to cell death (Filgueiras & Vanetti, 2006; Marchese et al., 2017; Y. Zhang et al., 2016). It was also reported that eugenol increased intracellular concentration of reactive oxygen species in *S. aureus*, which led to damage to DNA and other critical cellular components (Das et al., 2016). Inactivation of critical enzymes such as protease and ATPase have also been observed on eugenol-treated bacteria (Hyldgaard et al., 2012; Marchese et al., 2017; Wendakoon & Sakaguchi, 1995).

In addition to their antimicrobial properties, CA and EG are also reported to possess health-promoting benefits against diseases such as diabetes, obesity, cancer and depression (Chouhan et al., 2017; Friedman, 2017; Marchese et al., 2017). However, due to their intense aroma, negative organoleptic effects of EO components such as CA or EG may exceed the acceptable threshold of average consumers even at relatively low dosage. It has been reported that shrimp with addition of $> 0.9\%$ thyme oil significantly reduced the consumer acceptance of the product (Lv et al., 2011). This can limit the application of EO components to spicy foods where the sensory threshold is relatively high among consumers (Hyldgaard et al., 2012).

Table 1-8. Brief summary of the antimicrobial effect (minimum inhibitory concentration (MIC)) and antimicrobial mechanism of selected EO components (Adapted from (Hyldgaard et al., 2012))

Compound	Plant origin	Chemical classification	MIC (µg/mL)	Mechanism	References
Trans-cinnamaldehyde	Cinnamon	Phenylpropene aldehyde	*E. coli* (397–1322) *S.* Typhimurium (397) *L. monocytogenes* (3965) *S. aureus* (2.1–750)	Membranes permeabilization: leak cellular components (ATP/ nucleotides/ electrolytes) Inhibition: ATPase, histidine decarboxylase	(Ainsworth & Gillespie, 2007; K.-H. Bang et al., 2000; Chang et al., 2001; Di Pasqua et al., 2006; Domadia et al., 2007; Gill & Holley, 2006a, 2006b; Helander et al., 1998; Hemaiswarya et al., 2011; Kwon et al., 2003; Zemek & Ježo, 1987)
Eugenol	Clove Nutmeg Cinnamon Basil Bay leaves	Phenylpropene phenol	*E. coli* (800–3000) *S.* Typhimurium (3.18–500) *L. monocytogenes* (800- >1000)	Membranes permeabilization: leak cellular components (ATP/ nucleotides/ electrolytes); Disrupt proton motive force Inhibition: ATPase,	(Ainsworth & Gillespie, 2007; Chang et al., 2001; Gill & Holley, 2006a, 2006b; Walsh et al., 2003; Yamazaki et

				histidine decarboxylase and other enzymes	al., 2004; Zemek & Ježo, 1987)
Carvacrol	Oregano and thyme	Monoterpenoid phenol	*E. coli* (225–2500) *S.* Typhimurium (150-250) *L. monocytogenes* (450-1500)	Membranes permeabilization; Intracellular ATP depletion; ATPase inhibition	(Ahmad et al., 2011; Gill & Holley, 2006a, 2006b; Horváth et al., 2009; Ultee et al., 1999, 2002; J. Xu et al., 2008)
Thymol	Thyme	Monoterpenoid phenol	*E. coli* (225–5000) *S.* Typhimurium (56.25-150) *L. monocytogenes* (450)	Membrane damage (leak electrolytes and ATP); Increase membrane fluidity; ATPase inhibition	(Ahmad et al., 2011; Di Pasqua et al., 2006; Helander et al., 1998; Walsh et al., 2003; J. Xu et al., 2008)
Citral	Citrus fruits	Monoterpenoid aldehyde	*E. coli* (447-500) *S.* Typhimurium (500) *L. monocytogenes* (500)	Membrane damage	(S. M. Kim et al., 1995; Nazer et al., 2005; Onawunmi, 1989; Somolinos et al., 2010)
Vanillin	Vanilla	Phenylpropene phenolic aldehyde	*E. coli* (2282) *L. innocua* (5325)	Membrane permeabilization; Disrupt proton motive force; Impair cellular respiration;	(D. Fitzgerald, 2003; D.J. Fitzgerald et al., 2004; Daniel J.

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					Fitzgerald et al., 2005)

1.5 Statement of problem

Based on the extensive literature review performed, it is evident that

1) The low-a_w nature of LMFs does not guarantee the safety of foods. Many virulent foodborne pathogens, including *Salmonella* spp. and *E. coli* O157:H7 can survive for an extended time in low-a_w conditions. As a result, there have been multiple outbreaks of foodborne illnesses linked to LMFs contaminated with these pathogens. To improve the safety of LMFs, thermal processing is the mainstream technology used as the kill step during production, which utilizes heat to inactivate microorganisms and enzymes in foods and enhance both the safety and stability of the products. However, it is known that in a matrix with reduced a_w, pathogens such as *Salmonella* spp. tend to develop higher thermal resistance. Therefore, mild heat treatments that work very well in high-a_w foods can be less effective when applied to products with low a_w. Intensive thermal treatment with either higher temperature or longer treatment time can lead to damage to the nutritional and/ or organoleptic properties of the food.

2) The complexity of LMFs composition may introduce additional challenges to thermal treatment, as the different components in LMFs, including carbohydrate, protein and fat, can affect bacterial resistance during treatments. There have been some explorations on the thermal resistance mechanism of bacteria at low-a_w conditions. However, in-depth investigations on bacterial thermal resistance in LMF matrices with different compositions in both physiological and genetic levels is lacking.

3) As food-grade antimicrobial compounds, parahydroxybenzoates (parabens) and many essential oil (EO) components (e.g. cinnamaldehyde (CA) and eugenol (EG)) have shown promising antimicrobial effect in multiple studies. Previous studies have demonstrated

enhancement of thermal treatment by inclusion of plant-derived antimicrobial compounds in aqueous system, but very few have studied such effect in LMFs or at low-a_w conditions. Additionally, potential health concerns related to the use of parabens as well as the negative organoleptic effect introduced by the compounds have further limited their application in many food products.

To address these needs and gaps in the literature, the overall goals of this project were to 1) develop enhanced thermal treatments that use food-grade antimicrobial compounds, including paraben and EO components (such as CA and EG), to achieve a synergistic antimicrobial effect in LMFs; and 2) investigate bacterial thermal resistance in matrices with different compositions at low a_w and develop a deeper understanding of the matrix effect on the resistance mechanism at both physiological and genetic levels.

1.6 Specific objectives

1) Evaluate combination of butylparaben and heat treatment to enhance antimicrobial efficiency against *E. coli* O157:H7 and *S*. Typhimurium in MBM.

    Hypothesis: The antimicrobial effect from both BP and the heat treatment would act synergistically against pathogens in low a_w environment and improve treatment efficiency in MBM.

2) Investigate the effect of heat treatment with food-grade antimicrobial compounds against *S*. Typhimurium in starch, protein and lipid matrices at low a_w and understand bacterial response to these treatments at physiological level.

    Hypothesis: The effect of heat treatment with alternative food-grade antimicrobial compounds is dependent on both the a_w levels and the LMF components. Bacterial

resistance could be contributed by the varied metabolic activity levels of bacteria in low-a_w matrices and the altered membrane properties of bacteria during adaptation to low a_w.

3) Investigate the genetic response of *S*. Typhimurium during low a_w adaptation and antimicrobial treatments and identify its correlation with bacterial resistance in different LMF components.

Hypothesis: *S*. Typhimurium has varied expression levels of stress response genes in low-a_w matrices within different LMF components, which may partially explain the previously observed inactivation resistance and resistance mechanism.

2 Chapter 2 Evaluation of butylparaben-assisted heat treatment against *E. coli* O157:H7 and *S*. Typhimurium in MBM

Abstract

Heat resistant foodborne pathogens have been a concern in low-moisture foods and ingredients (LMFs). Due to low thermal conductivity of low moisture materials, thermal treatment is not efficient and may cause nutritional loss. This study investigated the enhancement of thermal treatment of meat and bone meal (MBM) at low water activity (a_w) by inclusion of butylparaben (BP) as a model antimicrobial compound. Stationary phase *Escherichia coli* O157:H7 (Shiga toxin-negative) or *Salmonella enterica* serotype Typhimurium was inoculated into MBM containing 0 -2000 ppm BP and incubated at 55 or 60 ºC for up to 5 h. A biphasic inactivation pattern was observed for both pathogens, indicating existence of potentially thermal resistant subpopulations. Addition of 1000 ppm BP to MBM (a_w = 0.4) significantly lowered the D-value at 55 °C for *E. coli* O157:H7 (2.6 ± 0.5 h) compared to thermal treatment alone (5.1 ± 0.6 h) during the treatment after the first 1 h ($P < 0.05$), indicating that addition of BP accelerated the inactivation of thermal-resistant subpopulation of *E. coli* O157:H7 in MBM. Interestingly, similar enhancement in thermal inactivation upon addition of BP was not observed in either the sensitive or resistant subpopulation of *S.* Typhimurium at a_w of 0.4 or 0.7, which is likely caused by the higher thermal resistance developed by *S.* Typhimurium within a low a_w environment ($a_w < 0.85$). These results suggest that addition of antimicrobial compounds can improve the thermal processing efficiency in LMFs, while their efficiency against different pathogens may vary.

2.1 Introduction

To ensure the safety of LMFs, pathogen levels are usually controlled via inactivation steps during manufacturing ("kill steps"), in addition to maintaining sanitary environment and avoiding cross contamination (Podolak & Black, 2017). Thermal pasteurization is an efficient and inexpensive technology that is commonly used to inactivate foodborne pathogens in a wide range of products (Silva & Gibbs, 2012). However, studies have found that the heat resistance of certain pathogens including *E. coli* O157:H7 and *S. enterica* increases as the a_w decreases (Archer et al., 1998; Beuchat et al., 2013; Beuchat & Scouten, 2002; Santillana Farakos et al., 2013).Thus heat treatment with higher temperature or longer treatment duration has to be adopted for LMFs to ensure food safety. Heat treatments at high temperatures or extended times can induce detrimental effect on product quality, which limits its usage in food industry (Leistner, 2000; Rodrigo et al., 2016; Trugo et al., 2000). Therefore, improved thermal treatment methods are needed to address the dilemma.

Parabens are esters of *p*-hydroxybenzoic acid. In the United States, methyl (21 C.F.R. § 184.1490, 2019) and propyl paraben (21 C.F.R. § 184.1670, 2019) are allowed in foods at maximum concentration of 0.1% each. Butyl paraben (BP) is regulated as a synthetic flavor compounds and adjuvants and can be used in accordance with good manufacturing practices (21 C.F.R. § 172.515, 2019). The relative antimicrobial effect of the parabens is proportional to the length of their alkyl chain (Aalto et al., 1953), where BP is more effective than methyl or propyl paraben. Although the detailed mechanism has not been fully revealed, studies have indicated that the disruption of bacterial and fungal cell membrane structure is likely one of the main factors contributing to their antimicrobial activity (Davidson, 2005; Lück & Jager, 1997; Tatsuguchi et al., 1991).

Recently, a number of these compounds have been found to enhance the thermal inactivation of foodborne pathogens, achieving shorter heating times or allowing lower processing temperatures (Ait-Ouazzou et al., 2011; Dock et al., 2000; Espina et al., 2012, 2014; Ruan & Buchanan, 2016). The parabens are among the compounds that have demonstrated significant enhancement of thermal inactivation at low treatment temperatures (Gao et al., 2020; Gurtler & Jin, 2012; Ruan & Buchanan, 2016). However, this work has been largely conducted at high water activity (a_w) in microbiological media or aqueous foods. The overall goal of the current project was to determine if such enhancement of thermal inactivation would be effective in LMF. BP was selected as a model compound for evaluating the possible enhancement of thermal inactivation in LMF, since it has been extensively studied, has a broad effective pH range, broad antimicrobial activity, and a reasonable solubility in both water (0.02%, w/w) and oil phase (4.7%, w/w). Meat and bone meal (MBM) was selected as the LMF to be studied due to its extensive use in human foods, pet foods, and animal feeds, and represents a significant challenge for control of microbiological contamination.

2.2   *Materials and Methods*

2.2.1   *Determination of MBM composition*

Moisture content of MBM (~1 g) was measured with Mettler Toledo HE53 Moisture Analyzer (Mettler Toledo, Columbus, OH, U.S.A.). The fat content of MBM (~1 g) was measured by weighing the compounds extracted via Soxhlet extraction method after nitrogen evaporation following a modified AOAC *Official Methods of Analysis* Method 920.39 with substitution of diethyl ether with petroleum ether. The ash content of MBM (~1.7g) was calculated from the weight of the residue after ashing MBM samples in a muffle furnace at 500 °C for 48 h.

2.2.2 *Sample preparation*

Butyl paraben (BP, 0-2000 ppm) (MP Biomedicals, OH, USA) was dissolved in 100% ethanol. 800 µL BP solution or pure ethanol (0 ppm controls) was mixed with 200 g MBM samples using a 350 W household food processor for 1 min (MC-KS, Magicook, China). Mixed samples were then stored uncovered inside a biosafety cabinet for at least 1 h to reduce ethanol residue inside the MBM before bacterial inoculation. Homogeneity tests were conducted by measuring the BP concentration in each sample (or MBM samples spiked with known levels of BP as standards) with a modified HPLC method. Briefly, BP in 2 g samples from three different locations in MBM spiked with 146 ppm BP were extracted by vigorously mixing with 6 mL ethyl acetate for 45 min. Extract was then evaporated under nitrogen stream reconstituted with 1 mL methanol. Residual lipid was removed by centrifuging the reconstituted extract at 15,000 g for 10 min (at 4 °C) after being stored at -20 °C for 1 h. BP concentration in the supernatant was measured with a Shimadzu LC2010 HPLC (Shimadzu, Kyoto, Japan) equipped with a Waters® Spherisorb™ ODS2 10 µm (4.6 × 250 mm) attached with a Phenomenex® SecurityGuard™ guard cartridge (C18 4 × 4.0 mm) and a UV-Vis detector (254 nm) running in isocratic mode (60% methanol, 1 mL/min). The final BP concentration in MBM was calculated based on the standard curve and expressed as parts per million after multiplying the dilution factor (ppm).
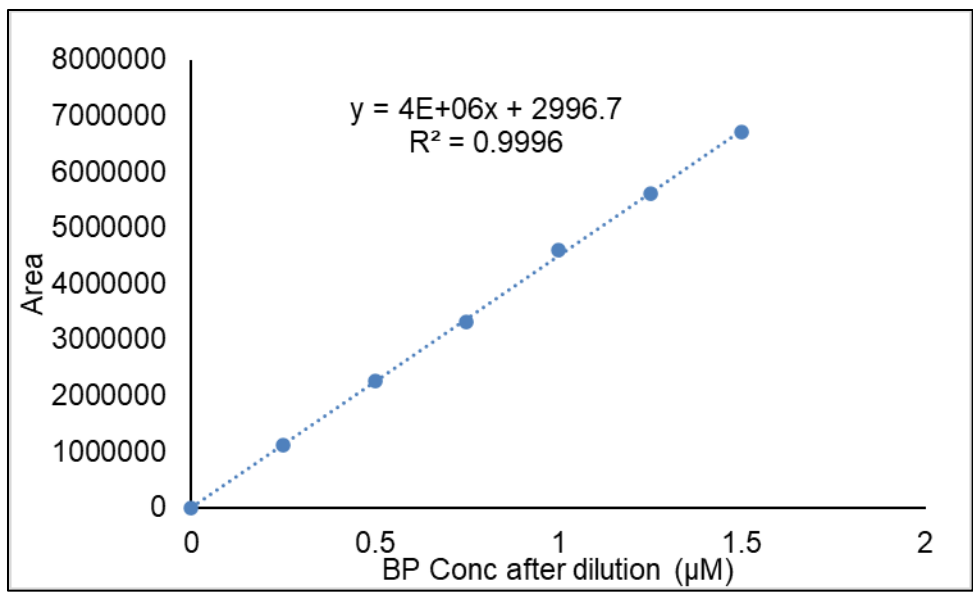
Figure 2-1. BP standard curve for HPLC analysis in the diluted extractant of MBM

2.2.3 *Bacterial culture and sample inoculation*

A rifampicin resistant, Shiga-toxin negative *Escherichia coli* O157:H7 strain (ATCC #700728) or a strain of *Salmonella enterica* serotype Typhimurium (CVM98) from the stock culture collection of the department of Nutrition and Food Science at University of Maryland, College Park was individually tested as a target pathogen in this study and prepared via our pre-established method with some minor modifications (Bastarrachea et al., 2017; Ding et al., 2018). Briefly, bacteria from frozen culture were recovered and inoculated onto Trypticase soy agar (TSA) (236920, Difco, BD, Franklin Lakes, NJ) plates, and stored at 4 °C for up to 3 weeks. Before each experiment, a single colony of *E. coli* O157:H7 or *S*. Typhimurium from the plates was inoculated in 25 mL fresh sterile Trypticase soy broth (TSB) (211825, Difco, BD, Franklin Lakes, NJ) overnight at 37 °C for 18-20 h to achieve the early stationary phase (approximately 9 log CFU/mL). It was then harvested and concentrated 100-fold through centrifugation at 7,197 *g* for 10 min and re-suspended into 2 mL of sterile 0.2% Buffered Peptone Water (BPW), with final concentration of ~11 log CFU/mL. 200 µl of the re-suspended culture was blended into 100 g afore-mentioned MBM samples (with or without BP) to reach a starting population of ~ 5 - 7 log CFU/g (for *E. coli* O157:H7) or ~ 4 - 6 log CFU/g (for *S*. Typhimurium) before applying the treatments described in the following section. Homogeneity tests were conducted by taking 1-g samples from three different locations (surface, middle and bottom) in the inoculated MBM at the beginning of each treatment.

2.2.4 *Sample equilibration and adaptation*

Before the start of the treatment, deionized water (200 – 1200 µL) was mixed into the inoculated MBM to adjust the a_w of the mixed product to the target levels. The exact a_w values were measured with a HygroPalm AW water activity meter (Rotronic Instrument, NY, USA)

after equilibration was achieved. Water activity values were adjusted to 0.4 a_w for samples inoculated with *E. coli* O157:H7 and 0.4 or 0.7 a_w for samples inoculated with *S.* Typhimurium. In experiments with additional adaptation periods, bacteria were inoculated in MBM samples without BP at 0.4/ 0.7 a_w following the afore-mentioned procedures, and subsequently exposed to the low a_w environment for additional 24 h before adding BP to the sample and re-adjusting a_w to 0.4/ 0.7.

2.2.5 *Heat treatment*

1 g of the inoculated MBM samples with 0 (ethanol control), 1000, or 2000 ppm BP at their desired a_w levels were transferred to capped aluminum sample containers (I.D. 54.6 mm × Height 12.7 mm). They were heated within a digitally-controlled natural convection incubator (Model 1545, VWR, Radnor, PA, U.S.A.) pre-heated to desired treatment temperatures (± 0.5 °C). Temperature of the sample was recorded with a data-logging thermometer (Traceable™ Excursion-Trac™, Fisherbrand, Pittsburgh, USA) inserted into a sample container holding 1g of uninoculated MBM and seated in the middle of the rack. After loading all the sample containers into the incubator (within 30 s), it took 25 min for sample temperature to reach its set value (55 / 60 °C) with more than 90% of increase in temperature (~ 30/ 35 °C increase in temperature) achieved within the first 10 min (Figure 2-2A). One sample container was taken out at each time point (0.5, 0.75, 1, 2, 3, 4, 5, and 6 h) for bacterial enumeration. A brief action of opening the incubator door to retrieve the sample (within 5 s) resulted in lowering temperature of the remaining samples by less than 0.5 °C, which was recovered within the next 10 min of treatment (Figure 2-2B). Samples with 0 (ethanol control), 1000, or 2000 ppm BP incubated at room temperature (20-22 °C) were included as control.

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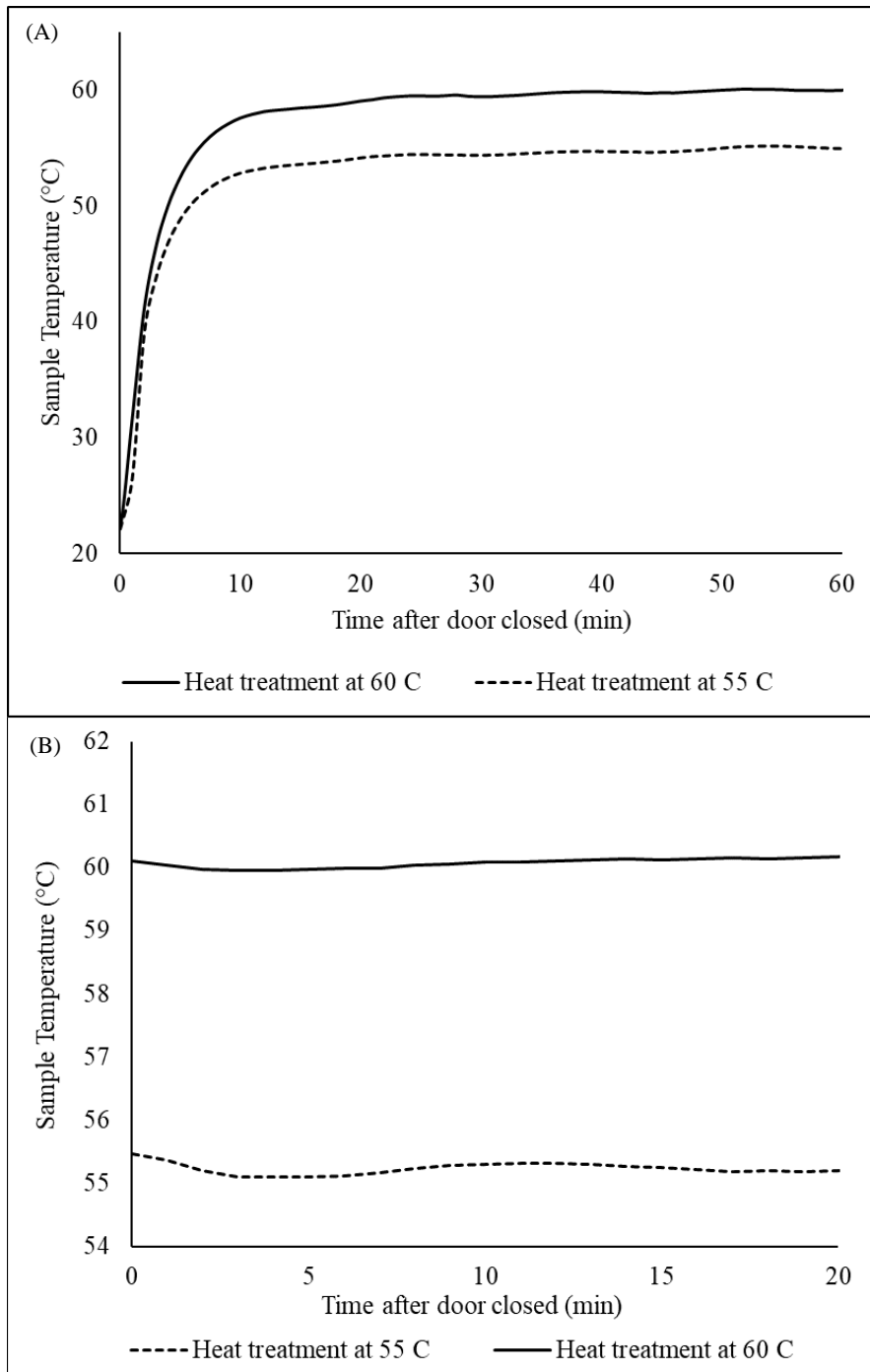
Figure 2-2. Temperature change of sample within container after being placed onto the middle rack of a pre-heated incubator and leave the door opened for 30 s at the beginning of the treatment (A), or after opening the door for 5 s during the treatment (B). Measurements were done separately from the actual treatments.

2.2.6 *Sample extraction and enumeration*

The surviving bacterial cells in samples were extracted by vortexing 1 g sample in 9 mL of sterile 0.2 % Buffered Peptone Water (BPW) for 20 s. 50 µL of sample was spiral plated onto duplicated TSA plates supplemented with 50 ppm rifampicin (RIF) (for *E. coli* O157:H7) or Bismuth Sulfite Agar (BSA) plates (for *S.* Typhimurium) after appropriate serial dilutions in 0.2% BPW using a spiral plater (Neutec Group Inc., Nr 10003700/113, Farmingdale, NY) at E50 mode. For samples with bacterial concentrations of < 2.3 log CFU/g, three aliquots of 333 µL 10-fold diluted sample were individually spread-plated onto three plates. BSA was chosen as the selective medium in this study because it demonstrated higher recovery efficiency for *S.* Typhimurium inoculated in MBM compared to Xylose Lysine Deoxycholate (XLD) agar in preliminary experiments, where the cells that grew on BSA were ~1 log CFU/ g higher than XLD after harvesting from the same sample with the BP-assisted heat treatment at 55 °C for 1 h. All plates were counted using an automatic plate counter (IUL Instruments S. A, Flash & Go, Königswinter, Germany) after incubation at 37 °C for 24 h and converted to Log_{10} (CFU/g) values. The minimum detection threshold for surviving bacteria in MBM was 1 log CFU/g. The treatment data were plotted as bacterial population reduction $\log N_t - \log N_0$, where N_0 is the log bacterial population at time zero and N_t is the bacterial population at a sampling time t hr. Detection limits for each figure were calculated by using the minimum detection threshold (1 log CFU/g) minus the lowest starting population (bacterial population at t_0, which varied from 6 to 4 log CFU/g based on adaptation conditions).

2.2.7   Data analysis

All experiments were performed in three independent trials (except for preliminary studies). The log-linear model was used to describe the microbial inactivation curve for both microorganisms (Bevilacqua et al., 2015):

$$\log N_t = log N_0 - \frac{t}{D} \quad (1)$$

At a given time t, the logarithm of the initial microbial population and the survivors is described as log N and log N_0. D is the decimal reduction time determining the required treatment time to reach a 1-log reduction within the microbial population at a specific temperature (Bevilacqua et al., 2015). The parameters in the log-linear model were obtained via the built-in linear regression method of Microsoft Excel (Seattle, WA, USA) and D values were calculated from the slope of the curves. The significance between different treatments was calculated at logarithmic scale via unpaired Student's t test using Microsoft Excel (Seattle, WA, USA) at a significance level of $\alpha = 0.05$.

2.3   Results and Discussions

2.3.1   Composition of MBM samples and BP/ inoculation homogeneity

MBM samples from different manufactures vary in their compositions and therefore we measured the composition of MBM samples we received. The MBM sample used in this study had an initial a_w of ~ 0.3, and contained 15.16 ± 0.66 % fat, 1.69 ± 0.13 % moisture, and 24.00 ± 0.38 % ash, with the rest ~60% being protein and carbohydrate. The carbohydrate content of MBM is typically low, being derived from post-rigor animal sources. Therefore, the majority of the remaining 60% is likely to be protein. A previous study has reported that MBM contains 48.7% gross protein (Cascarosa et al., 2012). Homogeneity and recovery test showed that BP was distributed evenly within MBM samples, where the BP levels were 142.18 ± 3.61 ppm,

which was 97.6 ± 0.02 % of the spiked concentration (145.7 ppm). The inoculation and mixing methods consistently yielded similar bacterial counts (~6 log CFU/g) from different locations within the samples (standard deviation value < 0.35 log CFU/g) for both bacteria, confirming their homogenous distribution within samples.

2.3.2 *Antimicrobial effect of BP assisted heat treatment against E. coli O157:H7 in MBM*

Preliminary experimental results indicated that heat treatment at 50 °C was not enhanced by 1000 ppm of BP against *E. coli* O157:H7 in MBM at 0.4 a_w (Figure 2-3), and 55 °C was chosen for all heat treatments against *E. coli* O157:H7 in this study.
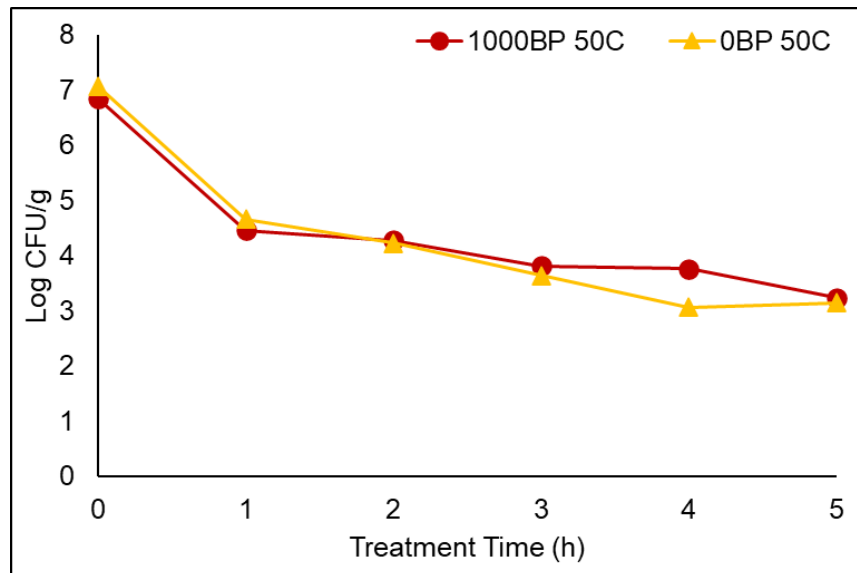
Figure 2-3. Inactivation kinetics for *E. coli* O157:H7 received 50 °C heat treatment at 0.4 a_w in the presence of BP at 1000 ppm without additional 24-h adaptation period. Numbers shown on the vertical axis represent survival population in corresponding time points.

Results in Figure 2-4A show that after a total of 5-h of heat treatment, the bacterial reduction of 4.9 ± 0.6 log (CFU/g) and 4.3 ± 0.5 log (CFU/g) was achieved with heat treatment with and without 1000 ppm BP, respectively. A greater reduction was achieved during the first 1-h of the heat treatment than the following 4 h with (3.3 ± 0.3 log CFU/g in the first hour vs. 1.6 ± 0.5 log CFU/g in the next four hours, $P < 0.05$) and without (3.4 ± 0.4 log CFU/g in the first hour vs. 0.9 ± 0.1 log CFU/g in the next four hours, $P < 0.05$) the addition of BP. Except for the room temperature controls, inactivation was biphasic, suggesting the presence of a thermal-resistant subpopulation, which is likely due to physiological heterogeneity within the inoculated bacterial population (Stringer et al., 2000). Similar biphasic inactivation kinetics has also been previously observed during thermal inactivation on *S. enteritidis* PT4 in nutrient broth, where the $D_{60°C}$ value of the more resistant, tail subpopulation was more than four times higher than the majority population (Humpheson et al., 1998). Instead of genotypic differences between the population, the tailing effect was contributed to the different ability to produce heat shock proteins at stressed conditions (Humpheson et al., 1998). Therefore, to take both resistant and sensitive bacterial subpopulations into account, the data were separated into two phases, and separate D-values calculated to compare the treatment efficiency (Table 2-1).
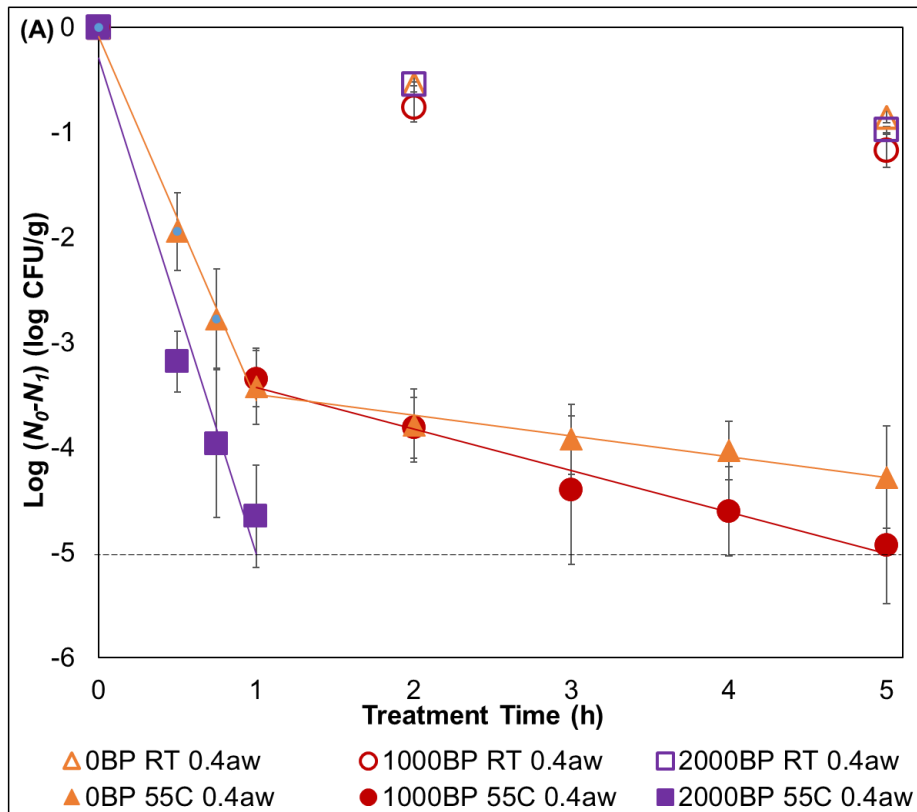
Figure 2-4A. Inactivation kinetics for *E. coli* O157:H7 received 55 °C heat treatment at 0.4 a_w in the presence of BP at different concentrations without additional 24-h a_w-adaptation period. Numbers shown on the vertical axis represent log-reduction values in corresponding time points by comparing to the initial bacterial population. Solid lines indicate the log-linear reduction model fitted to the corresponding data points. Average ± standard deviation (SD). Due to the varied initial population for treatments with or without 24-h adaptation, detection limit for bacterial reduction is at ~ 5 log CFU/g reduction (dotted line). Data points below the limit of detection were not shown on the figures.

Table 2-1. D-values (h) of heat treatment at 55 °C in the presence of 0/ 1000 ppm BP against *E. coli* O157:H7 in MBM at 0.4 a_w, with or without 24-h adaptation period before treatments. Values connected by the same letters indicate significant difference at $P < 0.05$ according to Student's t test. Dash indicates that a D-value could not be calculated due to poor model fitting ($R^2 < 0.8$) or lack of data.

24-h Adaptation	Duration	0BP + 55 °C	1000BP + 55 °C	2000BP + 55 °C
No	0 – 1 h	0.3 ± 0.0^{bc}	-	0.2 ± 0.0^{c}
	1 – 5 h	5.1 ± 0.6^{ab}	2.6 ± 0.5^{a}	-
Yes	0 – 1 h	0.3 ± 0.0	0.4 ± 0.0	-

In the first one hour, no significant difference was observed between the bacterial reduction induced by the heat treatment alone (3.4 ± 0.3 log CFU/g) or by the combined 1000 ppm BP and heat treatment (3.3 ± 0.3 log CFU/g) ($P > 0.05$). However, when the concentration of BP was raised to 2000 ppm, the first hour of treatment reduced the bacterial population by 4.6 ± 0.5 log CFU/g, which was significantly greater than the heat treatment in the absence or presence of 1000 ppm BP ($P < 0.05$). The D-value for heat treatment with 2000 ppm BP also significantly decreased from 0.3 ± 0.0 to 0.2 ± 0.0 h when compared to the treatment without BP ($P < 0.05$). Similarly, concentration-dependent enhancement in thermal inactivation from the addition of BP has also been reported in solution-based systems (Ruan & Buchanan, 2016).

In the subsequent four hours, heat treatment with 1000 ppm BP had a significantly lower D-value (2.6 ± 0.5 h) than the thermal treatment alone (5.1 ± 0.6 h) ($P < 0.05$). Bacterial counts in samples treated with 2000 ppm BP at 55 ˚C were below the detection limit after 1-h and thus no second phase D-value could be calculated for that treatment. The enhanced effectiveness of 1000 ppm BP against the thermally resistant subpopulation could be attributed to the fact that the effect of BP is largely based on its ability to disrupt cellular machinery, such as the membrane repair system, that protects the bacteria from heat stress (Denyer, 1995; Nes & Eklund, 1983). Since heat-sensitive subpopulation likely did not possess a strong defense system to protect itself against heat treatment (such as a slower production of heat shock protein (Russell, 2003)), heat treatment alone was highly effective and the incremental benefit from addition of BP could not be detected. However, since the heat-resistant subpopulation was likely to have a stronger defense to maintain its physiological functions, heat treatment alone was less efficient in achieving inactivation. The addition of BP could therefore exert its effect by weakening the membrane repair system that sensitizes the bacteria to the heat treatment. It is also possible that

due to the low a_w of MBM, where lipid, instead of water, is the dominant solvent, both bacterial cells (Schneider & Doetsch, 1974) and paraben molecules (Seki et al., 2003) had limited mobility. The time required for BP to reach bacterial cells (or vice versa) in MBM would likely to be much longer than in a solution-based system. Compared to the heat-sensitive subpopulation, the heat resistant subpopulation was exposed to BP for longer time, which increased the probability of BP interacting the cells and weakening their membrane.

The effect of 24-hr adaptation period for *E. coli* O157:H7 in low a_w environment prior to the heat treatment on the inactivation kinetics was also investigated. As seen in Figure 2-4B and Table 2-1, during the first 1 h, there was no significant difference between the D-values of heat treatments with (0.4 ± 0.0 h) or without 1000 ppm BP (0.3 ± 0.0 h) ($P > 0.05$). The D-value of heat treatment alone against bacteria receiving prolonged adaptation period did not increase as compared to treatment without the prolonged adaptation period ($P > 0.05$). The results indicated that the resistance of *E. coli* O157:H7 towards the heat treatment, with or without addition of BP, did not increase after the 24 h adaptation period. Therefore, the bacterial cells could adapt to the low-a_w environment and develop its resistance during the original equilibration period (30 - 45 min) and were not likely to increase its resistance as adaptation period was extended. Because *E. coli* O157:H7 in samples was reduced by 2.9 ± 0.2 log CFU/g during the 24 h adaptation period, bacterial counts in these samples decreased to below lowest detection limit after the first 1-h of heat treatment.
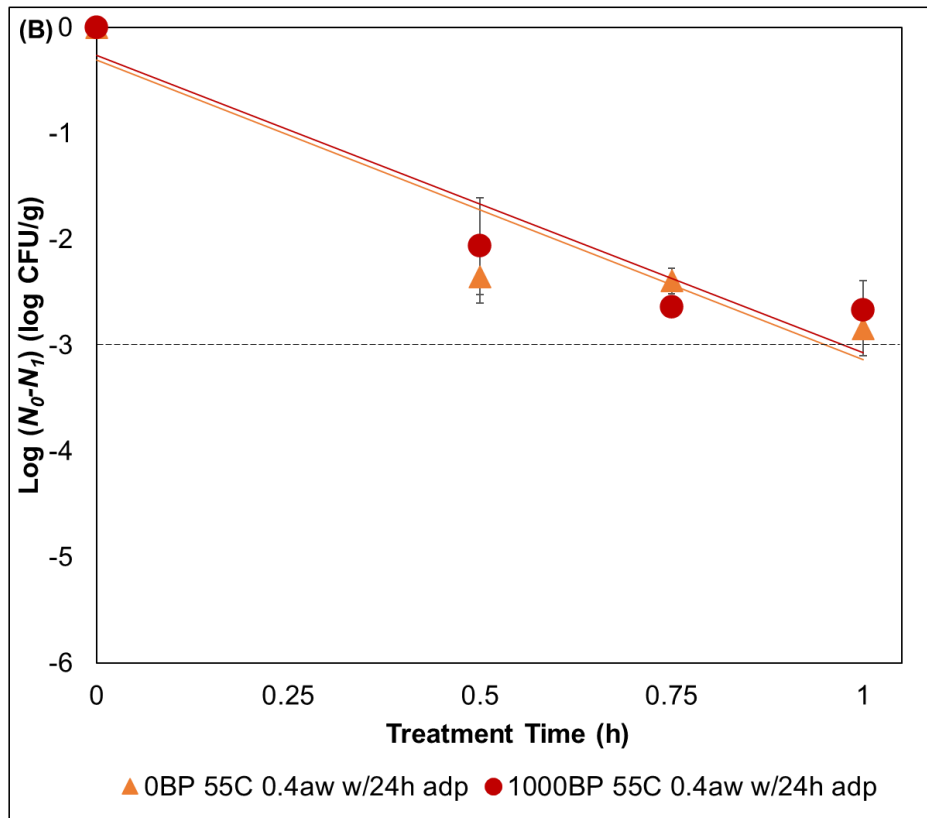
Figure 2-4B. Inactivation kinetics for *E. coli* O157:H7 received 55 °C heat treatment at 0.4 a_w in the presence of BP at different concentrations with additional 24-h adaptation period. Numbers shown on the vertical axis represent log-reduction values in corresponding time points by comparing to the initial bacterial population. Solid lines indicate the log-linear reduction model fitted to the corresponding data points. Average ± standard deviation (SD). Due to the varied initial population for treatments with or without 24-h adaptation, detection limit for bacterial reduction is at ~ 3 log CFU/g reduction (dotted line). Data points below the limit of detection were not shown on the figures.

2.3.3 *Antimicrobial effect of BP assisted heat treatment against S. Typhimurium in MBM*

Results from preliminary experiments at 55 ℃ indicated that heat treatment against *S.* Typhimurium in MBM at 0.4 a_w was not enhanced by 1000 ppm of BP (Figure 2-5). Since *S.* Typhimurium is known to be more heat resistant than *E. coli* O157:H7, higher temperature(s) was applied for heat treatment (Beuchat et al., 2013). During preliminary experiments, the combined treatments were tested at 65 °C in MBM at 0.4 a_w, but the bacterial count in samples decreased below limit of detection within the first 1 h and could not be used. Therefore, 60 °C was chosen for all heat treatments against *S.* Typhimurium in this study.

Figure 2-5. Inactivation kinetics for *S.* Typhimurium received 55 °C heat treatment at 0.4 a_w in the presence of BP at 1000 ppm without additional 24-h adaptation period. Numbers shown on the vertical axis represent log-reduction values in corresponding time points by comparing to the initial bacterial population. Solid lines indicate the log-linear reduction model fitted to the corresponding data points. Average ± standard deviation (SD).

S. Typhimurium population in MBM at 0.4 a_w was reduced by 3.8 ± 0.2 log CFU/g and 3.9 ± 0.3 log CFU/g after heat treatment with or without 1000 ppm BP in 4 h, respectively (Figure 2-6A). Like *E. coli* O157:H7, the inactivation of *Salmonella* was biphasic. Heat treatment with or without 1000 ppm BP caused a rapid reduction of *S.* Typhimurium during the first 30 min, with 2.3 ± 0.2 and 2.2 ± 0.2 log CFU/g, respectively. During the next 3.5 h, *Salmonella* population with and without 1000 ppm BP treatment declined an additional 1.5 ± 0.3 and 1.7 ± 0.1 log CFU/g, respectively. The log reduction rate in the first 30 min was significantly higher than the reduction rate in the subsequent 3.5 h, ($P < 0.05$), which suggested that a range of thermal resistance existed among the inoculated *S.* Typhimurium population as well.
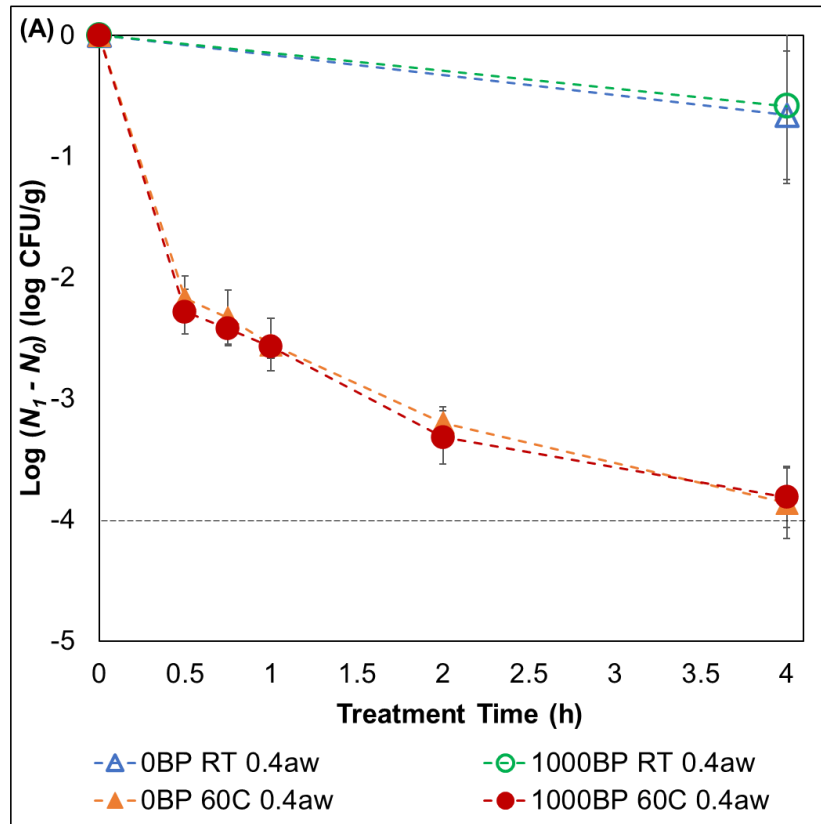
Figure 2-6A. Inactivation kinetics for *S.* Typhimurium received 60 ℃ heat treatment at 0.4 without additional 24-h adaptation period. Numbers shown on the vertical axis represent log-reduction values in corresponding time points by comparing to the initial bacterial population. Average ± standard deviation (SD). Due to the varied initial population for treatments with or without 24-h adaptation, detection limit for bacterial reduction is at ~ 4 log CFU/g reduction (dotted line). Data points below the limit of detection were not shown on the figures.

Since it was not possible to collect data between 0 and 30 min in our experimental set-up, a log linear model could not be built and therefore D-value was not calculated for this period. As a means of clarifying, the log reductions were compared in three treatment periods: 0 – 0.5 h, 0.5 – 1 h, and 0.5 - 4 h. To consider both resistant and sensitive subpopulations, the results were also separated into two parts. Addition of 0 and 1000 ppm BP to 0.4 a_w MBM did not significantly enhance the heat treatment either during the first 30 min or the subsequent 3.5 h ($P > 0.05$). This is likely caused by the high thermal resistance developed by *S.* Typhimurium at low a_w conditions, which has been observed in previous studies (Archer et al., 1998; Syamaladevi, Tadapaneni, et al., 2016; Syamaladevi, Tang, et al., 2016) and may have changed the fatty acid composition of the membrane (Chen, Golden, & Critzer, 2014) or enhanced the level of osmoprotectant (Mutz et al., 2020) so that the addition of 1000 ppm BP could not induce enough stress within the cells. In the preliminary experiment where 2000 ppm BP was added to the heat treatment at 0.4 a_w (Figure 2-7), no obvious enhancement on heat treatment efficiency against *S.* Typhimurium was observed over the treatment period of 1 h.
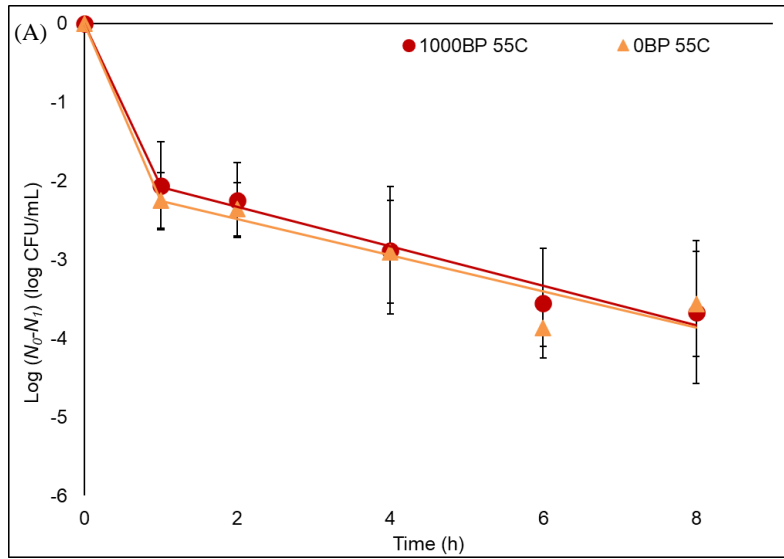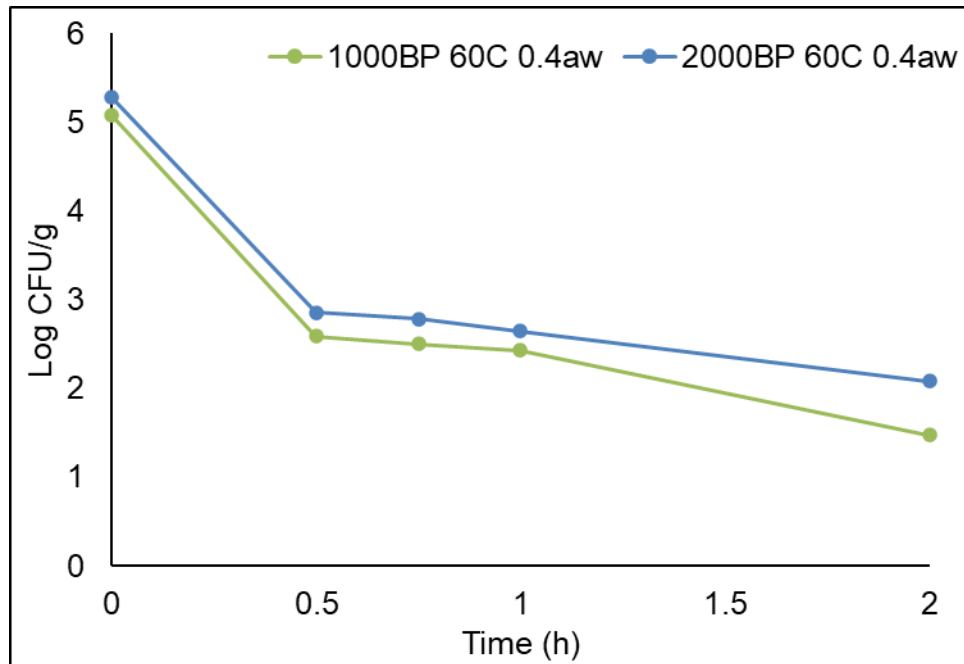
Figure 2-7. Inactivation kinetics for *E. coli* O157:H7 received 50 °C heat treatment at 0.4 a_w in the presence of BP at 1000 ppm without additional 24-h adaptation period. Numbers shown on the vertical axis represent survival population in corresponding time points. Results generated from 2 replicates.

To determine whether the bacterial thermal resistance at low a_w is a factor contributing to the absence of enhancement effect from BP, a_w of MBM was increased to 0.7 (Figure 2-6B). Such experiments at this higher water activity were not performed with *E. coli* O157:H7 since the enhanced antimicrobial effect of 1000 ppm BP was observed at a_w of 0.4. After 1 h, bacterial population was reduced to below the limit of detection and thus was not used for analysis. When compared to treatment conducted at 0.4 a_w, reduction induced by heat treatment alone at 0.7 a_w (2.8 ± 0.3 log CFU/ g) was not significantly different in the first 30 min ($P > 0.05$), which suggested that the heat-sensitive subpopulation of *S.* Typhimurium did not change its heat resistance even at a higher a_w. However, in the following 30 min, inactivation by heat treatment at 0.7 a_w (0.7 ± 0.1 log CFU/g) was significantly higher than at 0.4 a_w (0.4 ± 0.1 log CFU/g) ($P < 0.05$), indicating that the thermal resistance of the heat resistant subpopulation was slightly reduced after a_w was increased from 0.4 to 0.7.

Figure 2-6B. Inactivation kinetics for *S.* Typhimurium received 60 °C heat treatment at 0.7 a_w in the presence of BP at different concentrations without additional 24-h adaptation period. Numbers shown on the vertical axis represent log-reduction values in corresponding time points by comparing to the initial bacterial population. Average ± standard deviation (SD). Due to the varied initial population for treatments with or without 24-h adaptation, detection limit for bacterial reduction is at ~ 4 log CFU/g reduction (dotted line). Data points below the limit of detection were not shown on the figures.

Addition of 1000 or 2000 ppm BP to MBM at 0.7 a_w did not significantly accelerate microbial inactivation either in the first 30 minute or between 30 and 60 minute compared to heat treatment alone ($P > 0.05$). Therefore, by reducing bacterial heat resistance of *S.* Typhimurium, addition of BP at $\leq$ 2000 ppm did not enhance heat treatment in MBM. It is worth noticing that the bacterial reduction caused by heat treatment alone was lower than the reduction induced by the treatment with 1000/ 2000 ppm BP at 0.7 a_w in all three individual replicates at each tested time points, but significant difference was not observed ($P > 0.05$) among the average of those replicates because of the high standard deviation among trials. Thus, BP may have the potential to work synergistically with heat treatment in low-a_w environment at higher dose or temperature, albeit the effect may be marginal.

The difference of the treatment efficiency against the two target pathogens is likely resulted from the higher thermal resistance developed by *S.* Typhimurium at low a_w condition, which is consistent with previous findings in alfalfa seeds. Beuchat and Scouten have observed that heat treatment at 60 °C or above was much more efficient against *E. coli* O157:H7 than *Salmonella* spp., where treatment at 60 °C for 7 h has resulted in 0.03-0.58 log reduction among *Salmonella* spp. at 0.59 a_w, while a reduction of 2.30 log was detected among *E. coli* O157:H7 at 0.52 a_w (Beuchat & Scouten, 2002). Since both BP and heat treatment have similar cellular targets (such as membrane integrity), the enhancement effect of BP towards the heat treatment could be adversely affected by the higher level of thermal resistance among *S.* Typhimurium (Davidson, 2005; Russell, 2003; Tatsuguchi et al., 1991).

When a prolonged adaptation period for the inoculated *S.* Typhimurium was adopted prior to treatment, results on Figure 2-6C suggest that bacterial reduction from heat treatments at a_w of 0.4 (1.4 ± 0.3 (first 30 min)/ 1.1 ± 0.4 log CFU/ g (rest 3.5 h)) and 0.7 (2.4 ± 0.4 (first 30

min)/ 0.7 ± 0.1 log CFU/ g (rest 30 min)) a_w were not significantly different from the corresponding treatments on cells adapted for 30-45 min ($P > 0.05$). They indicate that the heat resistance of *S.* Typhimurium was not significantly affected during the extended adaptation period. Addition of 1000 ppm BP did not enhance the heat treatment on the cells that received prolonged adaptation at 0.4 or 0.7 a_w ($P > 0.05$).
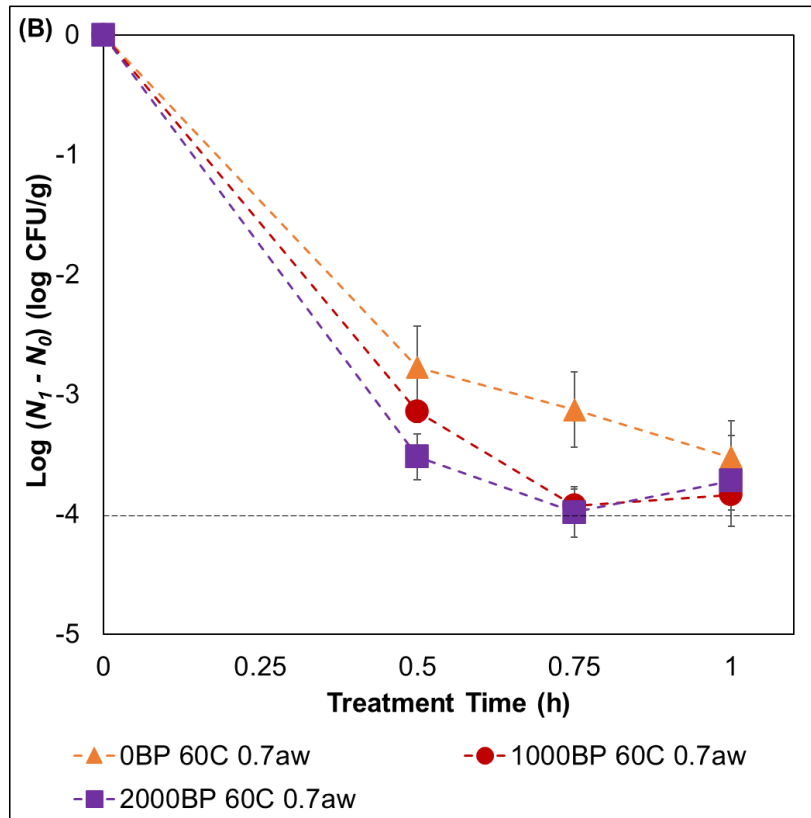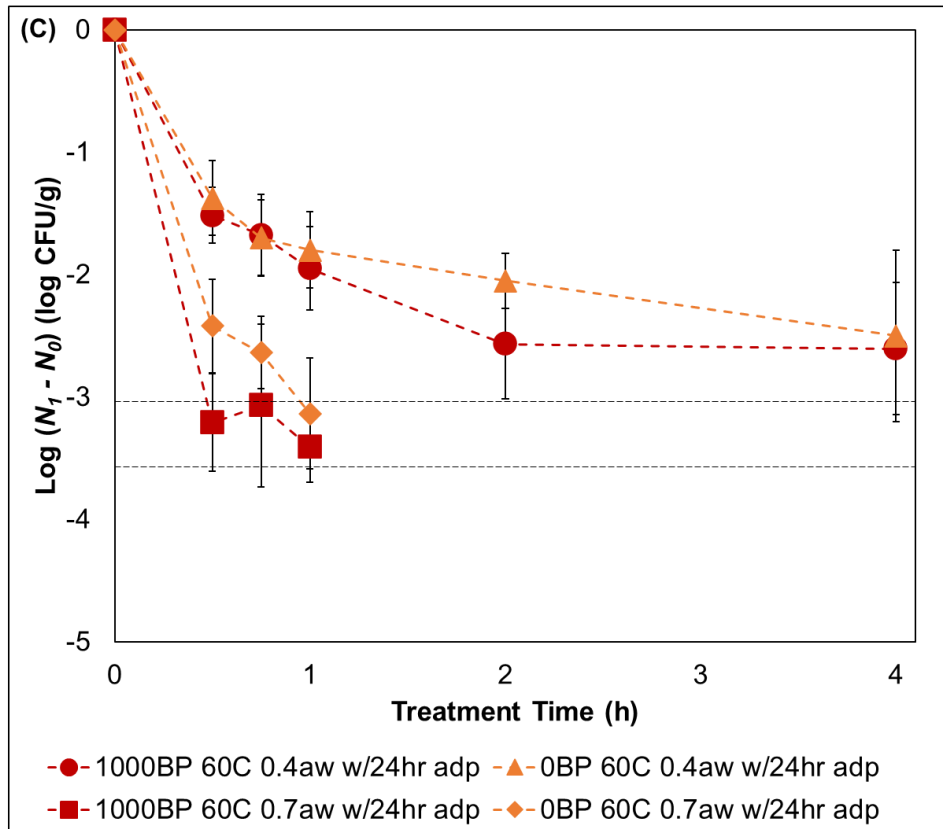
Figure 2-6C. Inactivation kinetics for *S*. Typhimurium received 60 °C heat treatment at 0.4 or 0.7 a_w in the presence of BP at different concentrations with or without additional 24-h adaptation period. Numbers shown on the vertical axis represent log-reduction values in corresponding time points by comparing to the initial bacterial population. Average ± standard deviation (SD). Due to the varied initial population for treatments with or without 24-h adaptation, detection limit for bacterial reduction is at ~ 3.0 (for treatment at 0.4 a_w) or 3.5 (for treatment at 0.7 a_w) log CFU/g reduction (dotted lines). Data points below the limit of detection were not shown on the figures.

It is also worth noting that during its adaptation to the low a_w environment (0.4 a_w) for 24-h, *S*. Typhimurium population was reduced by 1.9 ± 0.3 log CFU/g, which was significantly lower than the reduction of *E. coli* O157:H7 (2.9 ± 0.2 log CFU/g) after the same adaptation period. This finding corresponds to the higher tolerance of *S*. Typhimurium towards the desiccated environment as seen in other studies (Kimber et al., 2012; Koseki et al., 2015), which may have also contributed to the lower reduction of *S*. Typhimurium observed after the heat treatment in MBM with low a_w.

2.4   *Conclusion*

Addition of BP at ≥ 1000 ppm can significantly enhance the effect of heat treatment against *E. coli* O157:H7 in MBM at 0.4 a_w. Results with *S.* Typhimurium suggested that it was more heat resistant than *E. coli* O157:H7 and the addition of 1000 ppm BP was not able to enhance the effect of heat treatment at 0.4 a_w. Even when the heat resistance of *S.* Typhimurium was reduced by increasing the a_w to 0.7, addition of 1000 or 2000 ppm BP could not significantly enhance the thermal inactivation. A sub-population with higher thermal resistance existed in both bacteria. BP accelerated thermal inactivation of the thermal-resistant subpopulation in *E. coli* O157:H7 but not *S.* Typhimurium. Results from this study have shown the potential of using food-grade antimicrobial compounds to improve the antimicrobial effect of existing thermal treatment (such as "hot packing") or heat-assisted dehydration process for LMFs. It should be noted that BP was used in this study only as a model compound because of our prior results with this compound and its structural motif, a fatty acid chain esterified with an aromatic ring, to demonstrate the enhancement in thermal inactivation rate of select microbes. It demonstrates the possibility of using a wide variety of food-grade compounds to enhance the antimicrobial effect of heat processing for LMFs through their synergistic actions. Exploration into combination of heat treatment with compounds beyond BP to accelerate microbial inactivation of pathogenic bacteria in LMF would be needed. It is also necessary to investigate protection mechanism of *S.* Typhimurium against the combined BP and heat treatment at low a_w condition. The experiments in Chapter 3 will study the alternative antimicrobial compounds that can enhance heat treatments against *S.* Typhimurium at low a_w and the associated physiological changes that contribute to bacterial resistance. The molecular mechanism behind its resistance in LMFs will be studied in Chapter 4.

3 Chapter 3 Evaluate the effect of heat treatment with food-grade antimicrobial compounds against *S*. Typhimurium in starch, protein and lipid matrices at low a_w and the corresponding resistance mechanisms

Abstract

Food-grade antimicrobial compounds combined with mild heat treatments can accelerate inactivation to address this issue, but little is known on how different LMF components affect bacterial resistance to the combined treatments. Trans-cinnamaldehyde (CA, 1000 ppm) and eugenol (EG, 1000 ppm) can accelerate thermal inactivation of *Salmonella* Typhimurium in water. Their ability to induce similar effects in bacteria adapted to low a_w in different LMF components was evaluated. Although CA and EG significantly accelerated thermal inactivation (55 °C) of *S.* Typhimurium in whey protein (WP), corn starch (CS) and peanut oil (PO) at 0.9 a_w, such effect was not observed in bacteria adapted to lower a_w (0.4). The a_w-dependent matrix effect on bacterial thermal resistance was observed at 0.9 a_w and was ranked as WP > PO > CS. Metabolic activity was measured in *S.* Typhimurium following sub-lethal treatments. Regardless of a_w (0.4 or 0.9), bacterial metabolic activity was lowered with the 55 °C heat treatment in all tested LMF components ($P < 0.05$). Addition of CA/ EG to the heat treatment further reduced metabolic activity at both a_w in CS and PO ($P < 0.05$), while similar effect was absent in WP. Thus, bacterial metabolic response to thermal treatments differed at two a_w values and that the effect of CA/ EG treatments on bacterial metabolic activity was partially dependent on the food matrix. Membrane fluidity and membrane fatty acid compositions of *S.* Typhimurium were also measured with 1,6-diphenyl-1,3,5-hexatriene (DPH) assay and gas chromatography. Cells adapted to 0.4 a_w had lower membrane fluidity and unsaturated to saturated fatty acids ratio than bacteria at 0.9 a_w or in the stationary phase broth culture, suggesting that bacteria at low a_w can change its membrane composition to increase its rigidity and reduce its permeability, thus increasing resistance against both the heat and antimicrobial treatments. This study demonstrates the potential of using food-grade antimicrobials to complement thermal treatment in certain

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conditions and provides an insight into the resistance mechanism of *S*. Typhimurium at low a_w in various food matrices.

3.1   Introduction

Pathogens such as *S*. Typhimurium can develop increased thermal resistance in ~~at~~ low-a_w environments, thus requiring a treatment with higher temperature or longer duration to ensure food safety (Archer et al., 1998; Beuchat et al., 2013; Beuchat & Scouten, 2002; Santillana Farakos et al., 2013). Alternatively, to reduce the loss of nutritional and sensory quality in the final products, the inactivation efficiency of the existing heat treatment can be enhanced through the synergistic application of different preservative factors ("hurdles") at lower intensity (Leistner, 2000; Rodrigo et al., 2016; Trugo et al., 2000).

Many previous studies have demonstrated enhancement of thermal treatment by inclusion of food-grade antimicrobial compounds such as plant-derived terpenes/ essential oils (and their vapors) (Ait-Ouazzou et al., 2011; Corrêa et al., 2021; Espina et al., 2012, 2014; Sarbu et al., 2019; Y. Xu et al., 2021, 2022), long/ medium-chain fatty acids (Y. Xu et al., 2021; H. Zhang et al., 2019), benzoic acid derivatives (Ding et al., 2021; Dock et al., 2000; Ruan, 2016), and gaseous chlorine dioxide (Rane et al., 2021); but fewer studies have focused on such an effect in LMFs (Abdelhamid & Yousef, 2021; Xie et al., 2021; Y. Xu et al., 2021). However, bacteria adapted to a low-a_w environment may develop resistance against stress from antimicrobial compounds as well (Dubois-Brissonnet et al., 2011; Naïtali et al., 2009). In our previous study, although addition of BP at $\geq$ 1000 ppm can significantly enhance the effect of heat treatment against *E. coli* O157:H7 in MBM, it was not able to enhance heat treatment against *S*. Typhimurium at 0.4 a_w. Even when the heat resistance of *S*. Typhimurium was reduced by increasing the a_w to 0.7, addition of 1000 or 2000 ppm BP could not significantly enhance the thermal inactivation. Therefore, in this study, other food-grade antimicrobial compounds were explored for their ability to accelerate thermal inactivation of *S*. Typhimurium.

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Reduced metabolic activity (Gilbert et al., 2018; Vidal-Aroca et al., 2009) and changes in the fatty acid profile of cellular membrane (Di Pasqua et al., 2006; Dubois-Brissonnet et al., 2011) can all contribute to bacterial adaptation to treatments with antimicrobial compounds. Bacteria in food with different compositions, also developed different types and levels of resistances against both heat treatment (Syamaladevi, Tang, et al., 2016) and antimicrobial compounds (Wei Chen et al., 2015; Farbood et al., 1976). Nevertheless, most of the prior studies were conducted in individual LMF products with complex compositions, where the effect of individual components in foods (such as carbohydrate, protein and lipids) on bacterial resistance was not considered. Therefore, it is necessary to systematically evaluate bacterial resistance towards both heat treatment and antimicrobial compounds in different food components at low a_w.

Trans-cinnamaldehyde (CA) (21 C.F.R. § 182.60, 2019) and eugenol (EG) (21 C.F.R. § 184.1257, 2019) are among the food-grade antimicrobial (generally recognized as safe) compounds that demonstrated significant ability to accelerate thermal inactivation in some of the fore-mentioned studies, but the treatments were largely conducted at very high processing temperatures (> 70 °C) or in microbiological media or aqueous foods with high a_w. This study aims to determine (1) if addition of CA or EG would be effective to improve the inactivation efficiency during thermal processing of food with different components and a_w values, and (2) explore the mechanism behind the matrix/ a_w effect on bacterial resistance via measurement of bacterial metabolic activity and changes in bacterial membrane composition and fluidity following the treatments and adaptation.

3.2   *Materials and Methods*

3.2.1   *Bacterial culture and sample inoculation*

Corn starch (CS) (ACH Food Companies, Memphis, IN, USA), whey protein (WP) (Hilmar Ingredients, Hilmar, CA, USA) and peanut oil (PO) (Foodhold USA, Landover, MD, USA) were used to represent the dominant components in LMFs (carbohydrate, protein and lipids). *Salmonella enterica* serovar Typhimurium LT2 (ATCC 700720) was used as the target pathogen and raised according to a pre-established method (Ding et al., 2021). Briefly, *S.* Typhimurium from frozen culture was inoculated onto Trypticase soy agar (236920, Difco, BD, Franklin Lakes, NJ, USA) with additional 0.6% yeast extract (TSAYE) and stored at 4 °C for up to 3 weeks. Before each experiment, bacteria from the plates were inoculated into 100 mL sterile Trypticase soy broth (TSB) (211825, Difco, BD, Franklin Lakes, NJ) at 37 °C for 18-20 h to achieve the early stationary phase (approximately 9 log CFU/mL). It was then harvested via centrifugation at 7,197 *g* for 10 min and washed with sterile phosphate buffer saline (1× PBS) before being resuspended into 800 µL sterilized DI water. Inoculation in CS or WP was done by mixing 800 µL of the washed bacterial suspension (in water) with 20 g of CS or WP via hand massaging in a sterilized stomach bag for 10 min (B01064, Whirl-Pak, Nasco, Fort Atkinson, WI, USA). Inoculation in PO was conducted by re-suspending bacterial pellet that has been air dried under a biosafety cabinet for 20 min into 10 mL of PO with a handheld homogenizer at medium speed for 60 s.

3.2.2   *Antimicrobial compounds screening in aqueous solution*

Freshly harvested stationery-phase *S.* Typhimurium was directly suspended in DI water with different natural antimicrobial compounds (CA (A14690, Lot# 5018R23W, Alfa Aesar, Ward Hill, MA, USA) at 1000 ppm, EG (97530, Lot# A0382988, Acros Organics, Fair Lawn,

NJ, USA) at 1000 ppm, vanillin (Acros Organics, Fair Lawn, NJ, USA) at 1000 ppm, carvacrol

(TCI America, Portland, OR, USA) at 1000 ppm, citral (TCI America, Portland, OR, USA) at

500 ppm, thymol (Acros Organics, Fair Lawn, NJ, USA) at 500 ppm, octanoic acid (Acros

Organics, Fair Lawn, NJ, USA) at 500 ppm), respectively. 200 µL of the spiked sample was

immediately transferred to a capped 500 µL centrifugal tube for a 20-min heat treatment in a

water bath pre-equilibrated to 55 ± 0.3 °C. Additional samples with 0, 500 or 1000 ppm

antimicrobial compounds were also stored at room temperature (20-22 °C) as controls.

3.2.3 *Antimicrobial compounds screening in cocoa butter*

Air-dried bacterial pellet from 100 mL stationery-phase *S.* Typhimurium culture was

suspended into 10 mL of melted refined cocoa butter (CB) (Spectrum, New Brunswick, NJ)

(contains > 99% triglycerides) with a handheld homogenizer. The a_w of the inoculated CB was

adjusted to 0.5 for 48 hrs. The samples were then melted at 37 °C and spiked with individual

antimicrobial compounds of choice by mixing the inoculated samples with CB containing

individual antimicrobial compounds at 1:1 ratio to reach a final concentration of 1000 ppm.

Control samples with no antimicrobial compounds were also prepared. The samples were

immediately transferred to a capped 500 µL centrifugal tube for a 20-min heat treatment in a

water bath pre-equilibrated to 55 ± 0.3 °C.

3.2.4 *Sample equilibration and antimicrobial compounds spiking*

The a_w of the inoculated LMF matrices was adjusted to target levels by incubating the

inoculated samples inside a sealed desiccator containing deionized water or saturated $MgCl_2$

solution for 72 h at dark (22 ± 2 °C) prior to subsequent treatment (Greenspan, 1977). The

equilibrated samples were then spiked with CA or EG to reach a final concentration of 1000

ppm. CS and WP were spiked by mixing the suitable amount of the pure compound into the

samples via hand-stirring in a glass beaker. PO was spiked by vortexing uninoculated PO

containing corresponding antimicrobial compounds for 1 min before mixing it with inoculated

PO at 1:1 ratio. Control samples with no antimicrobial compounds were also prepared by either

stirring the samples (CS and WP) without addition of any antimicrobial compounds or mixing

the sample (PO) with un-spiked PO. A HygroPalm AW water activity meter was used to

determine the exact a_w values of the mixed samples (Rotronic Instrument, NY, USA). Water

activity values were measured to be 0.39 ± 0.03 (incubated with saturated $MgCl_2$ solution;

approximated as 0.4 a_w)/ 0.92 ± 0.02 a_w (incubated with saturated KNO_3 solution; approximated

as 0.9 a_w) for all samples.

3.2.5   *Heat treatment*

100 mg of the CS or WP samples or 200 mg of the PO with 0 or 1000 ppm antimicrobial

compounds at different a_w levels were individually transferred into a sealed stomach bag

(B01067, Whirl-Pak, Nasco, Fort Atkinson, WI, USA) (for CS and WP samples) or 500 µL

centrifuge tube (for PO and water samples). Heat treatment was conducted by submerging the

body (beneath bag seal/ tube cap) of the sample-containing bags/ tubes in a water bath pre-

equilibrated to desired treatment temperatures (± 0.3 °C). One bag or tube was removed for

analysis at each pre-determined sampling time points. Samples inside the tubes took ~2 min to

reach its set values (55 °C) upon submerging into the water. Additional samples with 0 or 1000

ppm antimicrobial compounds were also stored at room temperature (20-22 °C) as non-heated

controls.

3.2.6   *Sample extraction and bacterial enumeration*

The surviving bacterial cells in samples after the treatments were extracted by thoroughly

mixing 100 mg or 100 µL sample with 900 µL sterilized 0.2 % Buffered Peptone Water (BPW).

0.1% Tween-20 were added to BPW for the extraction of PO to enhance extraction efficiency. After appropriate serial dilutions in BPW, 50 µL of sample was spiral plated onto TSAYE or Xylose Lysine Deoxycholate agar (XLD) (BD Difco, DF0075-07-3, Franklin Lakes, NJ, USA) plates with a spiral plater at E50 mode (Neutec Group Inc., Nr 10003700/113, Farmingdale, NY, USA). The minimum threshold for detecting surviving bacteria in the LMF matrixes was 2 log CFU/g. Dilution and plating were conducted within one minute following the treatment to minimize the residual activity of heat treatment or antimicrobial compounds in samples. After incubation at 37 °C for 24 h, plates were counted with an automatic plate counter (IUL Instruments S. A, Flash & Go, Königswinter, Germany) and converted to Log_{10} (CFU/mL) values. The results on TSAYE plates were normalized by calculating bacterial population reduction ($\log N_t - \log N_0$) at each sampling time, where N_t is the bacterial population after treatment time t min and N_0 is the initial bacterial population. The microbial inactivation kinetics were fitted to the Weibull model with the nonlinear regression function of JMP Pro 15 software (Cary, NC, USA) (Dhaliwal et al., 2021; M. A. J. S. Van Boekel, 2002) using equation (1):

$$log \left(\frac{N_t}{N_0}\right) = -\frac{1}{2.303}\left(\frac{t}{\alpha}\right)^{\beta} \quad (1)$$

Where α is a scale parameter that describes the characteristic time (min) and β is a shape parameter of the inactivation curve. Based on the Weibull model, the time needed to reach a 2-log reduction (t_{D2} , min) was computed for each treatment using equation (2)

$$t_{D2} = \alpha(4.606)^{\frac{1}{\beta}} \quad (2)$$

Injured bacteria before and after the 72-h a_w equilibration period were determined by calculating the difference between results on TSAYE and XLD plates.

3.2.7 _Sample homogeneity for microbial load and concentration of compounds_

These tests were conducted by taking three 1-g samples from random positions in the inoculated LMF components prior to treatments. The bacterial homogeneity was measured with the enumeration method described above. Because the same spiking method was used for both CA and EG, only the concentrations of EG was measured to reflect the distribution homogeneity of compounds within the samples. The homogeneity of EG in CS and WP was measured by extracting the compound from spiked samples in 10 mL methanol for 12 h and then measuring the EG concentration in the solvent with a Shimadzu LC2010 High Pressure Liquid Chromatograph (HPLC) (Shimadzu, Kyoto, Japan) equipped with a Waters® Spherisorb™ ODS2 10um column (4.6 × 250 mm) and a UV-Vis detector (280 nm) operating in isocratic mode (60% methanol, 1 mL/min). The final EG concentrations in the matrix were calculated based on a standard curve and expressed as parts per million (ppm) (Figure 3-1). Homogeneity test was not performed in PO as high solubility of the spiked compounds in PO (> 1000 ppm) and the liquid state of the sample would ensure homogeneity after through mixing.
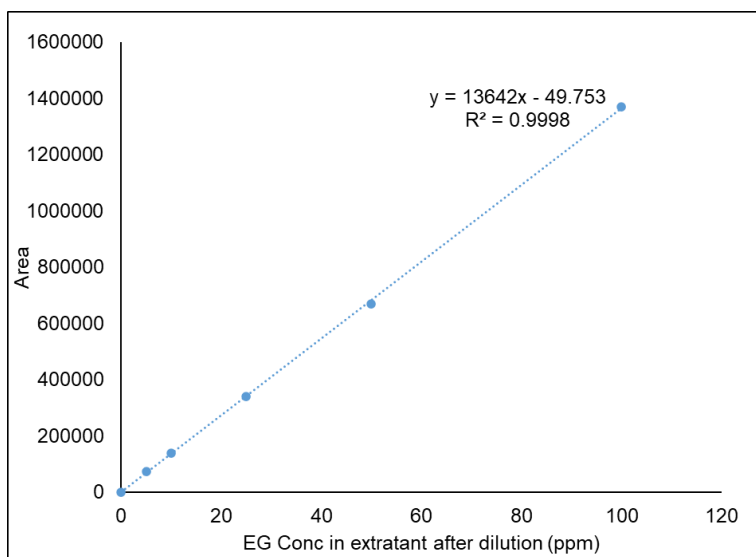
Figure 3-1. EG standard curve during HPLC analysis in the diluted extractant of CS and WP

3.2.8 *Measuring bacterial metabolic activity change in LMF matrixes after treatments using Resazurin assay*

Resazurin (alamar blue) is a non-fluorescent dye that can be metabolically reduced to a strongly-fluorescent compound (resorufin) in living cells (Duarte et al., 2009). The irreversible reaction is catalyzed by reductase enzymes such as intracellular diaphorase or NADH dehydrogenase (O'Brien et al., 2000; Zalata et al., 2002) and the resulting product is freely released into extracellular environment (Uzarski et al., 2017). Concentration of the produced resorufin is proportional to the metabolic activity of the cell population and can be measured via fluorescent spectrophotometry (McGaw et al., 2014; Uzarski et al., 2017). Resorufin is further reduced into non-fluorescent dihydroresorufin by bacterial reductase, causing the fluorescence intensity to decrease after reaching its peak (Pace & Burg, 2015).

Sample inoculation, equilibration and spiking were performed in the same manner as previously described. Heat treatment was conducted on 1 g sample inside a sealed stomach bag (for CS and PW) or 1.5 mL centrifugal tube (for PO) submerged in the pre-equilibrated water bath. Treatment time was limited to 3 min to minimize the effect of bacterial inactivation on the observed metabolic levels. Additional samples with 0 or 1000 ppm antimicrobial compounds were stored at room temperature (20-22 °C) as non-heated controls.

After the treatments, bacteria in PO samples were harvested by centrifugation at 10,000 g for 5 min. Bacteria in CS or WP samples were first extracted in BPW. Insoluble starch particles in the CS extract were removed by centrifugation at 100 g for 1 min, while bacteria were separated from the supernatant via a second centrifugation at 10,000 g for 5 min. Bacteria in the WP extract were pelleted by centrifugation at 10,000 g for 5 min. Bacterial pellets were subsequently washed thrice with sterilized 1×PBS. The final pellet was resuspended into sterilized TSB containing 50 µM resazurin and incubated at 37 ºC in dark. Fluorescence

measurements were taken periodically over a period of 12 h with a SpectraMax M5e microplate reader (Molecular Devices, Sunnyvale, CA) setting at excitation and emission wavelengths of 560 and 590 nm, respectively.

3.2.9 *Measuring bacterial membrane fluidity and membrane fatty acid compositions in bacteria adapted to different a_w*

Bacterial membrane fluidity was determined by measuring the polarization value of 1,6-diphenyl-1,3,5-hexatriene (DPH), a membrane inserted probe (Ding et al., 2018; Mykytczuk et al., 2007). The DPH probe can incorporate itself along the fatty acid side chains within the bacterial cytoplasmic membrane, where its rotational movement is restricted by the arrangement of the membrane lipids via interaction with the acyl chains and can reflect membrane fluidity (Adler & Tritton, 1988; Ding et al., 2018; Mykytczuk et al., 2007). DPH incorporated to membrane with higher fluidity has higher rotational freedom due to lower restriction from chain interaction, while its motion in membrane with lower fluidity may be more restricted. A low polarization value from DPH therefore indicates a high membrane fluidity and vice versa. Bacterial membrane fatty acid compositions were also determined with analytical gas chromatography (GC) using the method described below.

Stationary phase (20-h) *S.* Typhimurium cells were harvested from the original culture via centrifugation (7,197 *g* for 10 min) and washed with sterilized DI water. The final pellet was resuspended in 200 µL DI water and stored inside a sealed desiccator containing deionized water or saturated $MgCl_2$ solution for 72 h at dark (22 °C). The final a_w values of the desiccated samples were measured to be 0.4/ 0.9 with the HygroPalm AW water activity meter immediately before the analysis of the membrane properties. Stationary phase *S.* Typhimurium cells harvested from fresh culture (TSB) were also included as controls. Bacteria inoculated in the LMF

components were not included in the analyses due to the interference from the natural lipids in the food samples. DPH (Acros Organics, NJ, USA) was first dissolved in tetrahydrofuran (Fisher Scientific, NJ, USA) then diluted in sterilized PBS to make a 4 μM working solution. *S.* Typhimurium cells adapted to low-a_w environment or from fresh TSB culture were resuspended in 1 mL of the working solution and incubated at dark for 30 min. The fluorescence polarization values were measured on the SpectraMax M5e microplate reader at excitation and emission wavelengths of 358 and 428 nm, respectively. The emission intensity of light emitted both parallel (I_{para}) and perpendicular (I_{perp}) to the plane of excitation was measured by the integrated emission polarizers. The polarization value (mP) was calculated via the following formula:

$$mP = 1000 \times \frac{I_{para} - G \times I_{perp}}{I_{para} + G \times I_{perp}} \qquad (3)$$

Where G is the instrument dependent grating factor and was set to be 1 based on the instrumental settings.

Extraction and esterification of bacterial fatty acids was conducted based on MIDI Technical note #101 with some modifications (Sasser, 2006). Briefly, desiccation-adapted or freshly harvested *S.* Typhimurium cells were resuspended in 2 mL of 3.75 M NaOH in 50% methanol (HPLC Grade, Fisher Scientific, NJ, USA) solution (v/v). The mixture was transferred to a capped glass tube and heated in a boiling water bath for 30 min, with a brief (10 s) vortexing after the first 5 min of the process to ensure a complete saponification. 4 mL of 6.0 N HCl (Certified, Fisher Scientific, NJ, USA): methanol (13:11, v/v) mixture was added to the cooled mixture then heated for 10 minutes at 80 °C to induce methylation. The tube was cooled rapidly under tap water and 1.25 mL of hexane (HPLC Grade, Fisher Scientific, NJ, USA): methyl tert-butyl ether (HPLC Grade, Alfa Aesar, MA, USA) (1:1, v/v) mixture was added to extract the generated fatty acid methyl esters by tumbling the tubes for 15 min. After phase separation, the

lower phase was removed, and the organic phase was washed with 3 mL of 0.3 M NaOH. Analysis of fatty acid methyl esters was carried out on a Shimadzu GC-2014 equipped with an auto injector (AOC-20i), a SHRXI-5 ms capillary column (15 m × 0.25 mm × 0.25 μm) and a flame ionization detector. 1.0 μl of sample was injected into the system with the injector temperature set at 250 °C and carrier gas (Helium) flow rate at 15 psi. Column temperature was set to hold at 50 °C for 4 min and then increased to 250 °C at the rate of 4°C/ min. The detector temperature was set at 250 °C. The concentration of the five major saturated/ unsaturated fatty acids (C18:0, C18:1, C16:0, C16:1, C14:0) in *Salmonella* (Álvarez-Ordóñez et al., 2008) were calculated against the standard curves of fatty acid methyl ester standards (Figure 3-2) (Acros Organics, NJ, USA). A cyclic fatty acid (methylenhexadecanoic acid (cyc17)) was also identified from the extract by comparing the retention time with a standard cyc17 fatty acid (Avanti Polar Lipids, Alabaster, Alabama, USA) that has been esterified in our lab using the procedures described earlier. However, because we did not know the efficiency of esterification of cyc 17 fatty acid, the concentration of esterified cyc17 in the standard could not be calculated. Therefore, the concentrations of cyc17 in different samples were analyzed by comparing the percentage of the area under the curve for cyc17 on the respective chromatograms. Results were expressed as relative percentages of the identified fatty acids (except for cyc 17) in each sample as well as the ratio between unsaturated and saturated fatty acids, not including cyc 17.
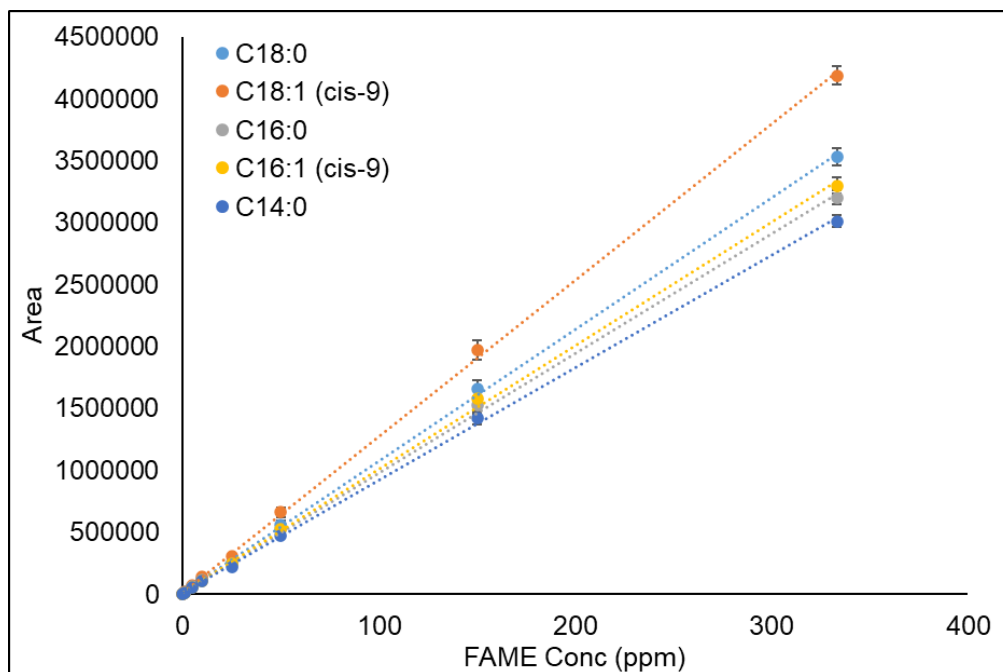
Figure 3-2. Standard curves for fatty acid methyl esters tested during GC analysis

3.2.10 Data analysis

The screening study with seven antimicrobial compounds in water was first performed

with two independent replicates, whereas triplicated tests were performed subsequently with the

two compounds of interests (CA and EG). Unless stated otherwise in the text, all other

experiments were performed in three independent trials. The significance levels were determined

with Student's t test using JMP Pro 15 software (Cary, NC, USA) at a significance level of $\alpha =$

0.05.

3.3   *Results and Discussions*

3.3.1   Sample homogeneity of inoculated bacteria and the spiked compounds

Homogeneity tests showed that the spiking methods evenly distributed EG in both CS

(944.2 ± 30.5 ppm) and WP (966.8 ± 38.4 ppm). The inoculation methods consistently yielded

similar bacterial counts from different locations within the same LMF matrix (standard deviation

value < 0.2 log CFU/g). These results confirmed the homogenous distribution for both bacteria

and the added compounds within the tested samples.

3.3.2   Antimicrobial effect of GRAS compounds assisted heat treatment against S. Typhimurium
*   in different LMF components*

The antimicrobial compounds for thermal treatments were selected based on a screening

study that tested the effectiveness of seven food-grade antimicrobial compounds at mild heating

temperature (55 °C) in both CB and water (Table 3-1). In CB at 0.5 a_w, no significant

enhancement to thermal inactivation was found on treatments with addition of the tested food-

grade compounds. However, in the aqueous solution, all tested compounds besides citral induced

an enhancement between 1.5 to 4 log in addition to thermal inactivation within 20 min (2.0 ± 0.1

log reduction). 1000 ppm of CA or EG significantly enhanced the effect of heat treatment

(55 °C) alone in the aqueous system ($P < 0.05$). The antimicrobial effect of CA and EG with relatively low doses ($< 0.2\%$) during extended storage (> 12 h) at room temperature have also been observed previously in both low and high a_w environments (Burt, 2004; Cava-Roda et al., 2021; Corrêa et al., 2021; Friedman, 2017; Ji et al., 2021). Therefore, subsequent tests in LMF components were conducted with CA (21 C.F.R. § 182.60, 2019) and EG (21 C.F.R. § 184.1257, 2019) at their maximum allowable dose in food (1000 ppm) to demonstrate the potential of food-grade antimicrobial compounds to enhance heat treatment effect in LMFs. CS, WP and PO were used as the representative carbohydrate, protein and lipid components of LMF, respectively. One thousand ppm of CA or EG did not induce significant bacterial inactivation at room temperature in all tested matrices at either 0.4 or 0.9 a_w (Table 3-2).

Table 3-1. *S*. Typhimurium reduction (compared to the initial bacterial population) (Log CFU/g) after 20 min of heat treatments (55 °C) in CB (0.5 a_w) or DI water, in the presence or absence of different potential antimicrobial compounds (data generated from duplicated trials).

Matrix		EG	CA	Vanillin	Carvacrol	Citral	Octanoic acid	Thymol	Ctrl (water/ CB)
CB (0.5 a_w)	#1	3.2	2.7	2.8	-	2.4	3.1	1.4	3.0
	#2	2.8	2.8	2.6	-	3.6	2.8	1.7	1.9
Water	#1	> 6	> 6	> 6	> 6	2.9	> 6	> 6	2.0
	#2	> 6	> 6	3.5	> 6	1.6	> 6	> 6	1.9

Table 3-2. Bacterial reduction (compared to the initial bacterial population) (Log CFU/g) after incubation in different LMF components at room temperature (22 °C) for 1 h, in the presence or absence of 1000 ppm CA or EG.

		Eugenol	Cinnamaldehyde	Control
PO	0.4a_w	0.03 ± 0.09	0.21 ± 0.14	-0.16 ± 0.11
	0.9a_w	-0.19 ± 0.16	0.00 ± 0.07	-0.17 ± 0.04
CS	0.4a_w	0.02 ± 0.07	-0.04 ± 0.05	-0.14 ± 0.06
	0.9a_w	0.23 ± 0.06	0.06 ± 0.10	-0.01 ± 0.07
WP	0.4a_w	0.10 ± 0.10	-0.06 ± 0.08	-0.21 ± 0.15
	0.9a_w	0.05 ± 0.08	0.28 ± 0.19	-0.16 ± 0.11

As seen in Figure 3-3, the presence of tails in the inactivation kinetics for some treatments made it difficult to compare treatment efficiency using linear regression model. Therefore, the Weibull model was used to fit the inactivation curve as it has been previously used to describe inactivation kinetics in both high and low (0.3 – 0.75) a_w environments (Dhaliwal et al., 2021). Except for PO at 0.4 a_w, the 2-log lethality time (t_{D2}) was computed from the fitted Weibull model and compared across different components to better account for the varied inactivation efficiency during different stages of treatments with biphasic inactivation kinetics (Table 3-3). For treatments in PO at 0.4 a_w, only < 1 log reduction was induced over the treatment time, which is too small for the model to accurately predict t_{D2} values (the models predict the values to be over 1000 min). A preliminary study (with single replicate) found that the heat treatment with CA (1.30 log reduction) or EG (1.22 log reduction) induced < 1.5 log reduction even when the treatment time was extended to two hours in PO at 0.4 a_w. Therefore, the comparison involves treatments in PO at 0.4 a_w were based on their log reduction values with the same treatment time.

Table 3-3. 2-log lethality time (t_{D2}, min) calculated from Weibull inactivation model of S. Typhimurium in different LMF components equilibrated to a_w 0.4 or 0.9, with 55 °C heat treatment in the presence or absence of 1000 ppm CA or EG. Values for treatments in PO at 0.4 a_w were not computed due to lack of inactivation data. Different letters indicate a statistically significant difference ($P < 0.05$). Average ± standard deviation (SD).

	WP		CS		PO	
	0.4 a_w	**0.9 a_w**	**0.4 a_w**	**0.9 a_w**	**0.4 a_w**	**0.9 a_w**
Heat treatment (55 °C) alone	122.9 ± 60.2 [a]	60.7 ± 11.9 [b]	90.2 ± 16.4 [a]	18.7 ± 1.7 [e]	-	23.3 ± 0.8 [d]
CA + Heat (55 °C)	65.9 ± 0.9 [ab]	40.4 ± 1.4 [c]	163.7 ± 66.1 [a]	13.9 ± 1.5 [f]	-	11.4 ± 0.8 [f]
EG + Heat (55 °C)	69.2 ± 2.5 [ab]	39.5 ± 4.5 [c]	149.0 ± 45.3 [a]	13.8 ± 1.1 [f]	-	11.9 ± 0.8 [f]

Figure 3-3. Inactivation kinetics for *S.* Typhimurium inoculated in WP/ CS/ PO at 0.4 or 0.9 a$_w$, with 55 °C heat treatment in the presence or absence of 1000 ppm CA or EG. Numbers shown on the vertical axis represent log-reduction values in corresponding time points by comparing to the initial bacterial population. Solid lines indicate the Weibull model fitted to the corresponding data points (R^2 > 0.8). Due to the varied initial population for treatments with or without 24-h adaptation, detection limit for bacterial reduction is at ~ 5 log CFU/g reduction for (a), ~ 6 log CFU/g reduction for (d), ~ 4.5 log CFU/g reduction for (b), ~ 3.5 log CFU/g reduction for (e), ~ 6.5 log CFU/g reduction for (c), and ~ 7.5 log CFU/g reduction for (f). Data points below the limit of detection were not shown on the figures.

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Effect of addition of 1000 ppm CA or EG on thermally induced microbial inactivation in WP, CS and PO equilibrated to 0.4 a_w is shown in Figure 3-3A, 2B, and 2C respectively. Interestingly, antimicrobial effect of heat treatment at 55 °C was not significantly accelerated by addition of either CA or EG regardless of the matrices tested. As indicated on Table 3-3, t_{D2} of the heat treatment alone was not reduced with the addition of CA or EG in either PO, WP or CS at 0.4 a_w ($P > 0.05$). When treatment temperature was increased to 70 °C, 1000 ppm CA (3.2 ± 0.2 log reduction) or EG (3.3 ± 0.1 log reduction) did not induce a significantly higher level of inactivation than the heat treatment alone (3.0 ± 0.1 log reduction) in PO at 0.4 a_w ($P > 0.05$). The initial population of *S.* Typhimurium in WP and CS were lower than PO after 72-h a_w adaption period and were reduced to below limit of detection within the first 30 min of heat treatment at 70 °C. Acceleration in thermal inactivation from the addition of either CA or EG at higher temperature (85 °C) in PO at 0.4 a_w was not observed (Figure 3-4). In summary, regardless of the compound or the matrix, neither EG or CA accelerated thermal inactivation of the bacteria at 0.4 a_w, although it is possible that PO specifically contained a significantly more thermally resistant sub-population than in CS and WP.
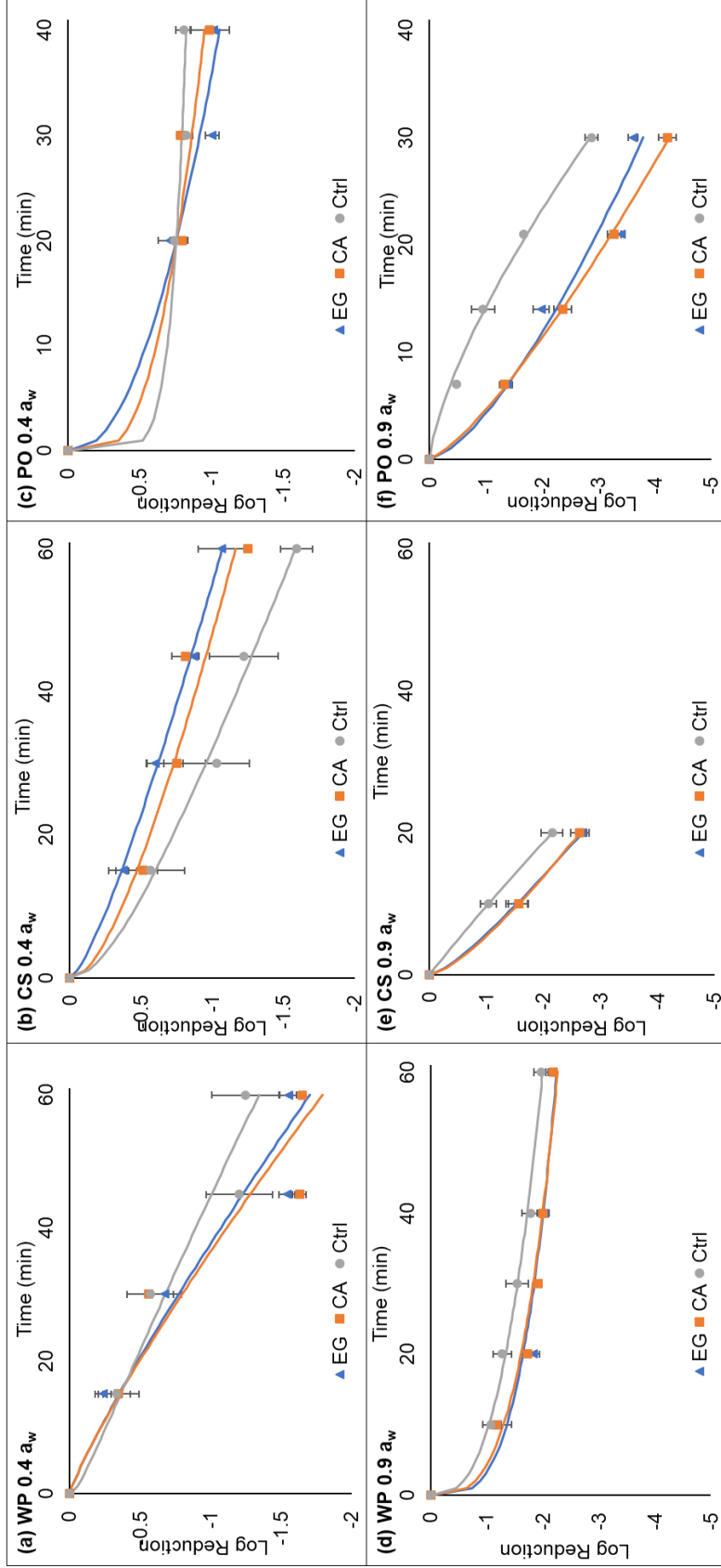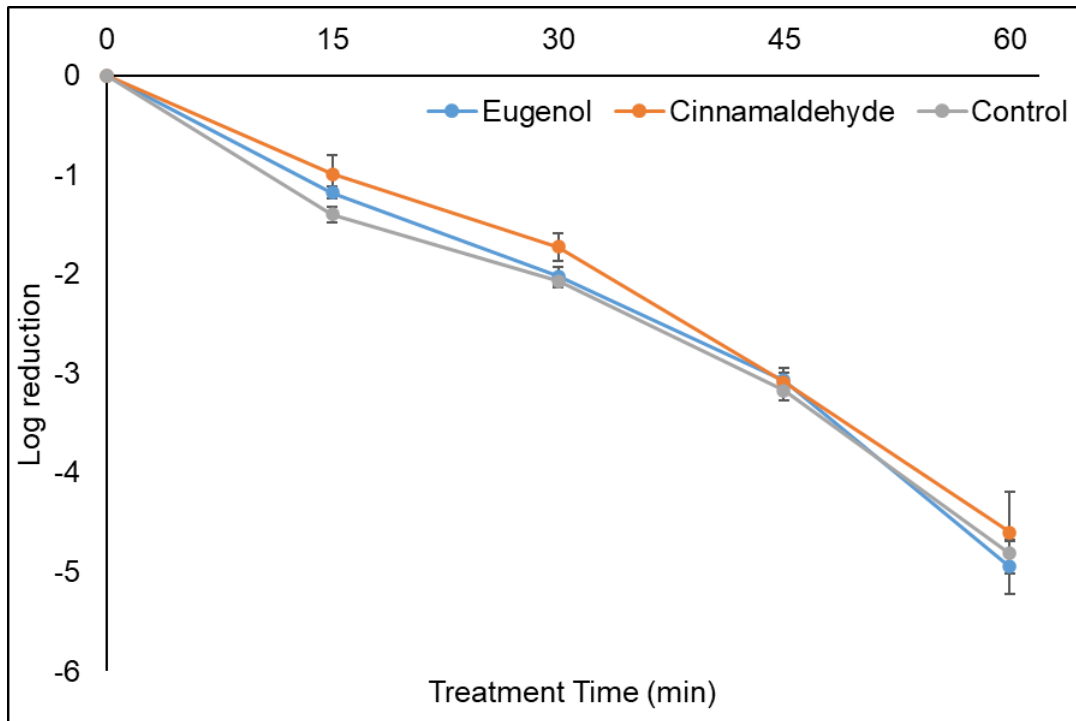
Figure 3-4. Inactivation kinetics for *S*. Typhimurium inoculated in PO at 0.4 a_w, with 85 °C heat treatment in the presence or absence of 1000 ppm CA or EG. Numbers shown on the vertical axis represent log-reduction values in corresponding time points by comparing to the initial bacterial population. Average ± standard deviation (SD).

In contrast to results at 0.4 a_w, when the a_w of the matrices was increased to 0.9, CA or EG significantly accelerated thermal inactivation in all three LMF components ($P < 0.05$) (Figure 3-3 (D-F); Table 3-3). In addition, the absolute t_{D2} of heat treatment was also significantly reduced when compared to treatment at 0.4 a_w in WP, CS and PO ($P < 0.05$) (Table 3-3), indicating lower bacterial resistance to inactivation. Similar results were reported in a recent study conducted in tahini (sesame seed paste) inoculated with *S*. Montevideo (Y. Xu et al., 2021). While there was synergistic effect between oregano oil or citric acid and heat treatment in tahini at high a_w (1.0 a_w), the effectiveness of the antimicrobial compounds was greatly reduced at lower a_w (~ 0.3 a_w). It was also reported that the enhancement of thermal inactivation from the addition of antimicrobial compounds in tahini at 0.3 a_w was either completely absent (with oregano oil) or could only be observed in the first 20 min of the treatment (with citric acid). Chen et al. also reported that the antimicrobial effect of CA at room temperature against *S*. Tennessee decreased when a_w reduced from 1 to 0.3 in a glycerol-sucrose model (Wei Chen et al., 2015). The apparent difference in the ability of the added compounds to accelerate thermal inactivation of *S*. Typhimurium at two a_w values can be partially explained by the lack of solvent (free water) to deliver the antimicrobial compounds close to bacterial membrane for uptake (Hua & Wang, 2014; U.S. Food and Drug Administration, 1984; Watkinson & Morgan, 1990). It is also known that the low a_w environment in LMF may induce higher expression of stress response genes in *S*. Typhimurium (Chen & Jiang, 2017; Rangel, 2011). Some of the upregulated genes, such as the *fabA*, which encodes the β-hydroxydecanoyl ACP dehydrase, can reduce cellular membrane fluidity by producing fatty acids with longer chain length and was observed in *S*. *enterica* after 6-day desiccation in peanut oil (0.52 a_w) (W Chen et al., 2014; Fong & Wang, 2016; Magnuson et al., 1993). This can enhance bacterial membrane rigidity and may serve as

cross-protection mechanism for both heat and membrane-disrupting agents such as CA and EG (Burt, 2004; Friedman, 2017). Furthermore, stress induced by essential oil compounds (including CA and EG) at lower dose are usually sublethal, which can cause higher inactivation when the cells were injured or under higher stress (Ait-Ouazzou et al., 2011; Ji et al., 2021). Because bacteria such as *S.* Typhimurium at low a_w are more resistant to heat treatment, they are less likely to be injured or significantly stressed. Therefore, it is possible for the effectiveness of CA or EG to be reduced at low a_w conditions.

It is also interesting to note that the t_{D2} values for the heat treatment in WP, CS or PO at 0.9 a_w were significantly different from each other (Table 3-3). The highest thermal resistance was observed in *S.* Typhimurium inoculated in WP and lowest thermal resistance was observed in CS (Table 3-3). Therefore, different food components can affect the heat resistance of *S.* Typhimurium in LMF matrices despite being at the same a_w. The effect of protein and lipid percentages in LMF matrices on bacterial thermal resistance has also been reported in a previous study, where the model predicted that the D- values of treatment at > 38.42 °C in high-protein matrices were larger than the high lipid matrices at 0.9 a_w (Jin et al., 2018). Although the impact of carbohydrate on bacterial resistance in LMF matrices has not been extensively studied, the protective effect from lipids in LMFs on bacteria against heat treatments at low a_w has been previously reported. In ground beef, pork sausage and poultry, *E. coli* O157:H7 had the lowest D-values when treated in media with the lowest fat content (3-7%) compared to media with higher fat content (11-30%) (Ahmed et al., 1995). Similar protective effect from fat was also observed in peanut butter (0.45 a_w) that has been inoculated with *Salmonella* Tennessee (Ma et al., 2009; Syamaladevi, Tang, et al., 2016). Some previous studies suggested that the matrix effect on bacterial thermal resistance was contributed by the varied desiccation stress presented

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during the a_w adaptation period or the thermal treatment in different food components with the same initial a_w (Finn, Condell, et al., 2013; Rangel, 2011; Sperber, 1983). Dhaliwal et al. (2021) suggested that the bacterial inoculation and a_w adaptation period in different LMF matrices promoted water expulsion from bacterial cells at different rate (Dhaliwal et al., 2021). Since water loss from bacterial cells can reduce protein mobility and strengthen the structure of heat sensitive protein (Liu et al., 2018), bacteria inoculated in different LMF components may develop varied thermal resistance despite being held at the same a_w. A study conducted by Syamaladevi et al. (2016) found that due to the different sorption isotherm profiles between LMF matrices, the actual a_w in the matrix would develop component-dependent changes as the temperature increased during thermal treatment, causing bacteria inoculated in different food matrices with the same a_w level at room temperature to be exposed to different desiccation stress during heat treatments, thus affecting thermal resistance (Syamaladevi, Tang, et al., 2016). Both findings can help to explain the observed matrix effect on bacterial thermal resistance. The matrix component effect on bacterial thermal resistance at 0.4 a_w was not compared due to lack of data.

To study the cause for the biphasic inactivation kinetics in WP at 0.9 a_w and PO at 0.4 a_w, bacterial injury levels before treatments were determined in these two matrices. The results showed that 1.2 ± 0.2 log bacteria in WP at 0.9 a_w were injured before the beginning of the treatment, which corresponded to the ~1 log inactivation within the first 10 min of thermal treatment. However, only ~0.1 log bacteria in PO at 0.4 a_w were injured before the start of the treatment, which still could not account for the rapid inactivation (~0.7 log reduction) observed within the initial 20-min treatment. Similar biphasic thermal inactivation patterns of *S.* Typhimurium at low a_w has been previously observed by our group in meat and bone meal (at

both 0.4 and 0.7 a_w) (Ding et al., 2021). A recent study has found that the presence of gaseous headspace in sample holder during thermal treatment on LMFs can lead to surface dehydration, which reduced the local a_w in samples and contributed to bacterial resistance increase (R. Yang et al., 2022). Since the sample holding vessel used during the heat treatment of this study did contain substantial gaseous headspace, it is likely that the bacterial thermal resistance has increased as the sample dehydrating during heat treatment, thus forming the observed biphasic inactivation pattern.

3.3.3   _Metabolic activity change of S. Typhimurium after treatments in different LMF_ _components_

Bacterial metabolic system is a vital component in cells that generates energy to support normal physiological activities, where stresses from antimicrobial treatments can adversely affect its function and eventually lead to inactivation (Jurtshuk, 1996). Therefore, bacterial metabolic activity was measured as an indicator of stresses experienced by the bacteria during treatments. Bacteria with higher initial metabolic activity would metabolize resazurin faster, thus producing peak fluorescence intensity faster (shorter peak time (t_{peak})), while bacteria with suppressed metabolic activity would require longer time to reach its peak intensity (longer t_{peak}). The bacteria were exposed to sub-lethal levels of treatments to not induce significant population change in the matrices, where no significant change to bacterial population was observed (Table 3-4). This avoided differing numbers of surviving cells affecting the measurement. However, due to the significant difference ($P < 0.05$) between the initial bacterial populations in different food matrices or at different a_w after the 72-h adaption periods (Table 3-4), values of t_{peak} were only compared within a specific matrix and a_w level.

Table 3-4. Initial bacterial population in different matrices after 72-h a_w adaptation and survival population after the 55 °C heat treatment with 1000 ppm CA or EG for 3 min (log CFU/g).

		WP	**CS**	**PO**
Initial population	0.4 a_w	7.0 ± 0.1	6.6 ± 0.1	8.6 ± 0.0
	0.9 a_w	7.9 ± 0.1	5.5 ± 0.1	9.6 ± 0.1
Survival population after CA-assisted treatment	0.4 a_w	6.7 ± 0.2	6.9 ± 0.1	8.4 ± 0.2
	0.9 a_w	7.7 ± 0.3	5.6 ± 0.2	9.7 ± 0.3
Survival population after EG-assisted treatment	0.4 a_w	6.4 ± 0.4	6.8 ± 0.3	8.6 ± 0.2
	0.9 a_w	7.4 ± 0.2	5.8 ± 0.1	9.6 ± 0.1

Results in Table 3-5 showed that the metabolic activity of *S.* Typhimurium in PO at 0.4 a_w (t_{peak} 95 ± 5 min) was lowered by heat treatment at 55 °C when compared to samples incubated at room temperature (t_{peak} 40 ± 3 min) ($P < 0.05$). The reduction of metabolic activity was also observed after heat treatment on WP and CS at 0.4 a_w ($P < 0.05$). Addition of 1000 ppm CA or EG to the heat treatment further reduced bacterial metabolic activity in CS and PO at 0.4 a_w ($P < 0.05$), but not in WP ($P > 0.05$). Similarly, at the higher a_w (0.9), the metabolic activity of *S.* Typhimurium in WP, CS, and PO was also lowered by the heat treatment at 55 °C ($P < 0.05$). The bacterial metabolic activity at 0.9 a_w was further reduced by heat treatment with the addition of 1000 ppm CA or EG in CS and PO ($P < 0.05$), but not in WP ($P > 0.05$). At room temperature, 1000 ppm of CA or EG reduced the metabolic activity of *S.* Typhimurium in CS at both 0.4 (t_{peak} 357 ± 5/ 348 ± 3 min) and 0.9 a_w (t_{peak} 525 ± 15/ 448 ± 6 min) ($P < 0.05$), but did not induce similar effect in WP or PO ($P > 0.05$). These results indicate that addition of CA or EG can suppress bacterial metabolic activity at mild heating temperature (55 °C) regardless of a_w in LMF matrices containing protein or oil. It is known that many antimicrobial compounds have the potential to impair bacterial metabolic system, which disturbs bacterial stress response and contributes to inactivation (Duarte et al., 2009; Gilbert et al., 2018; Hyun & Lee, 2021; Vidal-Aroca et al., 2009). Leakage of ATP and small ions such as sodium and potassium has been previously observed on bacteria treated with CA, which could disrupt the proton motive force and cause stress to the bacterial respiratory system (Di Pasqua et al., 2006; Nowotarska et al., 2017). The phenolic group of EG can also disrupt membrane structure and induce similar effect to bacterial metabolic activity (Burt, 2004). The apparent lack of effect by either compound in WP may result from the presence of hydrophobic functional groups on protein molecules that are known to have high binding capacity for many phenolic/ terpenoid-like compounds

(Baranauskienė et al., 2006; Pol et al., 2001). Additionally, previous bacterial inactivation data

indicated that neither CA nor EG could accelerate thermal inactivation at 55 C in any of the LMF

components at 0.4 a_w, which was not consistent with the observed metabolic activity changes. As

discussed in the previous section, it was possible that the additional stress from CA/ EG to the

bacterial metabolic system was only sub-lethal. The self-repairing mechanism of *S.*

Typhimurium, such as the expression of heat-shock proteins (e.g. chaperone protein (Hsp70) and

GrpE protein) at low a_w (Chen & Jiang, 2017), could help repair the damage from the stresses

and increase bacterial survivability.

Table 3-5. The fluorescent signal peak time (t_{peak}, min) from resazurin assay on comparison of metabolic activity for *S.* Typhimurium inoculated in PW/ CS/ PO at 0.4 or 0.9 a_w, with 55 °C heat treatment or at room temperature (RT, 22 °C), in the presence or absence of 1000 ppm CA or EG. Different letters indicate a statistically significant difference within the specific matrix and a_w levels ($P < 0.05$). Average ± standard deviation (SD).

	WP		CS		PO	
	0.4 a_w	**0.9 a_w**	**0.4 a_w**	**0.9 a_w**	**0.4 a_w**	**0.9 a_w**
RT control	244 ± 3.3 [b]	316 ± 11.8 [b]	257.3 ± 7.5 [d]	362.7 ± 8.2 [c]	40 ± 5.0 [c]	17.3 ± 1.9 [d]
CA at RT	241.3 ± 6.8 [b]	306.7 ± 10.0 [b]	357.3 ± 5.0 [b]	525.3 ± 14.7 [a]	38.7 ± 1.9 [c]	20 ± 0 [d]
EG at RT	248 ± 6.5 [b]	322.7 ± 10.5 [b]	348 ± 3.3 [b]	448 ± 6.5 [b]	50.7 ± 3.3 [c]	57.3 ± 10.5 [d]
Heat alone	281.3 ± 9.4 [a]	374.7 ± 24.1 [a]	293.3 ± 6.8 [c]	538.7 ± 13.6 [a]	94.7 ± 5.0 [b]	44 ± 0 [c]
CA + Heat	276 ± 0 [a]	381.3 ± 29.3 [a]	404 ± 5.0 [a]	> 1000	120 ± 5.0 [a]	54.7 ± 1.9 [b]
EG + Heat	292 ± 5.6 [a]	365.3 ± 16.4 [a]	392 ± 6.5 [a]	> 1000	114.7 ± 5.6 [a]	173.3 ± 7.5 [a]

3.3.4 *Change of membrane fluidity and fatty acid compositions in S. Typhimurium adapted to different a_w conditions*

Bacterial membrane is an important action site for both heat treatment and treatment with many antimicrobial chemicals (such as CA and EG) (Gharib et al., 2018; Los & Murata, 2004; Serio et al., 2010). To better understand the observed heat and antimicrobial resistance mechanism of *S*. Typhimurium at low a_w, membrane fluidity was measured to reflect membrane rigidity and permeability (Bose & Chatterjee, 1995; Lande et al., 1995). Results in Table 3-6 showed that *S*. Typhimurium adapted to 0.4 a_w had lower membrane fluidity (116.3 ± 6.5) than cells adapted to 0.9 a_w (96.2 ± 1.6) or the stationary phase broth culture (97.0 ± 0.8) ($P < 0.05$). It suggested that bacterial membrane became more rigid after adaptation to low-a_w environment, which may contribute to the development of thermal resistance and reduce the penetration of CA/ EG into the membrane or the cytoplasm.

Table 3-6. Composition of selected membrane fatty acids and membrane fluidity of *S.*

Typhimurium adapted at different conditions. Average ± standard deviation (SD).

Fatty Acid Compositions (Relative percentage)	Adaptation Condition		
	0.4 a$_w$	0.9 a$_w$	Broth culture
Unsaturated Fatty Acids (UFA)			
C16:1	9.95 ± 0.82	11.09 ± 0.52	13.11 ± 0.48
C18:1	13.26 ± 1.11	13.55 ± 1.08	12.47 ± 0.31
Saturated Fatty Acids (SFA)			
C14:0	13.94 ± 0.35	13.90 ± 0.41	12.81 ± 0.31
C16:0	61.74 ± 0.29	60.29 ± 1.12	60.75 ± 0.44
C18:0	1.11 ± 0.25	1.17 ± 0.17	0.86 ± 0.04
UFA/ SFA	0.31 ± 0.01	0.34 ± 0.01	0.37 ± 0.01
Membrane Fluidity (mP)	116.31 ± 6.53	96.16 ± 1.58	97.04 ± 0.81

As a major factor affecting bacterial membrane fluidity (Álvarez-Ordóñez et al., 2008), membrane fatty acid compositions were analyzed to help understand change of bacterial membrane property. Six of the major membrane fatty acids (accounts for ~79% of the total membrane fatty acids (Álvarez-Ordóñez et al., 2008)) were identified and the concentrations for five of them were compared quantitatively. As shown in Table 3-6, the percentages of all the identified unsaturated fatty acids (UFA) were lower in *S*. Typhimurium adapted to 0.4/ 0.9 a_w when compared to the stationary phase bacteria in broth culture ($P < 0.05$), while percentages of the identified saturated fatty acids (SFA) showed an opposite trend ($P < 0.05$). The UFA to SFA ratio of *S*. Typhimurium that adapted to 0.9 a_w (0.34 ± 0.01) was higher than bacteria at 0.4 a_w (0.31 ± 0.01) ($P < 0.05$) but was lower than the bacteria in broth culture (0.37 ± 0.01) ($P < 0.05$). No difference was found for the cyc17 fatty acid between 0.4 (0.21 ± 0.01) and 0.9 a_w (0.20 ± 0.02) ($P > 0.05$). The reduced UFA/SFA ratio corresponded to the decrease of membrane fluidity on bacteria at lower a_w. A similar membrane fatty acids composition change with an increased percentage of SFA and a decreased percentage of UFA was observed on *E. coli* cells exposed to desiccation stress in a previous study (Scherber et al., 2009). Decreased UFA/ SFA ratio was also found in *Salmonella* cultivated with higher temperature or reduced pH, which correlated with the expression of heat-shock response gene regulator RpoH and increased thermal resistance (Álvarez-Ordóñez et al., 2008; Y. Yang et al., 2014). The adjustment of bacterial membrane fatty acids composition and the reduced fluidity can improve the barrier function and permeability characteristics of membrane against permeation of extracellular antimicrobial compounds and provide protection from heat-induced cellular component leakage (Mejía et al., 1999). It is also worth noting that while their fatty acid profiles were different, no significant difference was found between the membrane fluidity of bacteria at 0.9 a_w and in stationary phase broth culture.

It is possible that some unidentified fatty acids (such as cyc19) may also have impact on the membrane fluidity, which has been reported previously (Álvarez-Ordóñez et al., 2008; Annous et al., 1999; Magnuson et al., 1993).

Together, the results of both bacterial metabolic activity and membrane profile correspond with the increased resistance to the combined heat and antimicrobial compounds treatments at low a_w. The lower membrane fluidity was likely one of the major resistance mechanisms expressed by S. Typhimurium at low-a_w environment that preserved its membrane integrity at high temperatures and reduced the incorporation and permeabilization of CA/ EG. As a result, less stress was posed to the bacterial metabolic system during the treatments at low a_w. Together with its self-repairing system (such as the expression of heat shock proteins), S. Typhimurium can minimize the disruption caused by the stresses and increase their chance of survival. This insight provides guidance for further development of enhanced thermal treatments for LMF in future, where antimicrobial compounds with higher ability to interact with bacterial membrane should be tested. Although a variation in treatment efficiency was observed between different LMF components, the effect of different LMF components on either the bacterial metabolic activity or the membrane fluidity/ composition was not fully analyzed due to limitations of the experimental setup. Whether bacterial metabolic activity or membrane properties could be affected by adaptation in different LMF components is yet to be discovered. This question may be answered by analyzing the expression of related stress-response genes from bacteria inoculated in different LMF components via transcriptional analysis.

3.4   Conclusion

  Addition of 1000 ppm CA or EG significantly accelerated thermal inactivation (55 °C) of *S.* Typhimurium in water and LMF components (whey protein (WP), corn starch (CS) and peanut oil (PO)) at 0.9 a_w, although similar effect was not observed in bacteria adapted to lower (0.4) a_w in any of those matrices. *S.* Typhimurium was generally more resistant to inactivation at 0.4 a_w compared to 0.9 a_w regardless of the matrix components. The matrix effect on bacterial thermal resistance was observed at 0.9 a_w and was ranked as WP > PO > CS. Biphasic inactivation patterns were also observed in both WP at 0.9 a_w and PO at 0.4 a_w, indicating the development of heterogeneous resistance among the inoculated cells in different matrices. To better understand the bacterial resistance mechanism in LMF components with different a_w levels, its metabolic activity and membrane property were analyzed. Regardless of a_w (0.4 or 0.9), metabolic activity was lowered by the 55 °C heat treatment in all tested LMF components. Addition of CA or EG to the heat treatment further reduced metabolic activity in CS and PO at both a_w, while similar effect was not observed in WP. It demonstrated that the effect of heat and CA/ EG treatments on bacterial metabolic activity was partially dependent on the food components. *S.* Typhimurium adapted to 0.4 a_w had lower membrane fluidity and UFA/SFA ratio than bacteria at 0.9 a_w or in the stationary phase broth culture. These results suggest that bacteria at low a_w can change their membrane composition to increase its rigidity and reduce its permeability, thus increasing resistance against both the heat alone and combination treatments. Results from this study have demonstrated the potential of using food-grade antimicrobial compounds to complement thermal treatment in LMF during processes that start with a relatively high a_w (such as dehydration). It provided an insight of bacterial resistance mechanism at low a_w environment and its correlation with food components. Further studies are necessary to investigate the transcriptional response

related to the resistance mechanism of *S*. Typhimurium against the combined heat treatment at low a_w conditions. Exploration into combination of heat treatment with a wider variety of compounds or non-thermal processing methods to enhance thermal treatment during existing heating process (such as heat-assisted dehydration or "hot packing") for LMFs would also be recommended. Experiments in Chapter 4 focused on the detection of changes in gene expression of low-a_w adapted *S*. Typhimurium with and without the CA-assisted heat treatment in different matrices.

4 Chapter 4 Investigate the genetic response of *Salmonella* Typhimurium during low a_w adaptation and trans-Cinnamaldehyde-assisted heat treatment and identify its correlation with bacterial resistance in different LMF components

Publication status

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Abstract

Results in the previously study showed that a_w- and matrix- dependent bacterial resistance of desiccation-adapted _Salmonella_ Typhimurium during trans-cinnamaldehyde (CA)-assisted heat treatment. It is reasonable to assume that the desiccation stress from a low water activity (a_w) environment can induce changes to the transcriptional profiles in bacteria, which ultimately lead to the increased resistance. Therefore, to better understand the molecular mechanism behind the bacterial resistance in different low a_w environments and during the antimicrobial-assisted heat treatment, gene expression analysis was conducted on _S._ Typhimurium adapted to low a_w conditions in different matrices with or without the CA-assisted heat treatment. Differential expression of nine stress-related genes were analyzed in the results. Alternative sigma factor _rpoH_ and the stress response gene _dnaK_ was upregulated in _S._ Typhimurium by the stress during bacterial adaptation to the low a_w environments and the combined heat treatment, which likely contributed to the bacterial resistance during the combined treatment. Although its link to desiccation response in bacteria is not fully understood, the downregulation of _ompC_ during the combined treatment also likely contributed to the treatment resistance. _RpoE, otsB_ and _proV_ in _S._ Typhimurium in some matrices was upregulated by the desiccation stress during incubation at the low a_w environment and likely contributed to desiccation resistance. However, their expression was poorly linked to bacterial resistance during the combined heat treatment. The upregulation of _fadA_ was induced during bacterial adaptation to low a_w as well and also likely contributed to the desiccation resistance, but their increased expression likely did not contribute to bacterial resistance during the combined heat treatment either. The observed upregulation of _fabA_ and downregulation of _ibpA_ could not be directly linked to bacterial resistance to either desiccation or the combined heat treatment. Differential

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expressions were also observed among different matrices at the same a_w, where the expression profiles of *rpoH*, *dnaK* and *ompC* were partially consistent to the a_w-or matrix-dependent bacterial resistance observed during the combined treatment in the previous study. The partial inconsistencies between their expression profiles and bacterial resistance during the combined treatment suggested the presence of additional stress-response pathways. The deeper understanding about the molecular mechanism behind the bacterial resistance at low a_w is valuable for food safety risk assessment and the development of more efficient pasteurization technology for LMFs in the future.

4.1 Introduction

To overcome the desiccation stress and survive at low water activity (a_w) environments in low moisture foods (LMFs), bacteria adapting to a low a_w environment can 1) reduce their intracellular a_w through production of osmoprotectants such as trehalose or betaine to reduce the damage caused by osmotic shock (Sperber, 1983); 2) modify the composition of its outer membrane through the expression of porins (Finn, Condell, et al., 2013); and 3) reduce their metabolic activity or switch to alternative energy source (such as fatty acid catabolism) as an alternative to generate ATP for essential cellular processes (Beuchat et al., 2013).

In the previous studies, we have observed that *Salmonella* Typhimurium was generally more resistant to the antimicrobial (butyl paraben, trans-cinnamaldehyde or eugenol) -assisted heat treatment at lower a_w in LMF matrices. The observed resistance was also affected by different matrix compositions, where bacterial resistance to the antimicrobial-assisted heat treatment in WP was higher than PO or CS. Lower membrane fluidity, as contributed by the reduced unsaturated to saturate fatty acids ratio among membrane fatty acids, was detected in cells adapted to low a_w as a potential resistance mechanism. The physiological resistance mechanisms behind the observed cross-protection against the combined treatment in desiccated cells includes 1) reduced molecular mobility of biomolecules in cells that protect ribosomal units (such as 30S and 50S ribosomal subunits) against irreversible damage caused by thermal energy (Syamaladevi, Tang, et al., 2016); 2) expression of heat shock proteins (Hsps), which can provide protection against damage from heat and other antimicrobial treatments by refolding or eliminating denatured proteins and enzymes (Abee, 1999; Lou & Yousef, 1997); and 3) increased synthesis of saturated fatty acids that enhance bacterial membrane rigidity and protected the bacteria from heat-induced cellular component leakage and improve the barrier

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function against incorporation of extracellular antimicrobial compounds (Di Pasqua et al., 2006; Dubois-Brissonnet et al., 2011; Mejía et al., 1995; Scherber et al., 2009). Part of the resistance mechanism was explained in the previous study, where cells adapted to lower a_w exhibited reduced unsaturated to saturate fatty acids ratio among membrane fatty acids and lower membrane fluidity.

Based on the current understanding of bacterial resistance mechanisms, it is reasonable to assume that the desiccation stress from a low a_w environment can induce changes to the transcriptional profiles in bacteria for increased resistance. A previous study that conducted transcriptomic analysis on dehydrated cells identified 90 upregulated and 7 downregulated genes in *S.* Typhimurium, including genes involved in protein biosynthesis, metabolism, energy production, and membrane synthesis (Gruzdev et al., 2012). To better understand the molecular mechanism behind the bacterial resistance in different low-a_w environments and during the antimicrobial-assisted heat treatment, gene expression analysis was conducted on *S.* Typhimurium adapted to low a_w conditions in different matrices with or without the combined heat treatment. The genes were selected based on their known or potential roles in bacterial resistance. It was hypothesized that the sub-lethal stresses during desiccation adaptation and the combined heat treatment would induce a_w- and matrix- dependent expression of target genes that might contribute to the bacterial resistance.

4.2   *Materials and Methods*

4.2.1   *Identification of bacterial strain with whole genome sequencing*

Salmonella Typhimurium from frozen culture was inoculated onto Trypticase soy agar (236920, Difco, BD, Franklin Lakes, NJ, USA) with additional 0.6% yeast extract (TSAYE) and stored at 4 °C for up to 3 weeks. One colony of from the plates were inoculated into 100 mL

sterile Trypticase soy broth (TSB) (211825, Difco, BD, Franklin Lakes, NJ) at 37 °C for 18-20 h

to achieve the early stationary phase (approximately 9 log CFU/mL). 1 mL of the growth culture

was then harvested via centrifugation at 7,197 g for 10 min. Bacterial DNA was extracted and

purified from the pellet using the DNeasy blood and Tissue Kits (69504, Qiagen, Hilden,

Germany). The process was performed automatically on a QIAcube instrument (Qiagen, Hilden,

Germany) using the protocol provided by the manufacture. The isolated DNA was then subjected

to library preparation and indexing with the Nextera XT DNA Library Preparation (FC-131-

1024, Illumina, San Diego, CA) and Nextera XT Index Kits (TG-131-2001/2002, Illumina, San

Diego, CA) following manufacture's instructions. The indexed sample libraries were loaded onto

the reagent cartridge of the MiSeq system prior to sequencing (Illumina, San Diego, CA). The

generated raw sequencing data (fastq files) was assembled with SPAdes on the Galaxy platform

(Bankevich et al., 2012). CFSAN SNP Pipeline on the galaxy platform was used to perform the

alignment of the samples to selected reference strains with known sequence data from the

National Center for Biotechnology Information (NCBI) gene database (Davis et al., 2015). The

reference strain with the least SNP distance from the sample was identified.

4.2.2   *Bacterial culture and sample inoculation*

Corn starch (CS) (ACH Food Companies, Memphis, IN, USA), whey protein (WP)

(Hilmar Ingredients, Hilmar, CA, USA) and peanut oil (PO) (Foodhold USA, Landover, MD,

USA) were used to represent the dominant components in LMFs (carbohydrate, protein and

lipids). Prior to inoculation, all the matrices were sterilized by autoclaving at 121 °C for 15 min

(WP or CS) or passing through a sterilized 0.2 µm disk filter (PO). *Salmonella enterica* serovar

Typhimurium LT2 (ATCC 700720) was used as the target pathogen and raised according to a

pre-established method (Ding et al., 2021). Before each experiment, bacteria from the plates

were inoculated into 100 mL sterile Trypticase soy broth (TSB) (211825, Difco, BD, Franklin Lakes, NJ) at 37 °C for 18-20 h to achieve the early stationary phase (approximately 9 log CFU/mL). It was then harvested via centrifugation at 7,197 g for 10 min and washed with sterile phosphate buffer saline (1× PBS). Inoculation in CS or WP was done by mixing 800 µL of the washed bacterial suspension (in water) with 20 g of CS or WP via hand massaging in a sterilized stomach bag (B01064, Whirl-Pak, Nasco, Fort Atkinson, WI, USA). Inoculation in PO was conducted by resuspending bacterial pellet that has been air dried under a biosafety cabinet for 20 min into 10 mL of PO with a handheld homogenizer.

4.2.3   *Bacterial adaptation to different a_w conditions*

To prepare *S.* Typhimurium cells adapted to different a_w levels in LMF matrices, the a_w of the inoculated LMF matrices was adjusted to target levels by incubating the inoculated samples inside a sealed desiccator containing deionized water or saturated $MgCl_2$ solution for 72 h at dark (22± 2 °C) prior to subsequent treatment (Greenspan, 1977). To prepare *S.* Typhimurium cells adapted to different a_w levels in pure bacterial suspension ("pure culture"), stationary phase *S.* Typhimurium cells were harvested from the growth culture via centrifugation (7,197 g for 10 min) and washed with sterilized DI water. The final pellet was resuspended in 200 µL DI water, spread onto a sterilized petri dish, and stored inside a sealed desiccator with the same condition as described above. The final a_w values of the desiccated samples were measured to be 0.4/ 0.9 with the HygroPalm AW water activity meter (Rotronic Instrument, NY, USA) immediately before the treatment or RNA extraction (for samples without the treatment).   Water activity values were measured to be 0.39 ± 0.03 (incubated with saturated $MgCl_2$ solution; approximate as 0.4)/ 0.92 ± 0.02 (incubated with saturated KNO_3 solution; approximate as 0.9) a_w for all samples.

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4.2.4 *Antimicrobial compounds spiking and sub-lethal heat treatment*

To prepare *S*. Typhimurium cells exposed to the sub-lethal antimicrobial-assisted heat treatment, inoculated LMF matrices or pure bacterial suspension equilibrated to target a_w levels were spiked with CA to reach a final concentration of 1000 ppm. CS and WP were spiked by individually mixing the suitable amount of the pure compound into the samples via hand-stirring in a glass beaker. PO was spiked by vortexing uninoculated PO containing corresponding antimicrobial compounds for 1 min before mixing it with inoculated PO at 1:1 ratio. 1 g of the CS or WP samples or 200 mg of the PO sample with 1000 ppm CA at different a_w levels were individually transferred into a sealed stomach bag (B01067, Whirl-Pak, Nasco, Fort Atkinson, WI, USA) (for CS and WP samples) or 2 mL centrifuge tube (for PO sample). Heat treatment was conducted by submerging the body (beneath bag seal/ tube cap) of the sample-containing bags/ tubes in a water bath pre-equilibrated to 55 ± 0.3 °C for 3 minutes, where no significant change to bacterial population was observed. No treatment was applied to the pure culture samples.

4.2.5 *RNA extraction and cDNA synthesis*

Bacterial RNA was extracted from the inoculated samples/ pure bacterial suspension after adaptation to different a_w levels for 72 h, with or without the sub-lethal antimicrobial-assisted heat treatment. RNA extraction was also performed on stationary phase *S*. Typhimurium cells directly harvested from fresh growth culture as a control. To stabilize the gene expression levels in the adapted / treated bacterial cells, samples were mixed with RNAprotect Bacteria Reagent (76506, Qiagen, Hilden, Germany) immediately after the adaptation period/ or the treatment to reduce the synthesis and degradation of RNA during extraction and the further processing steps (Fong & Wang, 2016). Briefly, RNAprotect Bacteria Reagent were added to samples at 1:5 (for

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PO and CS samples and pure culture samples) or 1:20 (for WP samples) ratio and incubated at

room temperature for 5 min after vigorous mixing. After which, incubated samples were

centrifuged at 40 g for 2 min to accelerate phase separation. The aqueous layer from each sample

was individually transferred to a new tube and centrifuged at 5, 000 g for 10 min. After

discarding the supernatant, cell pellets were stored at -80 °C for further processing (< 1 week).

Isolation and purification of RNA from the stabilized samples were performed with the RNeasy

Mini Kit (74104, Qiagen, Hilden, Germany). The process was performed automatically on a

QIAcube instrument (Qiagen, Hilden, Germany) using the protocol provided by the manufacture.

Concentrations of the extracted RNA was quantified with Qubit RNA BR Assay Kit (Q10210,

Molecular Probes, Invitrogen, Waltham, MA) on a Qubit 2.0 Fluorometer (Invitrogen, Waltham,

MA) according to manufacturer's instruction.

Complementary DNA (cDNA) synthesis from the purified RNA was performed with the

QuantiTect Reverse Transcription Kit (205311, Qiagen, Hilden, Germany) following the

manufacture's instruction. The kit contained gDNA Wipeout Buffer to remove contaminating

genomic DNA (gDNA) left in the RNA extractant at the beginning of the process. No RT

controls were also included to detect the potential presence of gDNA contamination in the

synthesized cDNA product, where molecular-grade water (7732185, Fisher Scientific, Waltham,

MA) was used to replace the reverse transcriptase mix during the cDNA synthesis.

4.2.6   *Preparation of bacterial gDNA templates*

Bacterial gDNA templates were prepared by the boiling method described in a previous

literature with modifications (Queipo-Ortuño et al., 2008). Briefly, stationary phase *S.*

Typhimurium cells were directly harvested from 25 mL fresh growth culture via centrifugation at

15,000 g for 10 min. The pellet was washed with 10 mL molecular-grade water and resuspended

in 1 mL molecular-grade water and heated in a boiling the water bath for 10 min. It was then

cooled in an ice bath and centrifuged at 15,000 g for 1 min. After decanting the supernatant, the

pellet was stored at -20 °C.

4.2.7   *Quantitative PCR*

Levels of nine target genes in the synthesized cDNA from each sample were measured

with real-time quantitative PCR (qPCR) (Table 4-1). Primer designs were obtained from

previous literatures using the same/ similar bacterial strains with a primer efficiency between 90

to 110% (I.-S. Bang et al., 2005; Birhanu et al., 2021; Carroll et al., 2016; Chen et al., 2014;

Chen & Jiang, 2017; Eaves et al., 2004; Fong & Wang, 2016; Kollanoor Johny et al., 2017; H. Li

et al., 2012; Li, Overall, et al., 2015). Designed primers (standard desalted) were purchased from

IDTDNA (Integrated DNA Technologies, Coralville, Iowa) and resuspended in molecular-grade

water to make individual working stock solutions at 8 µM. Primers were screened by conducting

a trial qPCR analysis with the gDNA template (reconstituted in 500 µL molecular-grade water)

from the bacteria, only the primers with a quantification cycle value (Cq) value < 37 were

selected to be used in the following experiments. Efficiency of the chosen primers were then

analyzed by making serial dilutions of the cDNA samples and calculated based on a method

described previously (Pfaffl, 2001). The real-time qPCR was performed in a C1000 Touch

Thermal Cycler coupled with a CFX96 Optical Reaction Module (Bio-Rad, Hercules, CA) using

the PowerTrack SYBR Green Master Mix (A46109, Molecular Probes, Invitrogen, Waltham,
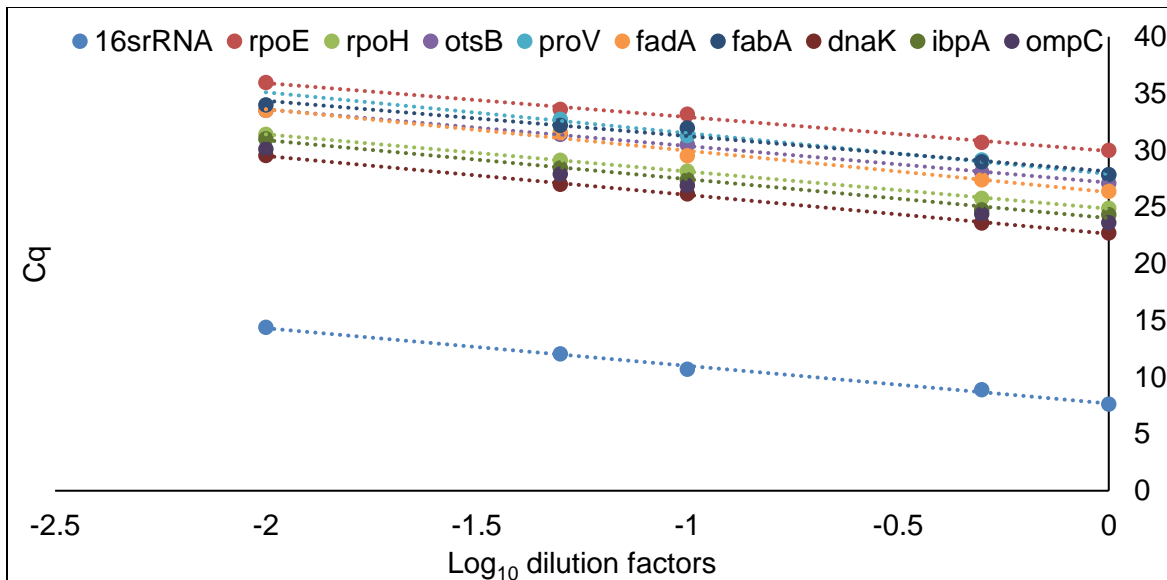
MA).

Figure 4-1. Primer efficiency standard curves

qPCR procedures (reaction mix volumes and thermal steps) were based on previous
literature and manufacture's recommendations with some modifications (Fong & Wang, 2016).
Briefly, individual 10 µL reaction mixture contains 5 µL Master Mix, 0.5 µL cDNA samples
(~150 ng), 7.15 µL forward and reverse primers (400 nM), 0.25 µL yellow sample buffer, and
3.75 µL molecular-grade water. The PCR reaction consisted of a total of 40 cycles after the
initial enzyme activation (95 °C for 2 min) with 5 s of denaturation (95 °C) and 30 s of
annealing/ extension (60 °C). A subsequent dissociation step was performed by increasing the
incubation temperature from 65 °C to 90 °C with a 0.1 °C/ s ramp. The generated melt curve was
analyzed for detecting presence of potential contamination or primer binding. The relative
expression of each target genes in samples were calculated using the Pfaffl method by using the
16S ribosomal RNA (16s rRNA) as the reference gene and the stationary phase *S.* Typhimurium
cells directly harvested from fresh growth culture as the calibrator sample (untreated control)
(Pfaffl, 2001). The equation was shown below:

$$Relative\ Exresion\ Ratio = \frac{\left(E_{target\ gene}\right)^{\Delta Cq,target\ gene\ (calibrator\ sample-test\ sample)}}{\left(E_{reference\ gene}\right)^{\Delta Cq,reference\ gene\ (calibrator\ sample-test\ sample)}}$$

Where E stands for the amplification efficiency of the primers, ΔCq stands for the

difference between the Cq values of the reference or target genes in the calibrator and test

samples. The Cq values used for the analysis were all less than 35.

Table 4-1. Screened genes and their primer sequences used in this study. Dash line in the efficiency column indicated that the primers had a Cq > 38 in the qPCR tests using the bacterial gDNA template and were not used in further qPCR analysis of the samples. F-Forward, R-Reverse.

Gene		Primer Sequence (5' – 3')	Primer efficiency (%)	Reference
16S rRNA	F-	CGATCCCTAGCTGGTCTGAG	100	(Fong & Wang, 2016)
	R-	GTGCAATATTCCCCACTGCT		
rpoS	F-	CAAGGGGAAATCCGTAAACCC	-	(Chen & Jiang, 2017)
	R-	GCCAATGGTGCCGAGTATC		
rpoE	F-	GTCTACAACATGACAAACAAAAACAAATGC	116	(Fong & Wang, 2016)
	R-	CCTTTTCCACTATCCCGCTATCGTCAACGC		
rpoH	F-	GATAAAGAGTGGGTGATATTCTCGTT	102	(Li, Overall, et al., 2015)
	R-	ACCGTCAGCGAGCAACAAC		
otsB	F-	ACCTTGATGGCACATTGGCAGA	104	(Chen & Jiang, 2017)
	R-	ACGCCCTGAAATCAATGCCA		
proV	F-	CCACAATGGTACGCCTTCTCA	90	(Chen & Jiang, 2017)
	R-	GCATGAGCGCAAATGACTGGA		
fadA	F-	ATCTCTCCGCCCACTTAATGCGTA	88	(Chen & Jiang, 2017)
	R-	AGCCTTGCTCCAGCGTTTGTTGTA		
fabA	F-	ACTCCCTGCGCCGAACATGC	109	(Chen et al., 2014)
	R-	CACTTCGCCCACGCCCAGAG		
dnaK	F-	CGATTATGGATGGAACGCAGG	96	(Chen & Jiang, 2017)
	R-	GGCTGACCAACCAGAGTT		
ibpA	F-	GCCGCCAACCGTTTCA	95	(Carroll et al., 2016)
	R-	TGCTTACCGTGAGCGTTCCT		
acrA	F-	CGCAGTACTATGTCGGTGAATTTACAGGCG	-	(Birhanu et al., 2021)
	R-	CGCGGATCCGTCTTAACGGCTCCTGTTTAA		
acrB	F-	GACGTCCTATTTTCG	-	(Eaves et al., 2004)
	R-	CGAAGACGCCTCTGT		
ompC	F-	ACGCTGCTGCATAAAGTTGTCA	101	(Kollanoor Johny et al., 2017)
	R-	CCGATGTTCTGCCGGAGTT		

4.2.8   Data analysis

The qPCR tests were performed with cDNA generated from three independently treated/ adapted samples, each with two on-plate replicates. The significance levels were determined with Student's t test using JMP Pro 15 software (Cary, NC, USA) at a significance level of $\alpha = 0.05$.

4.3   Results

4.3.1   Identification of bacterial strain used in the study and examination of primer efficiency

Based on the results on the pairwise SNP distance results, the lowest SNP difference (n = 2) was found between the sequence of *Salmonella* Typhimurium LT2 (ATCC#700720) in the NCBI database (SRR9641505) and that of the bacterial strain used in this study. The strain identity was used to locate literatures that used the same (or closely related) strains as references for primer designs in the subsequent qPCR studies. The chosen primers were screened by conducting a trial qPCR analysis with the bacterial gDNA template and primer efficiency tests. The results are listed in Table 4-1. The primer efficiency of *rpoE*, *rpoH*, *otsB*, *proV*, *fadA*, *fabA*, *dnaK*, *ibpA*, and *ompC* were between 88% to 115%. They were used as the target genes during the qPCR analysis of the samples (Table 4-1). The Cq for the primers of *rpoS*, *acrA* and *acrB* were > 38 in the qPCR tests using the bacterial gDNA template and were not included as target genes during further qPCR analysis.

4.3.2   Effect of a$_w$, matrix composition and antimicrobial compounds-assisted heat treatments on the gene expression of alternative sigma factors

4.3.2.1   Expression of rpoE

RpoE is a gene that encodes for the alternative sigma factor σ^E, which regulates the expression of genes involved in response to various stress factors such as heat, desiccation, and oxidation (Li, Overall, et al., 2015; McMeechan et al., 2007). The expression of σ^E directs the

RNA polymerase to express the stress-response genes within its regulon, including various periplasmic proteases and folding factors (Li, Nakayasu, et al., 2015; Miticka et al., 2003).

The results of *rpoE* expression are shown on Figure 4-2A. Before the combined heat treatment was applied, upregulation of *rpoE* was only detected from *S*. Typhimurium in the pure culture at 0.4 a_w, when compared to the bacteria from fresh growth culture without a_w adaptation (referred to as "the unadapted control" in later texts) as indicated by the asterisks. In the pure culture, the expression level of *S*. Typhimurium at 0.4 a_w was higher than 0.9 a_w ($P < 0.05$), suggesting that the desiccation stress induced the upregulation of *rpoE*. Interestingly, while downregulation of *rpoE* was observed in PO at both 0.4 and 0.9 a_w ($P < 0.05$), its expression levels remained unaltered in CS and WP with respect to the unadapted control, regardless of water activity. At the same a_w, the expression of *rpoE* in cells from PO was significantly lower than CS or WP ($P < 0.05$).

After the combined treatment, neither up-or downregulation of *rpoE* was detected among the three matrices when compared to the unadapted control. *RpoE* expression was not affected by different a_w in any of the matrices tested. Under the same a_w, expression level was not affected by matrix composition either. However, when compared to samples before the treatment, a higher expression level of *rpoE* was induced in *S*. Typhimurium in PO by the combined heat treatment at both 0.4 and 0.9 a_w ($P < 0.05$), while such effect was not observed in CS or WP ($P > 0.05$).
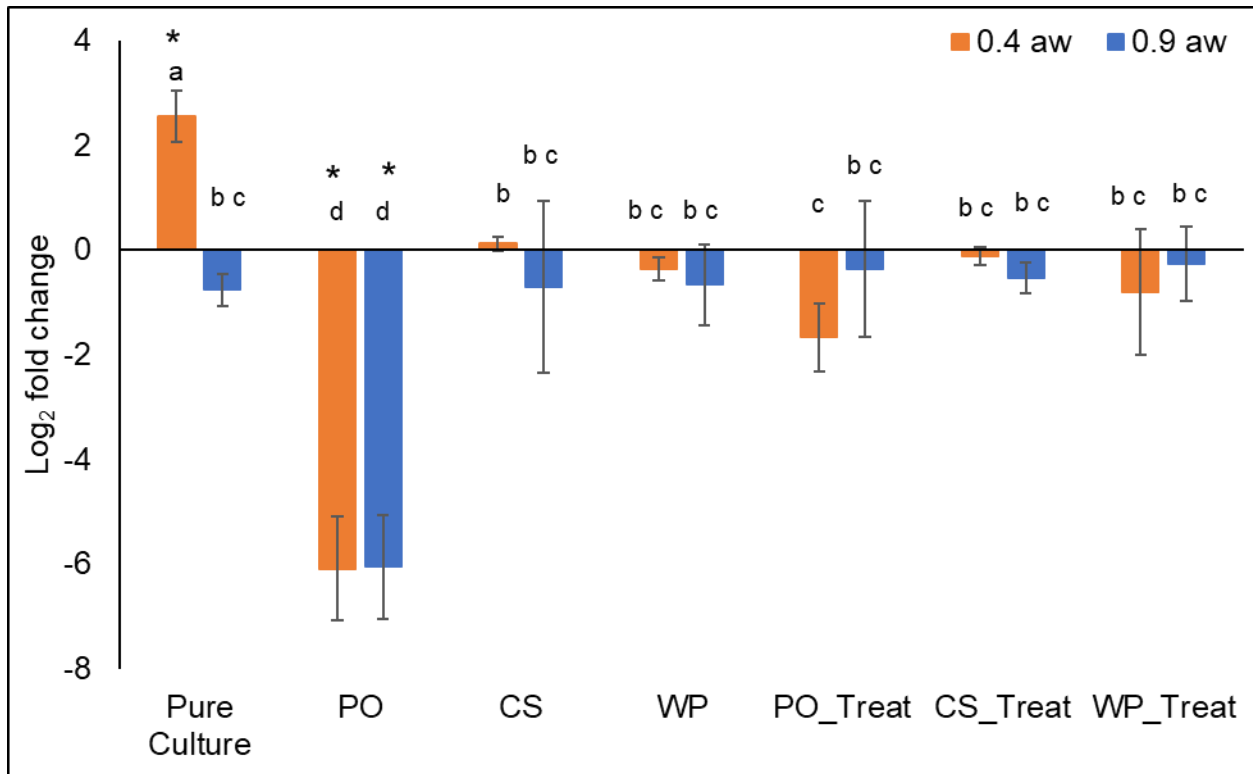
Figure 4-2A. Log$_2$ fold change in the relative expression of *rpoE* in *S*. Typhimurium adapted to 0.4 (orange) or 0.9 a$_w$ (blue) in pure culture or different LMF matrices, with or without the CA-assisted heat treatment (55 °C) for 3 min. The different letters indicate significant difference between different samples shown on the figures at $P < 0.05$. The asterisks indicate the significant difference between the corresponding sample and the unadapted control (bacteria from fresh growth culture) at $P < 0.05$. Average ± standard deviation (SD).

4.3.2.2 Expression of rpoH

RpoH encodes the alternative sigma factor σ^H, which directs the RNA polymerase to express more than 30 heat-shock proteins (HSPs) including molecular chaperones and proteases, which provides protection against cytoplasmic thermal stress (Spector & Kenyon, 2012).

Results of *rpoH* expression was shown in Figure 4-2B. In the samples without the combined treatment, *rpoH* was upregulated in pure culture, CS and WP at both water activity values but not in PO when compared to the unadapted control ($P < 0.05$). Unlike in other matrices, *rpoH* was downregulated in PO at 0.9 a_w when compared to the unadapted control ($P < 0.05$). The expression levels in the pure culture and the PO at 0.4 a_w were higher than at corresponding levels at 0.9 a_w ($P < 0.05$). However, the effect of a_w was not observed within CS or WP. The expression levels in the PO were lower than CS and WP at both 0.4 and 0.9 a_w ($P < 0.05$), while no difference was observed between CS and WP.

When the combined treatment was applied, upregulation of *rpoH* was detected in all samples except for PO at 0.9 a_w when compared to the unadapted control ($P < 0.05$). The expression levels at 0.4 a_w were higher than 0.9 a_w regardless of the matrices ($P < 0.05$). The expression level in the PO was higher than WP at 0.4 a_w but lower than WP at 0.9 a_w ($P < 0.05$). No difference was observed between CS and WP at either a_w. The combined treatment increased the expression of *rpoH* in PO at 0.4 a_w and 0.9 a_w ($P < 0.05$), when compared to corresponding untreated samples. The expression levels in CS and WP were not affected by the combined treatment.

Figure 4-2B. Log$_2$ fold change in the relative expression of *rpoH* in *S.* Typhimurium adapted to 0.4 (orange) or 0.9 a$_w$ (blue) in pure culture or different LMF matrices, with or without the CA-assisted heat treatment (55 °C) for 3 min. The different letters indicate significant difference between different samples shown on the figures at $P < 0.05$. The asterisks indicate the significant difference between the corresponding sample and the unadapted control (bacteria from fresh growth culture) at $P < 0.05$. Average ± standard deviation (SD).

4.3.3 *Effect of a_w, matrix composition and antimicrobial compounds-assisted heat treatments on the expression of stress response genes*

4.3.3.1 *Expression of otsB*

OtsB is a gene that encodes trehalose-6-phosphate synthase, which assists in the production of trehalose, an osmoprotectant that will accumulate inside cytoplasm to limit the loss of water from bacterial cells exposed to desiccated environments (Finn, Condell, et al., 2013).

Results of *otsB* expression are shown in Figure 4-3A. In the samples without the combined treatment, upregulation of the gene was only detected in pure culture and PO at 0.4 a_w when compared to the unadapted control ($P < 0.05$). *S.* Typhimurium in PO at 0.4 a_w had higher expression level than at 0.9 a_w ($P < 0.05$). However, the effect of a_w was not observed in CS or WP. Under the same a_w (both 0.4 or 0.9), the expression levels in PO were higher than CS or WP ($P < 0.05$), while no difference was observed between CS and WP.

After the combined treatment was applied, upregulation of *otsB* was again only detected in PO at 0.4 a_w when compared to the unadapted control ($P < 0.05$). However, downregulation of *otsB* was observed in CS at 0.9 a_w ($P < 0.05$). The expression levels in PO and CS at 0.4 a_w were higher than corresponding levels at 0.9 a_w ($P < 0.05$), but similar effect was not observed within WP. At 0.4 a_w, bacteria in PO had higher expression level than CS or WP, while at 0.9 a_w, bacteria in PO and WP had higher expression than CS ($P < 0.05$). When compared to corresponding samples without the treatment, the combined treatment reduced the expression of *otsB* in PO at both 0.4 and 0.9 a_w and in CS at 0.9 a_w but increased the expression in WP at 0.9 a_w ($P < 0.05$).
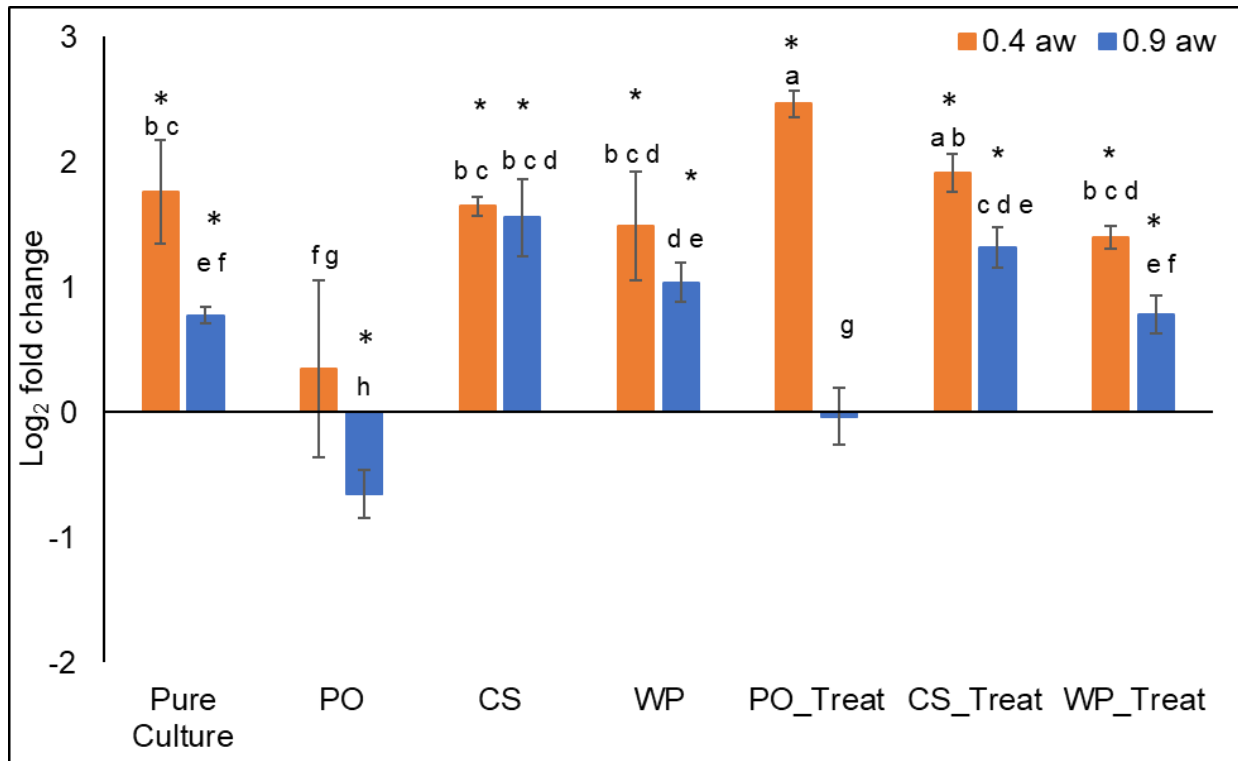
Figure 4-3A. Log$_2$ fold change in the relative expression of *otsB* in *S*. Typhimurium adapted to 0.4 or 0.9 a_w in pure culture or different LMF matrices, with or without the CA-assisted heat treatment (55 °C) for 3 min. The different letters indicate significant difference between different samples shown on the figures at $P < 0.05$. The asterisks indicate the significant difference between the corresponding sample and the unadapted control (bacteria from fresh growth culture) at $P < 0.05$. Average ± standard deviation (SD).

4.3.3.2 Expression of proV

ProV encodes a high-affinity transport system (ProU) for glycine betaine (also referred to as trimethylglycine), which is another osmoprotectant that accumulates to high intracellular concentrations to provide cells with protection during desiccation (Cairney et al., 1985; Finn, Condell, et al., 2013).

Results of *proV* expression are shown in Figure 4-3B. Without the combined treatment, upregulation of this gene was only detected in pure culture at 0.4 a_w when compared to the unadapted control ($P < 0.05$). However, downregulation of *proV* was observed in PO at both 0.4 and 0.9 a_w ($P < 0.05$). At 0.4 a_w, expression level in the pure culture was higher than at 0.9 a_w ($P < 0.05$). However, the effect of a_w was not observed in PO, CS or WP. The expression levels in PO were lower than CS and WP at both a_w ($P < 0.05$), while no difference was observed between CS and WP.

After the combined treatment was applied, upregulation of *proV* was only detected in WP at both 0.4 and 0.9 a_w when compared to the unadapted control ($P < 0.05$). The effect of a_w on *proV* expression was not observed in any of the matrices after the combined treatment ($P > 0.05$). At the same a_w, the expression level of *proV* in WP was higher than both PO and CS ($P < 0.05$). When compared to corresponding samples without treatment, the combined treatment increased the expression of *proV* in PO at both 0.4 and 0.9 a_w but did not induce significant change in CS or WP.
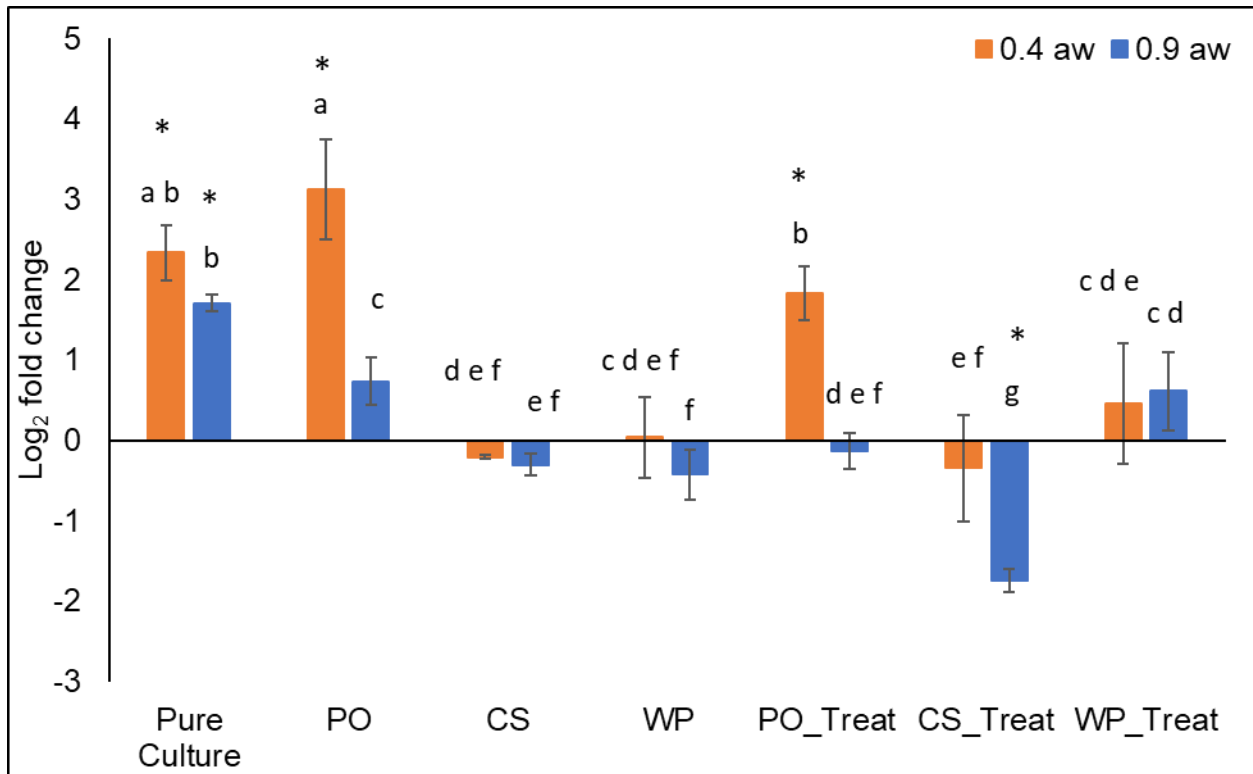
Figure 4-3B. Log$_2$ fold change in the relative expression of *proV* in *S*. Typhimurium adapted to 0.4 or 0.9 a$_w$ in pure culture or different LMF matrices, with or without the CA-assisted heat treatment (55 °C) for 3 min. The different letters indicate significant difference between different samples shown on the figures at *P* < 0.05. The asterisks indicate the significant difference between the corresponding sample and the unadapted control (bacteria from fresh growth culture) at *P* < 0.05. Average ± standard deviation (SD).

4.3.3.3   Expression of fadA

FadA is a gene that encodes 3-ketoacyl-coA thiolase. It is a key enzyme involved in the fatty acid β-oxidation pathway that catabolizes long-chain fatty acids into acetyl-CoA for the production of ATP in the tricarboxylic acid cycle (H. Li et al., 2012).

Results of *fadA* expression are shown in Figure 4-3C. Without the combined treatment, upregulation of the gene was detected in all matrices except for PO at 0.9 a_w and CS at either 0.4 or 0.9 a_w when compared to the unadapted control ($P < 0.05$), while downregulation of *fadA* was observed in PO at 0.9 a_w ($P < 0.05$). The effect of a_w was only observed in PO, where *S.* Typhimurium at 0.4 a_w had higher expression level than at 0.9 a_w ($P < 0.05$). At 0.4 a_w, *fadA* expression in PO and WP was higher than in CS ($P < 0.05$), while no difference was observed between PO and WP. At 0.9 a_w, the expression levels in PO, CS and WP were different from each other.

After applying the combined treatment, upregulation of *fadA* was detected in all matrices when compared to the unadapted control ($P < 0.05$). The expression levels in PO and CS at 0.4 a_w were higher than at 0.9 a_w ($P < 0.05$), but the effect of a_w was not observed in WP. Higher *fadA* expression was observed in WP at 0.9 a_w when compared to PO or CS at the same a_w ($P < 0.05$), while no difference was observed between the three matrices at 0.4 a_w. When compared to the samples without the treatment, higher expression of *fadA* was induced by the combined treatment in CS at 0.4 a_w and in all matrices at 0.9 a_w ($P < 0.05$).
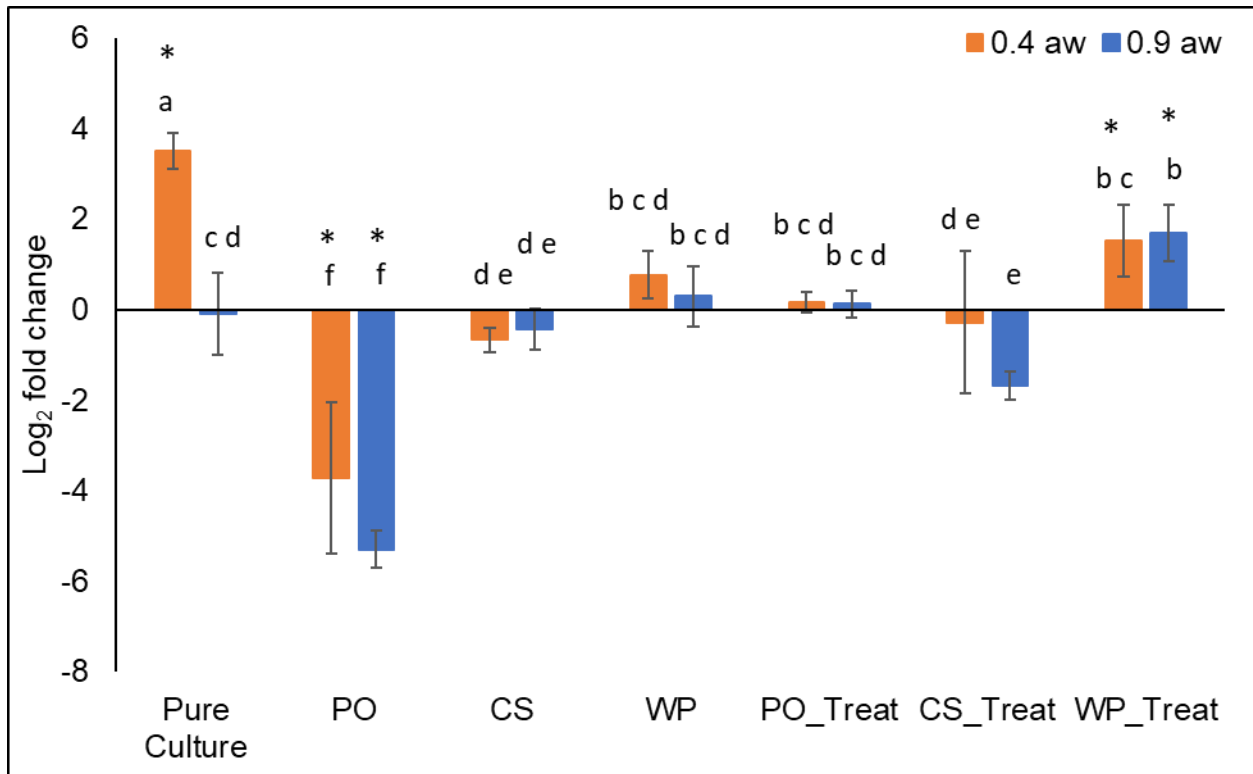
Figure 4-3C. Log$_2$ fold change in the relative expression of *fadA* in *S*. Typhimurium adapted to 0.4 or 0.9 a$_w$ in pure culture or different LMF matrices, with or without the CA-assisted heat treatment (55 °C) for 3 min. The different letters indicate significant difference between different samples shown on the figures at $P < 0.05$. The asterisks indicate the significant difference between the corresponding sample and the unadapted control (bacteria from fresh growth culture) at $P < 0.05$. Average ± standard deviation (SD).

4.3.3.4   Expression of fabA

  FabA encodes β-hydroxydecanoyl ACP dehydrase, which catalyzes the unsaturated fatty acid synthesis during fatty acid biosynthesis elongation by introducing the double bond to the growing fatty acid chain (Chen et al., 2014; Magnuson et al., 1993).

  Results of *fabA* expression are shown in Figure 4-3D. Without the combined treatment, *fabA* upregulation was observed in all matrices except pure culture at 0.9 a_w or PO at either 0.4 or 0.9 a_w when compared to the unadapted control ($P < 0.05$), while downregulation of *fabA* was observed in PO at both 0.4 and 0.9 a_w ($P < 0.05$). Compared to 0.9 $a_{w,}$ the expression of *fabA* was higher in pure culture and PO at 0.4 a_w ($P < 0.05$). The effect of a_w on *fabA* expression was not observed in CS or WP. At both 0.4 and 0.9 a_w, *fabA* expression in PO was lower than in CS or WP ($P < 0.05$), while no difference was observed between PO and WP at the same $a_{w.}$

  After applying the combined treatment, upregulation of *fabA* was only detected in WP at both 0.4 and 0.9 a_w when compared to the unadapted control ($P < 0.05$). The effect of a_w on *fabA* expression was not observed in any of the tested matrices ($P > 0.05$). *fabA* expression levels in PO, WP and CS were different from each other at 0.9 a_w ($P < 0.05$). When compared to the samples without the treatment, higher expression of *fabA* was only induced by the combined treatment in PO ($P < 0.05$), while no difference was observed in CS or WP.
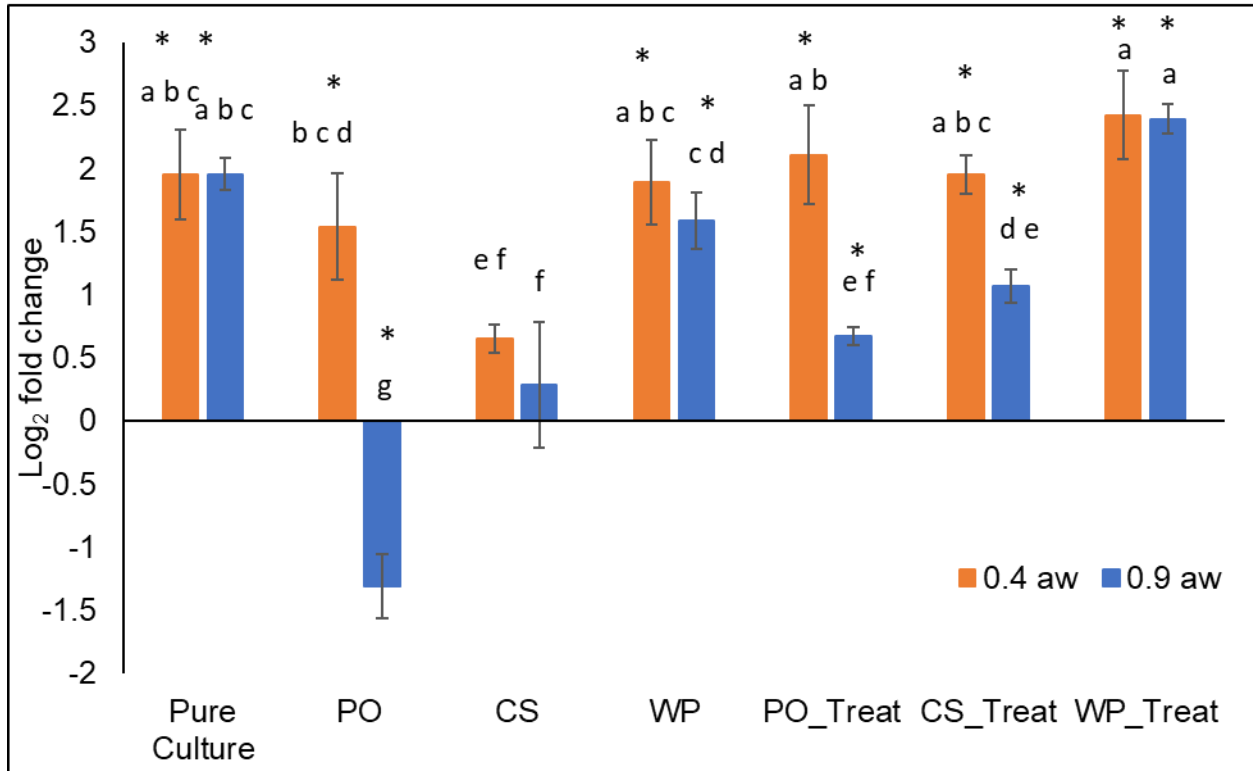
Figure 4-3D. Log_2 fold change in the relative expression of *fabA* in *S*. Typhimurium adapted to 0.4 or 0.9 a_w in pure culture or different LMF matrices, with or without the CA-assisted heat treatment (55 °C) for 3 min. The different letters indicate significant difference between different samples shown on the figures at $P < 0.05$. The asterisks indicate the significant difference between the corresponding sample and the unadapted control (bacteria from fresh growth culture) at $P < 0.05$. Average ± standard deviation (SD).

4.3.3.5 Expression of dnaK

DnaK is a gene that encodes a chaperon protein (Hsp70) that helps to stabilize proteins affected by heat stress by solubilization and refolding of misfolded or aggregated proteins (Ben-Zvi et al., 2004; Chen & Jiang, 2017; Mayer, 2013).

Results of *dnaK* expression are shown on Figure 4-3E. Without the combined treatment, upregulation of *dnaK* was observed in CS and WP at both 0.4 and 0.9 a_w and downregulation of *dnaK* was observed PO and pure culture when compared to the unadapted control ($P < 0.05$). The expression of *dnaK* was higher in PO at 0.4 a_w than at 0.9 a_w ($P < 0.05$). The effect of a_w on *dnaK* expression was not observed in CS, WP or pure culture. At the same a_w, *dnaK* expression in PO, CS and WP were significantly different from each other ($P < 0.05$).

After applying the combined treatment, upregulation of *dnaK* was detected in PO at both 0.4 and 0.9 a_w, CS at 0.4 a_w and WP at 0.4 a_w when compared to the unadapted control ($P < 0.05$). *S*. Typhimurium had higher *dnaK* expression in CS at 0.4 a_w than 0.9 a_w ($P < 0.05$). Higher expression levels were also detected in PO at both 0.4 and 0.9 a_w when compared to the WP ($P < 0.05$). When compared to the samples without the treatment, the combined heat treatment induced higher expression of *dnaK* in PO ($P < 0.05$). Interestingly, the same treatment reduced *dnaK* expression in CS ($P < 0.05$) but did not affect WP.
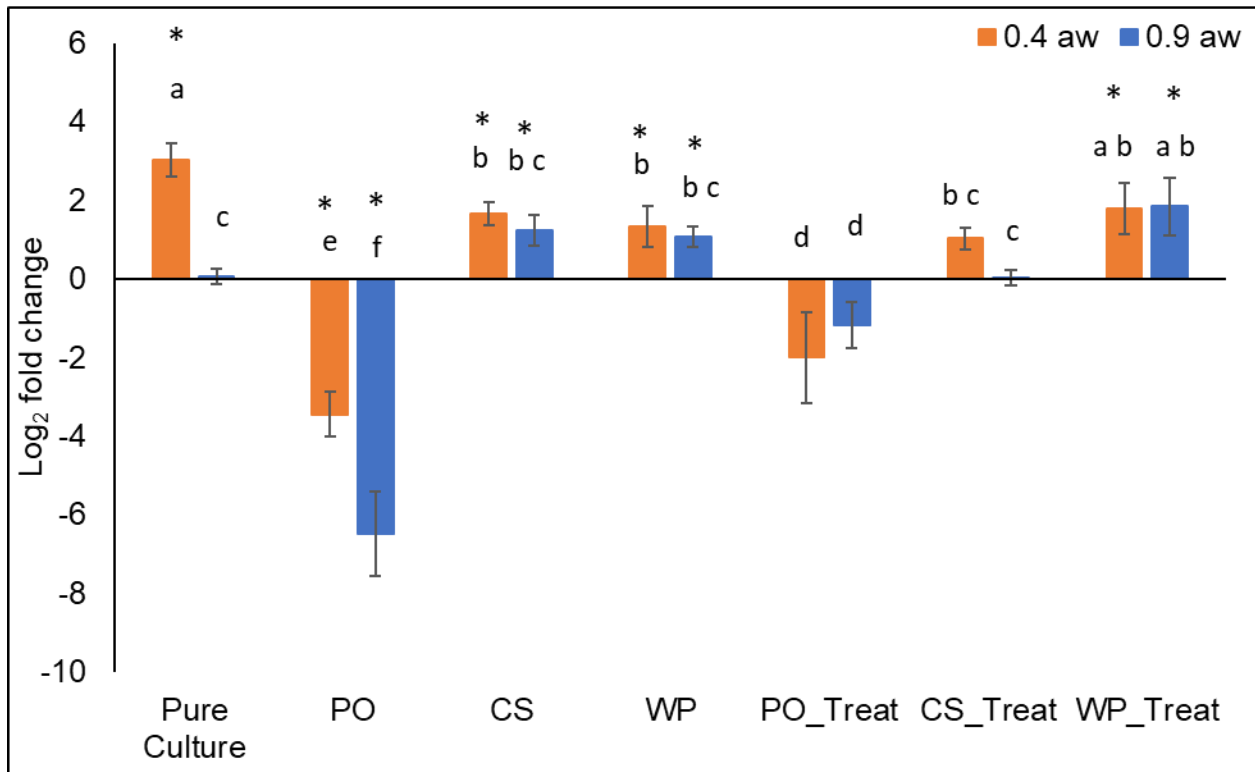
Figure 4-3E. Log$_2$ fold change in the relative expression of *dnaK* in *S*. Typhimurium adapted to 0.4 or 0.9 a$_w$ in pure culture or different LMF matrices, with or without the CA-assisted heat treatment (55 °C) for 3 min. The different letters indicate significant difference between different samples shown on the figures at $P < 0.05$. The asterisks indicate the significant difference between the corresponding sample and the unadapted control (bacteria from fresh growth culture) at $P < 0.05$. Average ± standard deviation (SD).

4.3.3.6   *Expression of ibpA*

IbpA is a gene that encodes a small Hsp that can bind to denatured proteins to stabilize the aggregates and prevent further aggregation, which improves the efficiency of other Hsps (such as Hsp70) to disaggregate and refold affected proteins (Carroll et al., 2016; Matuszewska et al., 2005).

Results of *ibpA* expression are shown on Figure 4-3F. Without the combined heat treatment, downregulation of *ibpA* was observed in all matrices tested when compared to the unadapted control ($P < 0.05$). The expression of *ibpA* was higher in pure culture at 0.4 a_w than at 0.9 a_w ($P < 0.05$). The effect of a_w on *ibpA* expression was not observed in PO, CS or WP. At the same a_w, *ibpA* expression in PO was significantly lower than CS or WP ($P < 0.05$), while no difference was observed between CS and WP.

After applying the combined treatment, downregulation of *ibpA* was also detected in all matrices when compared to the unadapted control ($P < 0.05$). The effect of a_w on *ibpA* expression was not observed in any of the tested matrices. At 0.4 a_w, *ibpA* expression in PO was significantly lower than in CS or WP ($P < 0.05$), while no difference was observed between CS and WP at either 0.4 or 0.9 a_w. When compared to the samples without the treatment, the combined heat treatment did not induce significant change to the expression of *ibpA* in any of the matrices.
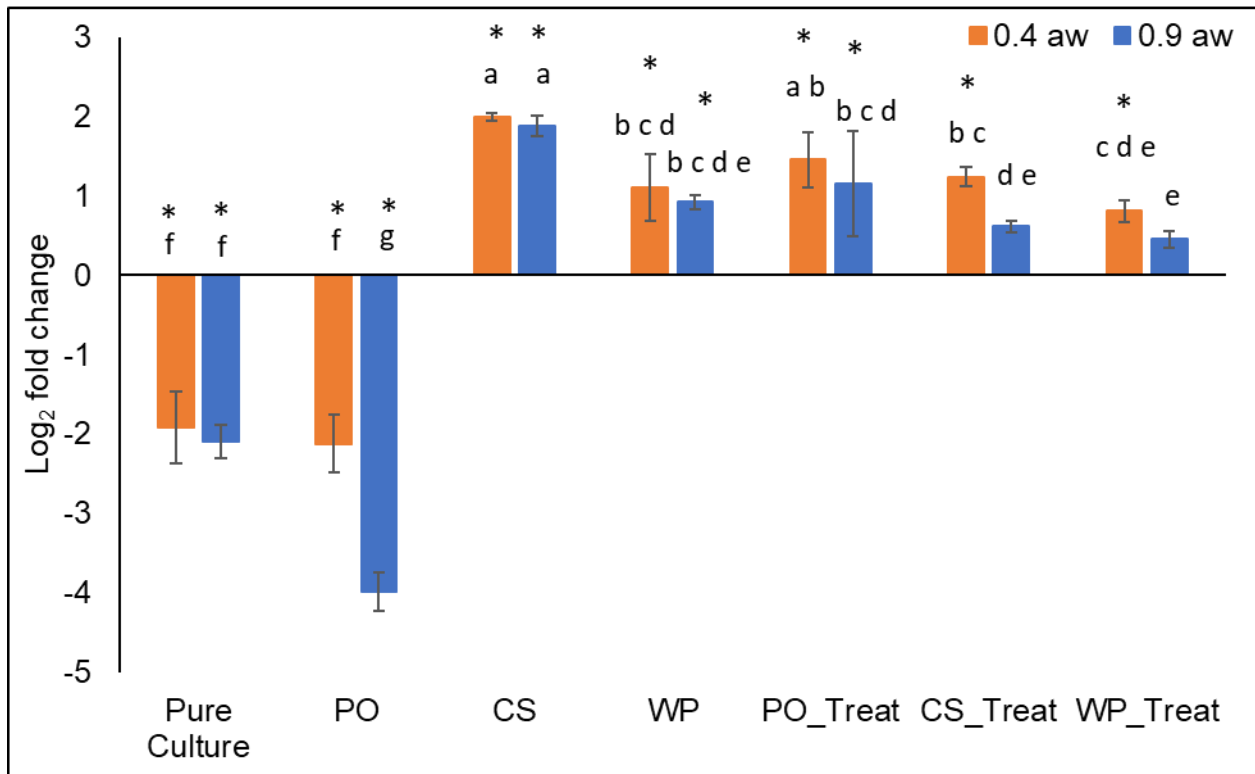
Figure 4-3F. Log$_2$ fold change in the relative expression of *ibpA* in *S*. Typhimurium adapted to 0.4 or 0.9 a$_w$ in pure culture or different LMF matrices, with or without the CA-assisted heat treatment (55 °C) for 3 min. The different letters indicate significant difference between different samples shown on the figures at $P < 0.05$. The asterisks indicate the significant difference between the corresponding sample and the unadapted control (bacteria from fresh growth culture) at $P < 0.05$. Average ± standard deviation (SD).

4.3.3.7   Expression of ompC

OmpC is a gene that encodes an outer membrane porin that is involved in the passive diffusion of osmoprotectants (such as proline) in bacteria under desiccation conditions in order to reach osmolarity balance (Finn, Condell, et al., 2013; Kempf & Bremer, 1998).

Results of *ompC* expression are shown in Figure 4-3G. Without the combined treatment, downregulation of *ompC* was observed in all matrices when compared to the unadapted control ($P < 0.05$). The expression of *ompC* was higher in pure culture at 0.4 a_w than at 0.9 a_w ($P < 0.05$). The effect of a_w on *ompC* expression was not observed in PO, CS or WP. However, at the same a_w, *ompC* expression levels in PO, CS and WP were significantly different from each other ($P < 0.05$). Expression levels were lowest in PO, followed by WP and then CS.

After applying the combined treatment, downregulation of *ompC* was also detected in all matrices when compared to the unadapted control ($P < 0.05$). *S.* Typhimurium had lower *ompC* expression in PO at 0.4 a_w than 0.9 a_w ($P < 0.05$). However, opposite trend was detected in CS, where the expression level at 0.4 a_w was higher than at 0.9 a_w ($P < 0.05$). Lower expression levels were also detected in PO when compared to CS or WP at the same a_w ($P < 0.05$), while no difference was detected between CS and WP. When compared to the samples without the treatment, the combined heat treatment induced higher expression of *ompC* in PO at 0.9 a_w ($P < 0.05$). Interestingly, the same treatment reduced *ompC* expression in CS ($P < 0.05$) and had no effect in WP.
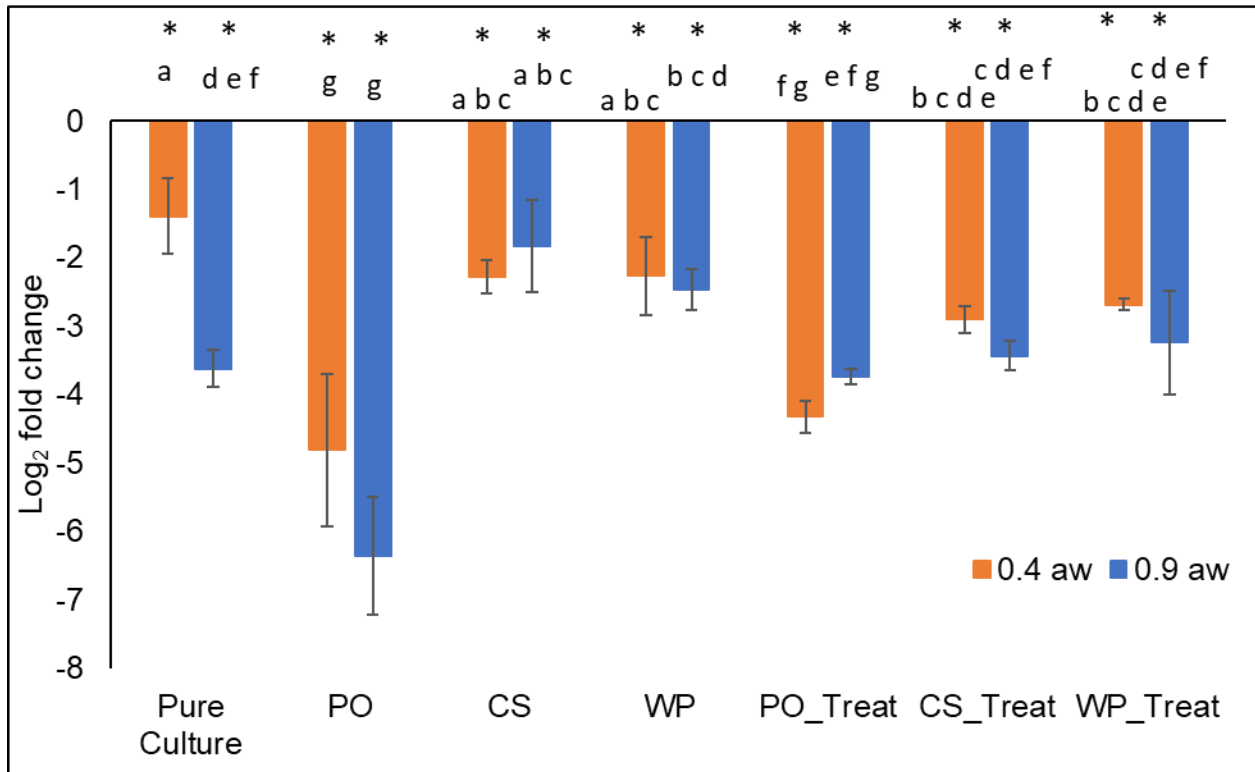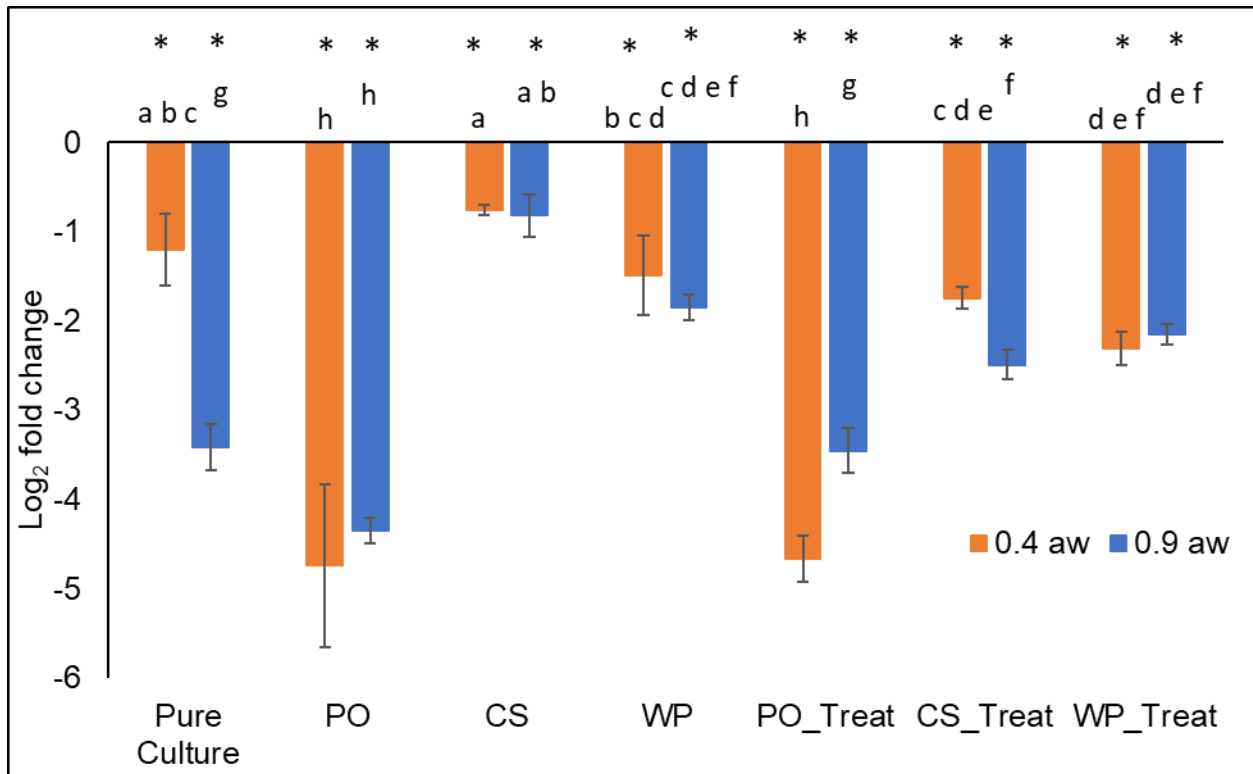
Figure 4-3G. Log$_2$ fold change in the relative expression of *ompC* in *S*. Typhimurium adapted to 0.4 or 0.9 a$_w$ in pure culture or different LMF matrices, with or without the CA-assisted heat treatment (55 °C) for 3 min. The different letters indicate significant difference between different samples shown on the figures at $P < 0.05$. The asterisks indicate the significant difference between the corresponding sample and the unadapted control (bacteria from fresh growth culture) at $P < 0.05$. Average ± standard deviation (SD).

4.4   Discussion

In the previous study, we have reported that *S.* Typhimurium was generally more resistant to the CA assisted heat treatment at 0.4 a_w compared to 0.9 a_w, regardless of the matrix components. In addition, effect of matrix on bacterial thermal resistance was also observed under the same a_w (0.9), where bacterial resistance to the CA-assisted heat treatment in WP was higher than PO or CS. We further observed that *S.* Typhimurium adapted to 0.4 a_w had lower membrane fluidity and lower unsaturated fatty acid to saturated fatty acid ratio than bacteria at 0.9 a_w, suggesting that bacterial resistance at lower a_w may be partially contributed by the increase of membrane rigidity. In order to gain further insight into the molecular mechanisms behind bacterial resistance at low a_w and the effect from different matrix components, we have extended the study and identified genes, which may be differentially expressed in *S.* Typhimurium that has adapted to low a_w conditions in different matrices. Besides PO, CS and WP, which represent the major components in LMFs, pure bacterial cells ("pure culture") that was dehydrated together with the other matrices to reach the same a_w was also included. Its results were used to reflect the effect of a_w alone, without interference from matrix-specific factors (Gruzdev et al., 2012; H. Li et al., 2012; Li, Overall, et al., 2015).

Sigma factors are dissociable protein subunits of prokaryotic RNA polymerase that regulate the process of transcription initiation. The association of different sigma factors with core RNA polymerase contributes to DNA strand separation and helps direct the RNA polymerase to recognize specific promoter DNA sequences (Kazmierczak et al., 2005). Apart from the primary sigma factor that is expressed in cells under normal conditions (such as σ^D), alternative sigma factors, such as σ^E, σ^H and σ^S, are usually overexpressed under stressful conditions (Burgess, 2017).

Upregulation of *rpoE* and *rpoH* in *S*. Typhimurium from different desiccated environments has been previously reported, where higher expression level was observed on cells adapted to lower a_w in air-dried pure culture (40% RH) or peanut oil (0.52 a_w) when compared to bacteria in fresh broth culture (Fong & Wang, 2016; Gruzdev et al., 2012; McMeechan et al., 2007). A previous study also found that the $\Delta rpoE$ mutants of *S*. Typhimurium had reduced survivability at low-a_w environment when compared to the wild-type bacteria (Finn, Handler, et al., 2013). These results corresponded to the upregulation of both *rpoE* and *rpoH* and the observed effect of a_w on their expression levels in the pure culture. However, the observed downregulation of *rpoE* in PO at 0.4 a_w when compared to the unadapted control and the lack of differential *rpoE* or *rpoH* expression in CS or WP between different a_w levels were not consistent with the results from the previous reports mentioned above. This can be possibly attributed to the protection from different microenvironments in which the bacteria reside, such as higher localized a_w or lower cellular dehydration rate during equilibration, which are both affected by the matrix compositions (Dhaliwal et al., 2021; H. Li et al., 2014). In addition, different methods used to induce the desiccation environment, such as the dispersing of bacterial pellet with homogenizer in PO versus the hand-mixing method in CS and WP, could also pose different levels or types of stress to bacteria and affect the expression levels (Fong & Wang, 2016). The use of stationery-phase culture as the unadapted control might also contribute to the lack of differential expression of *rpoE* in CS and WP when compared to the unadapted control, since the stress from nutrient limitation and accumulation of toxic chemicals during stationery-phase can induce increased *rpoE* expression as well (Testerman et al., 2002).

Upregulation of *rpoE* and *rpoH* in *S*. Typhimurium after heat treatment has also been previously documented (Fong & Wang, 2016; M. Morita et al., 1999; M. T. Morita et al., 1999;

Nonaka et al., 2006), which was consistent to the treatment effect on the expression levels of both *rpoE* and *rpoH* detected in PO but not in CS or WP.

The expression of *rpoE* is negatively controlled by a membrane-bound antisigma factor RseA, where extracellular stressors (such as heat and osmotic stress) can activate a proteolytic cascade that releases σ^E from the sequestration, therefore upregulate its expression (Li, Overall, et al., 2015; Österberg et al., 2011). Although osmotic stress during desiccation has not been directly linked to the upregulation of *rpoH*, a previous study found that starvation stress was able to induce upregulation of *rpoH* in *E. coli* (Jenkins et al., 1991). It is therefore possible that the lack of nutrient may contribute to the expression of *rpoH* in *S*. Typhimurium during extended adaptation to the desiccated environment. Upregulation of *rpoH* in bacteria during heat treatment has been reported more frequently (M. Morita et al., 1999; M. T. Morita et al., 1999; Nonaka et al., 2006). When at an elevated temperature (> 42 °C) induced by the combined treatment, the intramolecular hydrogen bonding was weakened, which opens the secondary structure of *rpoH* mRNA for ribosomal binding and translation (Spector & Kenyon, 2012; Takashi Yura & Nakahigashi, 1999). Additionally, expression of *rpoH* is also down-regulated by σ^H-regulated protease and the DnaK-DnaJ chaperone complex (Guisbert et al., 2004; M. Morita et al., 1999). Since both Hsps will bind to the aggregated or misfolded proteins generated during the combined treatment, their negative feedbacks to the expression of *rpoH* will likely be lifted.

The increased expression of *rpoH* in the matrices with lower a_w after the combined treatment corresponded to the higher bacterial resistance developed at lower a_w shown on the inactivation kinetics from our previous study. The observed effect of matrix composition on *rpoH* expression at the same a_w might also be contributed by the varied microenvironments in different matrices. The differential expression levels observed between PO and WP at 0.9 a_w

were consistent with the previously observed matrix effect on bacterial resistance at the same a_w, where *S.* Typhimurium inoculated in WP had higher resistance than PO. Therefore, *rpoH* was likely a contributor to the bacterial resistance during the combined heat treatment. Interestingly, neither desiccation nor the combined treatment induced a higher expression of *rpoE* in CS or WP. No significant difference in expression level was observed between the two matrices either. *RpoE* therefore might not be the major contributor to bacterial resistance during the combined heat treatment. A previous study has also reported that the gene was not essential for the viability and resistance of *S.* Typhimurium under certain stresses (Amar et al., 2018; Miticka et al., 2003). Other sigma factors, such as *rpoS*, might also be differentially expressed in different matrices during adaptation to low-a_w environments and contributed to bacterial resistance (Chen & Jiang, 2017).

Since the regulon of a single sigma factor like σ^E or σ^H may contain promotors for multiple downstream genes, it may regulate the expression of different stress-response genes and enable cells to become more resistant to multiple stress factors (Kazmierczak et al., 2005). Among the seven stress response genes tested in this study, upregulation of *otsB*, *proV*, *fadA* and *fabA* was detected in the pure culture and some tested matrices after adaptation to 0.4 a_w when compared to the unadapted control.

As a gene involved in the production for osmoprotectant, upregulation of *otsB* in *S.* Typhimurium from different desiccated environments has been previously reported, where higher expression level of *otsB* was observed on cells adapted to lower a_w in peanut oil (0.52 a_w), or on paper disk (0.11 a_w) compared to bacteria from fresh culture (Chen & Jiang, 2017; Finn, Handler, et al., 2013; H. Li et al., 2012). Upregulation of *otsB* in bacteria has been previously observed in PO after heat treatment at 45 °C, which corresponded to the observed effect of the

combined heat treatment in WP at 0.9 a_w in this study (Fong & Wang, 2016). The expression of

otsA/B in *S*. Typhimurium has been reported to provide protection to thermal stress with

trehalose biosynthesis (Howells et al., 2002). However, conflicting results were observed in both

PO and CS at 0.9 a_w, where expression of *otsB* was lowered after heat treatment.

The expression of *otsB* is known to be dependent on the alternative sigma factor *rpoS*

(Finn, Condell, et al., 2013; Kempf & Bremer, 1998). Although the expression of *rpoS* was not

studied in this experiment, its upregulation after both osmotic and heat shock has been

documented, which likely contributes to the upregulation of *otsB* in both treated and untreated

samples (Chen & Jiang, 2017; Shiroda et al., 2014). However, there has not been known links

between the expression of otsB and bacterial resistance to the combined heat treatment.

Therefore, instead of being a major contributor to bacterial resistance to the combined heat

treatment, *otsB* expression was likely just a result of *rpoS* upregulation in the treated cells.

As another gene involved in the desiccation defense mechanism of bacteria, upregulation

of *proV* in *S*. Typhimurium from different desiccated environments has been previously reported,

where higher expression level of *proV* was observed on cells adapted to lower a_w in broiler

chicken liter (0.81 a_w) when compared to bacteria from fresh culture (Chen & Jiang, 2017).

However, except for the pure culture, results observed on PO, CS and WP did not show the

effect of a_w. A previous study found that *kdp*, which is also an osmoprotectant transporter gene in

S. Typhimurium, had different expression levels in solution with different solutes (sodium

chloride and sucrose) at the same osmotic stress level (Balaji et al., 2005). Additionally, although

the ProU transport system for glycine betaine may be necessary for the survival of *S*.

Typhimurium at some desiccation environments, it may not be the case when bacteria adapted to

the low a_w condition under the conditions used in this study due to the existence of different osmoprotectant synthesis pathway (Finn, Condell, et al., 2013).

Similar to otsB, the effect of heat treatment on the expression of *proV* has not been reported previously. Previous studies have hypothesized that the induction of *proV* was dependent on the initial increase of certain osmoprotectants in cells (Booth & Higgins, 1990; Finn, Condell, et al., 2013). It is therefore possible that the expression of *proV* is indirectly affected by the expression of alternative sigma factors, which can be induced under either desiccation or thermal stress. However, there has not been enough evidence to link treatments with heat or antimicrobial compounds to the expression of *proV*. Discrepancy also existed between the gene expression and bacterial resistance in different matrices/ a_w levels as observed in the previous study. Therefore, *proV* is likely not the dominant contributor to bacterial resistance to the combined treatment either.

Upregulation of *fadA* in *S*. Typhimurium from different desiccated environments has been previously reported, with higher expression level observed on cells adapted to lower a_w in peanut oil (0.52 a_w) and on paper disk (0.11 a_w) compared to bacteria from fresh culture (Finn, Handler, et al., 2013; H. Li et al., 2012). The production of trehalose and many other osmoprotectants under desiccation stress requires glucose input, an alternative energy source is therefore needed to support necessary cellular activities (Finn, Condell, et al., 2013; H. Li et al., 2012). The FadA-catalyzed catabolism of fatty acids can serve as a cost-effective energy producing mechanism, and was therefore upregulated during adaptation to low-a_w environments (Finn, Condell, et al., 2013). However, the absence of the a_w-induced change on *fadA* expression in all matrices (except for PO) was not in agreement with findings in previous literatures. In addition to the matrix-specific effect, it is also possible that starvation occurred during the a_w

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adaptation process, where fatty acid catabolism may be needed to supply enough energy to bacteria incubated at higher a_w as well. The increased expression of various *fad* genes has been observed in *S*. Typhimurium during its response to starvation stress (Spector et al., 1999; Spector & Kenyon, 2012).

Upregulation of *fadA* in bacteria has been observed in PO after heat treatment of 45 °C, which has also been observed in PO, CS and WP in this study after the combined heat treatment when compared to the samples before the treatment (Fong & Wang, 2016). The combined treatment even induced higher *fadA* expression in matrices at 0.9 a_w when compared to samples without the treatment. However, the degradation of fatty acid in bacterial membrane induced by the expression of *fadA* may negatively affect membrane fluidity and permeability, leading to increased leakage of critical cellular components and the intake of extracellular antimicrobial compounds (Fong & Wang, 2016; Mejía et al., 1995). In other words, upregulation of *fadA* may reduce bacterial resistance to the combined heat treatment. The mechanism that triggered the upregulation of *fadA* during the combined heat treatment was not clear.

It was previously detected that membrane fluidity was lower in *S*. Typhimurium cells incubated at lower a_w. It is possible that other stress-response genes may have more prominent effect than *fadA* on membrane fatty acid composition that contributes to the reduced membrane fluidity, such as the genes on the *fab* locus (Mansilla et al., 2004). The higher expression of fadA at lower a_w and the lower expression in CS when compared to WP at 0.9 a_w were inconsistent with the a_w- or matrix effect on bacterial resistance observed in the previous study. Therefore, *fadA* was likely not a contributor to bacterial resistance during the combined treatment.

Upregulation of *fabA* in *S*. entertidis in desiccated environment has been previously reported, with higher expression level observed on cells adapted to lower a_w in granulated sugar

(0.50 a_w) and was associated with increased bacterial survival at low-a_w conditions (Chen et al., 2014). As increased level of unsaturated fatty acids in the bacterial membrane increases membrane fluidity (Álvarez-Ordóñez et al., 2008), the upregulation of *fabA* in *S*. Typhimurium at low a_w seems to be conflicting with the changes in bacterial membrane property in pure culture observed in the previous study, where both the unsaturated fatty acid percentage and membrane fluidity and was reduced at 0.4 a_w when compared to 0.9 a_w. Its expression profile at different a_w levels or in different matrices was also inconsistent with the previously observed results on bacterial resistance. Therefore, *fabA* was likely not a contributor to bacterial resistance during the combined treatment either.

However, besides *fabA*, various other genes from the *fab* locus also participated in the biosynthesis of unsaturated fatty acids and saturated fatty acids. After a double bond is introduced in the fatty acid acyl chain by FabA, it may be further elongated by the addition of two-carbon units as catalyzed by FabB or FabF, both of which are 3-ketoacyl-acyl carrier protein synthase enzymes (Mansilla et al., 2004). The elongation process may also yield saturated fatty acids with the help of *fabF* (Mansilla et al., 2004). The expression of *fabB* and *fabF* may increase the percentage of long-chain fatty acids and saturated fatty acids in membrane, therefore contribute to the decrease of membrane fluidity and increase bacterial resistance.

Upregulation of *fabA* in *S*. Typhimurium during heat treatment has not been previously studied. It has been suggested that the activity of *fab* enzymes was temperature-dependent in *E. coli*, but the thermal regulation effect was not linked to expression of *fabF* gene (Garwin et al., 1980; Mansilla et al., 2004). The mechanism that triggered the upregulation of *fabA* during the combined heat treatment in WP was not clear.

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Interestingly, unlike the genes discussed above, upregulation of *dnaK* was only detected in CS and WP but not in the pure culture when compared to the unadapted control. Upregulation of *dnaK* in *S*. Typhimurium from different desiccated environments has been previously reported, with higher expression level observed on cells adapted to lower a_w in both air-dried cells (40% RH) and broiler chicken liter (0.81 a_w) when compared to bacteria from fresh culture (Chen & Jiang, 2017; Gruzdev et al., 2012). Besides fixing heat-induced damages, the expression of Hsps at low a_w can also protect and fix proteins during other stress, including dehydration (Aertsen & Michiels, 2004; Gruzdev et al., 2012). During bacterial adaptation to low a_w conditions, the desiccation-induced carbon limitation (starvation) may lead to reduced protein synthesis (Georgopoulos, 1992). Therefore, the structural maintenance of the existing protein via chaperons such as Hsp70 is needed for the development of bacterial desiccation resistance (Rockabrand et al., 1998). However, some studies observed downregulation of *dnaK* in *S*. Typhimurium inoculated in PO (0.52 a_w) or reduced expression during prolonged low-a_w adaptation in PO (0.30 a_w) (X. Deng et al., 2012; Fong & Wang, 2016). These observations correlate with the observed difference of *dnaK* expression in different matrices at the same $a_{w,}$ which is likely caused by the matrix-specific protection effect discussed before. Upregulation of *dnaK* in *S*. Typhimurium during heat treatment has been previously reported (Berk et al., 2005; Fong & Wang, 2016; Sirsat et al., 2011). A previous study also found increased *dnaK* expression in *S*. Entertidis after treatment with CA (0.01%) or EG (0.04%) in Luria-Bertani broth at 37 °C for 30 min (Kollanoor Johny et al., 2017), suggesting that Hsp70 may also help repair the damage caused by the addition of antimicrobial compounds.

As the expression of *dnaK* is mainly promoted by RNA polymerase containing σ^H, the observed *dnaK* upregulation in matrices with or without the combined heat treatment was likely

induced by the expression of *rpoH* due to heat stress or starvation (T. Yura et al., 1993).

Additionally, as discussed above, the increased expression of *dnaK* also suggested that more

Hsp70 chaperons were mobilized to fix damaged proteins, which reduced the DnaK-DnaJ

chaperone complex and promoted the expression of *rpoH*. As a result, similar expression

patterns can be found between *dnaK* and *rpoH* in most of the tested conditions by comparing

results in Figure 4-2B and 4-3E. The different expression profiles between the two genes in

dehydrated pure culture without treatment and PO at 0.9 a_w with the combined treatment were

possibly contributed by the expression of other Hsps regulated by σ^H, such as *grpE*, which may

also be matrix-dependent and has been previously detected in *S*. Typhimurium cells dehydrated

in chicken liter (Chen & Jiang, 2017; X. Deng et al., 2012).

The higher expression of *dnaK* in WP with lower a_w after the combined treatment

corresponded to the higher bacterial resistance in WP at 0.4 a_w when compared to 0.9 a_w as

observed in the previous study. Therefore, *dnaK* expression likely contributes to bacterial

resistance at low a_w. However, the absence of expression difference in PO or CS between

different a_w , as well as the lower expression in WP when compared to PO at 0.9 a_w after the

combined heat treatment, were not consistent with the previously-observed results on bacterial

resistance. It suggested that other resistance factors have also contributed to the bacterial

resistance during the combined heat treatment.

Interestingly, different matrix effect on the expression of *dnaK* was observed between

samples with and without the combined heat treatment. This could partially be linked to the

composition-dependent a_w changes during the heat treatment. A study conducted by Syamaladevi

et al. (2016) found that due to the different sorption isotherm profiles of different matrix

components, the actual a_w in the matrix would develop matrix-dependent changes as the

temperature increased during thermal treatment, causing bacteria inoculated in different food matrices with the same initial a_w level at room temperature to be exposed to different stress during heat treatments (Syamaladevi, Tadapaneni, et al., 2016). The varied stress levels may result in the differential changes to gene expression levels observed in this study.

Downregulation of *ibpA*, and *ompC* was detected in all matrices when compared to the unadapted control. Expression of *ibpA* has not been previously observed in desiccated *S*. Typhimurium cells. The downregulation of *ibpA* in different matrices when compared to the unadapted control in this experiment was possibly an energy-saving strategy in response to the starvation stress due to the potential redundancy (Spector & Kenyon, 2012). Other cochaperones, such as GrpE, was reported to be upregulated in *S*. Typhimurium during low-a_w adaptation and may act as alternative to IbpA to promote protein structure rearrangement with chaperons such as DnaK (Chen & Jiang, 2017; Harrison, 2003).

Increased expression of *ibpA* was detected in *S*. Typhimurium after heat treatment and in *S*. Enteritidis after treatment with CA (0.01%) or EG (0.04%) in Luria-Bertani broth at 37 °C for 30 min (Carroll et al., 2016; Kollanoor Johny et al., 2017), which was not consistent with the results observed in this study. The lack of change to *ibpA* expression in the matrices after the combined heat treatment could also be contributed by energy deprivation (Matuszewska et al., 2005). Due to the lack of correlation between *ibpA* expression and bacterial resistance data from the previous study, as well as the lack of differential expression between different a_w levels or in different matrices after the combined heat treatment, it may not be a significant contributor to bacterial resistance.

Upregulation of *ompC* in *S*. Typhimurium in high osmolality solution (0.6 – 1.8 Osmol/kg) has been previously reported (Balaji et al., 2005; Chakraborty & Kenney, 2018). Higher

OmpC protein synthesis was also detected in *Cronobactor sakazaki* cells that have been air dried for 72 h (Riedel & Lehner, 2007). The osmolarity stress in cells at low a_w environment can promote the membrane-bounded EnvZ sensor kinase to phosphorylate OmpR, which will bind to low affinity binding sites of the *ompC* and induce the upregulation of OmpC as one of the predominant porins (Batchelor et al., 2005; Kempf & Bremer, 1998; Spector & Kenyon, 2012). However, the downregulation of *ompC* observed in this study when compared to the unadapted control was not consistent to the previous finding. Some other previous studies using air-dried cells or cells inoculated in peanut oil also did not detect increased *ompC* expression in *S.* Typhimurium during desiccation (X. Deng et al., 2012; Gruzdev et al., 2012; H. Li et al., 2012). The effect of desiccation on *ompC* expression in *S.* Typhimurium has not been fully understood.

A previous study reported that change of *ompC* expression in *S.* Typhimurium after heat treatment (42 °C for 14 h) was not observed (Uddin et al., 2019). However, another study found reduced *ompC* expression in *S.* Entertidis after treatment with CA (0.01%) or eugenol (0.04%) in Luria-Bertani broth at 37 °C for 30 min (Kollanoor Johny et al., 2017). Similar effect was also observed on *C. sakazaki* after treatment with CA (750 µM) (Amalaradjou & Venkitanarayanan, 2011). These results were consistent with the reduced *ompC* expression observed in CS at 0.9 a_w after the CA-assisted treatment in this study. Since OmpC porin is an outer membrane passive diffusion channel that helps the diffusion of various compounds, the reduced expression may reduce the uptake of extracellular antimicrobial compounds such as CA (Gil et al., 2009; Kollanoor Johny et al., 2017). The higher *ompC* expression in PO at 0.4 a_w after the combined heat treatment corresponded to the higher bacterial resistance at lower a_w observed in the previous study. However, the expression in PO at 0.9 a_w was lower than WP, which was not consistent with the higher bacterial resistance observed in WP at 0.9 a_w from the previous study.

It suggested that other transcriptional factors also contributed to bacterial resistance during the combined heat treatment.

Similar to the results observed with the alternative sigma factors, when compared to the unadapted control, the lack of differential expression of *otsB* and *proV* in CS and WP, the downregulation of *fadA*, *fabA* in PO, and the downregulation of *dnaK* in pure culture were likely also contributed by the matrix-specific protective effect, the different inoculation methods, or the use of stationery-phase culture as the unadapted control as discussed earlier.

Additionally, some mRNA in bacteria either have a short half-life or may serve as a nutrient source for bacteria under stress (Deutscher, 2006; Finn, Condell, et al., 2013). In this study, gene expression analysis on the LMF matrices were carried out after extended adaptation period (72 h). The detected signals therefore might only reflect the remaining mRNA at the sampling point, rather than all the mRNA that had been synthesized during the desiccation period. Therefore, genes presented in samples earlier may have degraded before being detected.

4.5   *Conclusion*

This study showed differential expression of alternative sigma factors and stress-repose genes in *S*. Typhimurium as a function of different a_w levels, matrix compositions, and the CA-assisted heat treatment. Some of the differentially expressed genes were linked to the cross-protection of desiccation-adapted bacteria against the combined treatment.

The upregulation of alternative sigma factor *rpoH* and the downstream stress response gene *dnaK* in *S*. Typhimurium from many of the tested LMF matrices was induced by the stress during bacterial adaptation to the low a_w environments and the combined heat treatment, which contributed to the bacterial resistance during the combined treatment. Although its link to the desiccation response in bacteria is not fully understood, the downregulation of *ompC* during the

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combined treatment also partially contributed to the treatment resistance. The upregulation of alternative sigma factor *rpoE* and the stress-response genes *otsB* and *proV* in *S*. Typhimurium in some of the tested matrices was induced by the desiccation stress during incubation at the low a_w environment and contributed to desiccation resistance. However, their expression had poor correlation with bacterial resistance during the combined heat treatment at different a_w/ matrices from the previous study and were therefore though to not be major contributors to the resistance during the combined treatment. The upregulation of *fadA* was induced during bacterial adaptation to low a_w as well and also contributed to the desiccation resistance, but their increased expression was not consistent to bacterial resistance during the combined heat treatment from the previous study either. The observed upregulation of *fabA* and downregulation of *ibpA* could not be directly linked to either bacterial resistance to desiccation or the combined heat treatment. Differential expressions were also observed among different a_w levels or in different matrices at the same a_w, where the expression profiles of *rpoH*, *dnaK* and *ompC* were partially consistent to the a_w- or matrix-dependent bacterial resistance observed during the combined treatment in the previous study. The inconsistencies between their expression profiles and bacterial resistance during the combined treatment suggested the presence of additional stress-response pathways.

Deeper understanding of the molecular mechanisms behind the bacterial resistance in low-a_w environments can be highly valuable for food safety risk assessment and the development of more efficient pasteurization technology for LMFs in the future. Based on the findings in this study, besides the stress from the combined treatment itself, the various factors in LMFs such as the a_w levels and matrix compositions can also act as stimuli to induce differential expression of different stress response factors, including alternative sigma factors and heat shock proteins. Their expression may contribute to both the resistance to desiccation and the cross-protection

against further antimicrobial-assisted heat treatments. Due to the scope limit of this study, expression analysis was only conducted on a selected range of genes. The molecular mechanisms behind the observed antimicrobial resistance, which results from interaction between multiple stress response genes, was not thoroughly understood. Future studies can apply transcriptomic analysis coupled with qPCR to gain more insights on changes to whole bacterial transcriptional profiles during desiccation adaptation and the treatment in different LMF matrices.

5 Chapter 5 Overall conclusions and future work

5.1 *Overall Conclusions*

The effect of heat treatment against *E. coli* O157:H7 was significantly enhanced by addition

of BP in MBM at 0.4 a_w. Results with *S*. Typhimurium suggested that it was more heat resistant

than *E. coli* O157:H7 and the addition of BP was not able to enhance the effect of heat treatment

at 0.4 a_w. Even when the heat resistance of *S*. Typhimurium was reduced by increasing the a_w of

MBM to 0.7, addition of BP could not significantly enhance the thermal inactivation. A sub-

population with higher thermal resistance existed in both bacteria. BP accelerated the

inactivation of the thermal-resistant subpopulation in *E. coli* O157:H7 but not *S*. Typhimurium.

These results have shown the potential of using food-grade antimicrobial compounds to improve

the antimicrobial effect of existing thermal treatment or heat-assisted dehydration process for

LMFs through their synergistic actions. Further studies were conducted to identify alternative

antimicrobial compounds to enhance thermal treatment against *S*. Typhimurium in LMF matrices

and investigate the resistance mechanisms.

CA and EG were selected from a screening study to assist thermal treatment against *S*.

Typhimurium adapted to different a_w in whey protein (WP), corn starch (CS) or peanut oil (PO).

Addition of CA or EG significantly accelerated thermal inactivation of *S*. Typhimurium in water

and LMF components (PO, WP and CS) at 0.9 a_w, although similar effect was not observed in

bacteria adapted to lower (0.4) a_w in any of those matrices. *S*. Typhimurium was generally more

resistant to inactivation at 0.4 a_w compared to 0.9 a_w regardless of the matrix components. The

matrix effect on bacterial thermal resistance was observed at 0.9 a_w and were ranked as WP >

PO > CS. Biphasic inactivation patterns were also observed in both WP at 0.9 a_w and PO at 0.4

a_w, indicating the development of heterogeneous resistance among the inoculated cells in

different matrices. Regardless of a_w (0.4 or 0.9), bacterial metabolic activity was lowered by the heat treatment in all tested LMF components. Addition of CA or EG to the heat treatment further reduced metabolic activity in CS and PO at both a_w, while similar effect was not observed in WP. It demonstrated that the effect of heat and CA/ EG treatments on bacterial metabolic activity was partially dependent on the food components. *S.* Typhimurium adapted to 0.4 a_w had lower membrane fluidity and lower unsaturated to saturated fatty acid ratio than bacteria at 0.9 a_w. It suggested that bacteria at low a_w can change their membrane composition to increase its rigidity and reduce its permeability, which contributes to resistance against both the combined heat treatments. The results have demonstrated the potential of using food-grade antimicrobial compounds to complement thermal treatment in LMF during processes that start with a relatively high a_w (such as dehydration). It also provided an insight of bacterial resistance mechanism at low a_w environment and its correlation with food components. Based on the a_w- and matrix-dependent bacterial resistance and physiological changes observed in the previous studies, transcriptional analysis was conducted to further understand the molecular mechanism behind the bacterial resistance.

Differential expression of alternative sigma factors and stress-repose genes in *S.* Typhimurium that adapted to different a_w in LMF components were observed with or without the CA-assisted heat treatment. The upregulation of alternative sigma factor *rpoH* and the downstream stress response gene *dnaK* in *S.* Typhimurium from many of the tested LMF matrices was induced by the stress during bacterial adaptation to the low a_w environments and the combined heat treatment, which contributed to the bacterial resistance to both desiccation and the combined treatment. Although its link to the desiccation response in bacteria is not fully understood, the downregulation of *ompC* during the combined treatment also partially

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contributed to the treatment resistance. The upregulation of alternative sigma factor *rpoE* and the stress-response genes *otsB, proV* and *fadA* in *S.* Typhimurium in some of the tested matrices was induced by the desiccation stress during incubation at the low a_w environment and contributed to desiccation resistance. However, their expression was not a major contributor to the resistance during the combined treatment. The observed upregulation of *fabA* and downregulation of *ompC* and *ibpA* could not be directly linked to either bacterial resistance to desiccation or the combined heat treatment. Differential expressions were also observed among different a_w levels or in different matrices at the same a_w, where the expression profiles of *rpoH*, *dnaK* and *ompC* were partially consistent to the a_w- or matrix-dependent bacterial resistance observed during the combined treatment in the previous study. The inconsistencies between their expression profiles and bacterial resistance during the combined treatment suggested the presence of additional stress-response pathways. These findings suggested that besides the stress from the combined treatment itself, the various factors in LMFs such as the a_w levels and matrix compositions can also act as stimuli to induce differential expression of different stress response factors, including alternative sigma factors and heat shock proteins. Their expression may contribute to both the resistance to desiccation and the cross-protection against further antimicrobial-assisted heat treatments, which should be considered for food safety risk assessment and the development of more efficient pasteurization technology in LMFs.

5.2   *Future Work*

The potential of applying CA or EG to complement thermal treatment in LMF matrices can be studied during processes that start with a relatively high a_w (such as heat-assisted dehydration) after optimizing treatment parameters such as treatment temperature and dosage of antimicrobial compounds. The sensory and nutritional quality of the treated products may be examined as well. In addition, since bacterial resistance is usually contributed by interactions between multiple stress response genes, deeper understanding in the changes to the whole transcriptional profiles of bacteria during desiccation adaptation and treatments in different LMF matrices may help us to better understand the molecular mechanisms behind the resistance. Transcriptomic analysis coupled with qPCR may be conducted in future to provide insights of the global transcriptional response in bacteria. It may also be worthwhile to explore the synergistic combination between heat treatment and non-thermal processing technologies, such as high pressure processing or incubation with ozone to enhance antimicrobial efficiency against resistant pathogenic bacteria in LMF matrices without the need to raise heat treatment intensity.
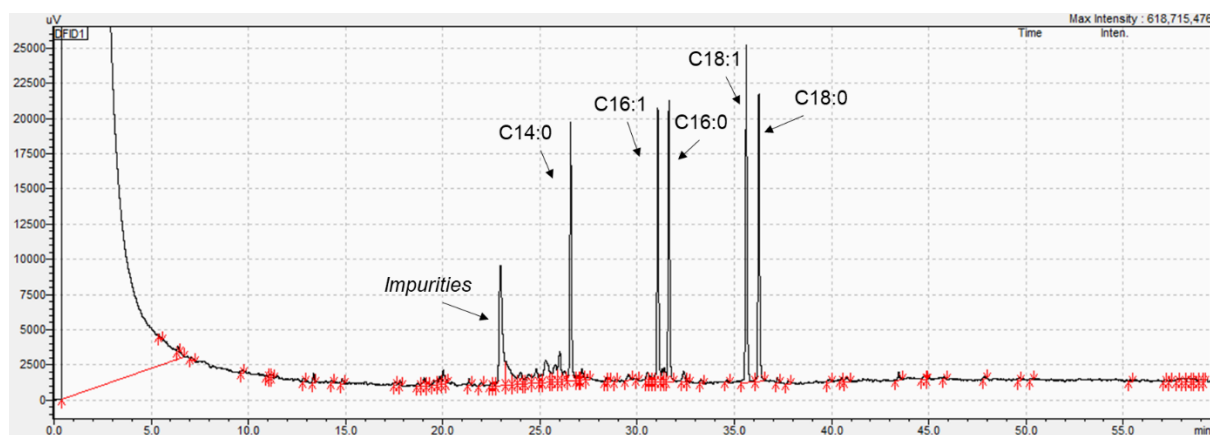
6 Appendix A Chromatograph during GC analysis

Figure SA-1. GC chromatogram of fatty acids methyl ester standards (except for cyc17)

Table SA-1. Retention time of different membrane fatty acids methyl ester identified in the study

Fatty acid methyl esters	Retention time (min)
C18:0	36.27
C18:1 (cis-9)	35.6
Cyc17	33.7
C16:0	31.65
C16:1 (cis-9)	31.07
C14:0	26.59

7   Appendix B Investigation of bacterial distribution in inoculated lipid matrix with fluorescence microscopy imaging

Methods

The distribution of bacteria in the inoculated PO was analyzed by fluorescent microscopy imaging. To track bacteria in the inoculated samples, instead of the *S.* Typhimurium strain described above, PO for this assay was inoculated with a strain of S. Typhimurium with a green fluorescent protein gene inserted (ATCC 14028GFP). Fluorescein disodium salt (Exi/ Emi: 425/ 515 nm) is insoluble in hydrophobic solvent, while Nile red (Exi/ Emi: 559/ 635 nm) has high partition coefficient (~ 200 at 4 ℃) (Greenspan and Fowler, 1985). They were therefore added to the inoculated samples separately to identify the water and oil phase in the matrix. Fluorescence microscopy observation of the samples was conducted using an inverted fluorescence microscope (ECLIPSE Ti-U, Nikon Instruments Inc, Melville, U.S.A.) paired with the NIS Elements BR software. Green and red fluorescence images were captured with the installed GFP (470/ 535 nm) and Texas Red (560/ 630 nm) filter cubes. Images were also taken at the bright field mode for comparison and size-analysis using the ImgeJ software.

Results

Microscopic images of samples inoculated with the GFP-labelled *S.* Typhimurium (Figure SB-A) suggested that after inoculation and the subsequent a_w-adjustment period, bacteria aggregated and formed small clumps inside the oil sample. The average size of the clumps (n = 42) was determined to be 325 ± 183 µm. As shown in the green (Figure SB-B) and red fluorescent images (Figure SB-C), the signal from the water-soluble fluorescein within the clumps was higher than the background and the signal from the oil-soluble Nile red within the clumps was lower than the background. This indicates that the clumps that hosted the bacteria were formed

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with mostly the water inside the matrix. The formation of bacterial-water cluster may have prevented the access of the antimicrobial compounds dissolved in the oil phase to the bacteria due to the reduced contact surface area, therefore partially inhibited their antimicrobial effect.

Figure SB-1. Fluorescent images of inoculated PO. A1-A6: Images of samples inoculated with GFP bacteria taken at bright field/ GFP mode; B1-B6: Images of samples (inoculated with non-GFP bacteria) with addition of fluorescein taken at bright field/ GFP mode; C1-C6: Images of samples (inoculated with non-GFP bacteria) with addition of Nile red taken at bright field/ Texas-red mode.
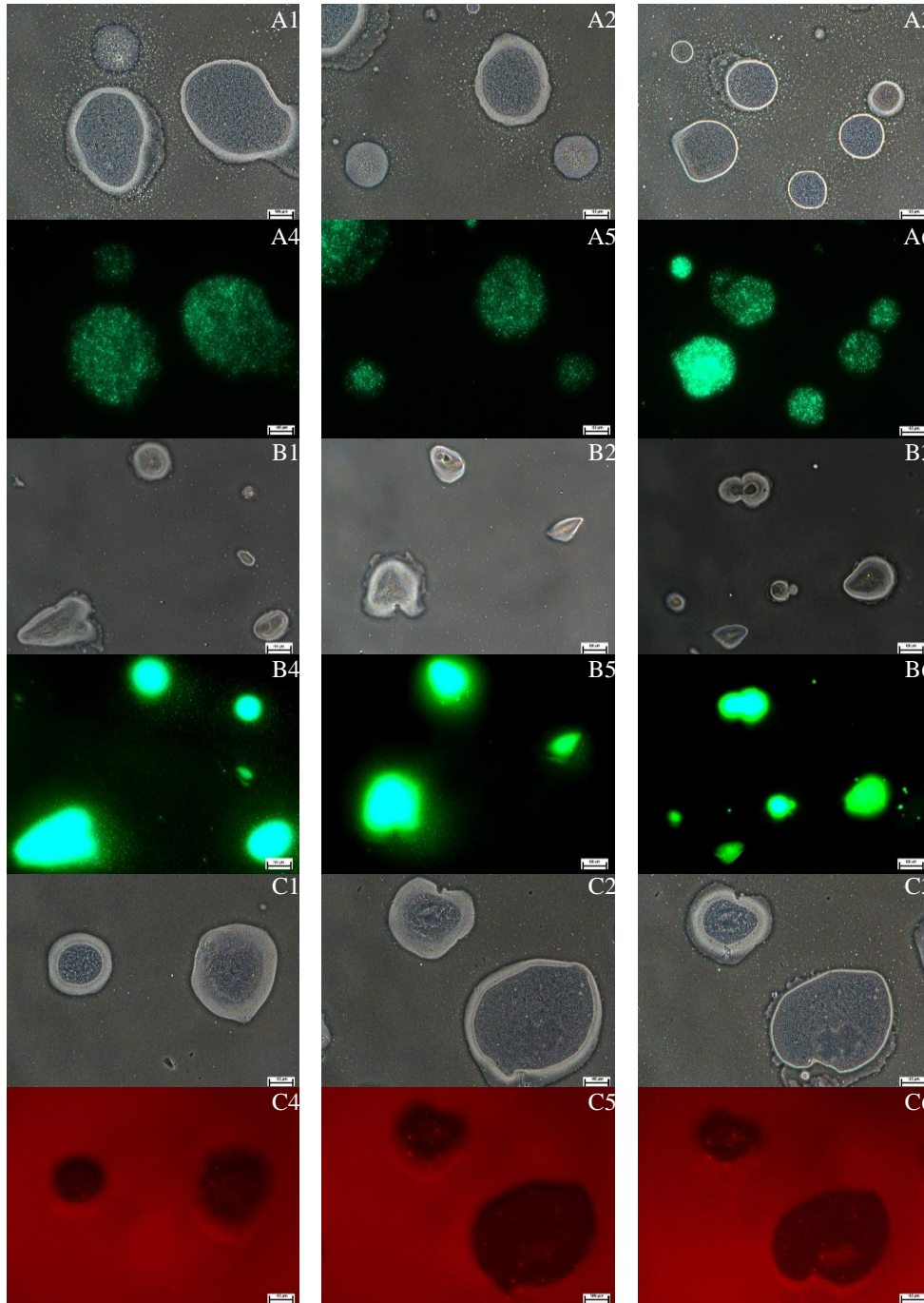
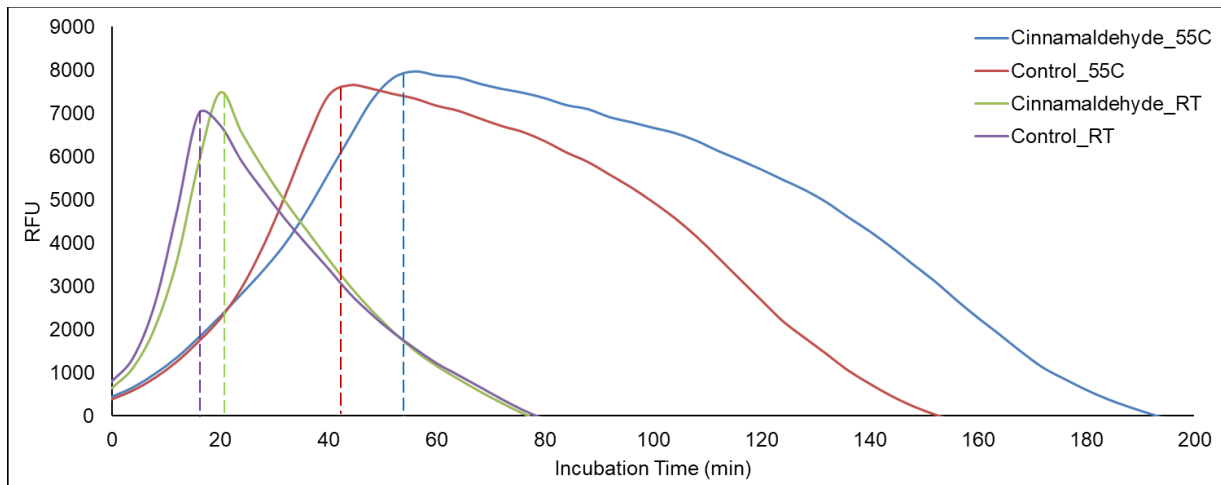# 8   Appendix C Example of fluorescence signal changes during Resazurin assay

Figure SC-1. Change of fluorescence signal intensity during incubation of *S*. Typhimurium harvested from PO at 0.4 a_w (with or without treatments) in TSB containing resazurin at 37 °C.

9 Appendix D Validation of Resazurin assay with sodium azide treatment

The inhibitory effect of sodium azide (NaN_3) on bacterial respiration activity has been previously reported (K Bore et al., 2017; Lichstein, 1944). It was therefore chosen to be used as a positive control to determine whether the fluorescence peak time detected during the Resazurin assay could effectively detect bacterial metabolic activity changes. 100 ppm sodium azide was added to *S*. Typhimurium suspension (7 log CFU/mL) in sterilized DI water. The solution was then incubated for 5 min before applying the Resazurin assay as described above. No significant bacterial reduction was induced during the treatment as confirmed by plating results. The results suggested that *S*. Typhimurium treated with sodium azide (194 ± 4 min) had significantly higher peak time than the cells incubated without the inhibitor (128 ± 0 min) ($P < 0.05$), suggesting that the peak time of resazurin assay effectively detected changes in the metabolic activity of bacteria.

10 Appendix E Investigation of bacterial membrane integrity with propidium iodide assay

Methods

Treatment effect on bacterial membrane integrity has also been investigated using propidium iodide. Propidium iodide (PI) is a fluorescent intercalating agent that can be used to measure cellular membrane damage after treatments. The fluorescence signal from PI increases when it penetrates cells with damaged cytoplasmic membrane and intercalates to DNA, which is proportional to the level of membrane damage (Berney et al., 2007; Ding et al., 2018; Stiefel et al., 2015).

Cells from 1 mL of both CA-assisted heat-treated and room-temperature control samples were harvested by centrifugation (10,000 g for 5 min), which were subsequently washed three times with phosphate buffer saline (PBS). Bacterial pellets were resuspended in 1 mL of 5 μM PI solution and incubated at room temperature for 15 min. The pellets were subsequently resuspended in 500 μL PBS after an additional wash with PBS. The fluorescence intensity of samples was measured at excitation and emission wavelengths of 535 and 617 nm, respectively, with a SpectraMax M5e microplate reader (Molecular Devices, Sunnyvale, CA).

Results

Results on Figure SE showed that after being treated with the heat treatment alone for 3 min, *S.* Typhimurium cells in both PO (0.4 a_w) (2.03 ± 0.31 RFU) and DI water (1.52 ± 0.02 RFU) uptake a higher level of propidium iodide than the room temperature controls (1 ± 0.06/ 1 ± 0.01 RFU) (*P* < 0.05). It suggested that heat treatment was able to induce membrane damage to the cells.

It has been previously reported that CA could cause membrane leakage on bacteria and yeasts in aqueous solution with exposure time up to 2 h (Di Pasqua et al., 2006; Nowotarska et al., 2017;

Smid et al., 1996). However, although addition of CA during the heat treatment in DI water induced higher degree of membrane damage than the heat treatment alone ($P < 0.05$), similar enhancement effect was not observed bacteria inoculated in PO at 0.4 a_w ($P > 0.05$). Additionally, no significant difference was observed between water and PO at 0.4 a_w after the heat treatment with or without 1000 ppm CA ($P > 0.05$), which was not consistent with the higher bacterial resistance observed in PO with lower a_w in the previous study. It suggested that the results from PI assay could not effectively reflect changes on bacterial membrane properties that might contribute to bacterial resistance at low a_w conditions. The fluorescence signals from PI will only increase when bacterial membrane has undertaken substantial damages from the treatments that allows the entrance of the PI molecules (Stiefel et al., 2015). The PI assay results may not be able to effectively reflect more subtle changes on membrane properties that may also contribute to bacterial resistance, such as change of membrane fatty acids profiles or membrane fluidity. Therefore, the PI assay was not adopted in this study.
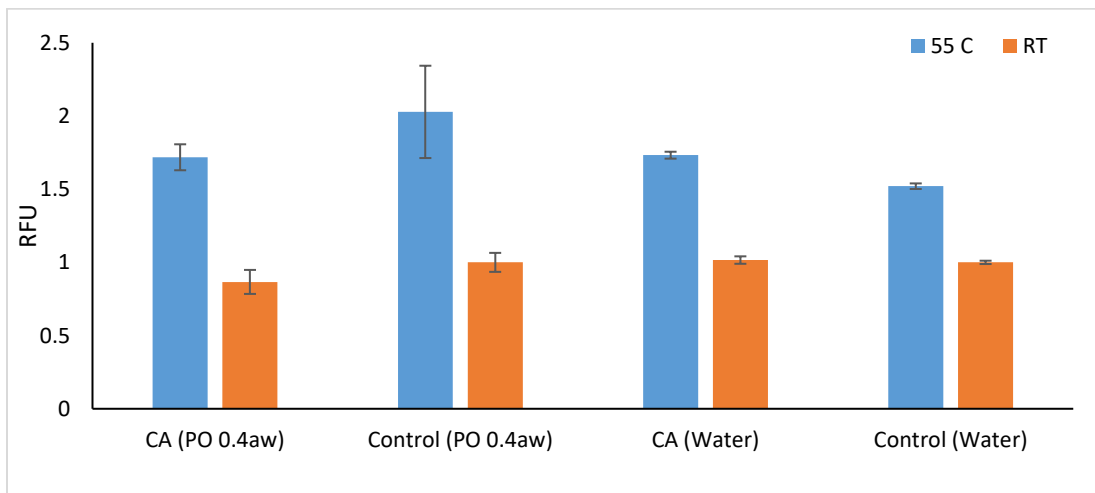

Figure SE: Bacterial membrane damage measured by PI assay.

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