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Comparative Study Of The D-values of *Salmonella* spp. and *Enterococcus faecium* in Wheat Flour

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COMPARATIVE STUDY OF THE D-VALUES OF *Salmonella* spp. AND *Enterococcus faecium* IN WHEAT FLOUR

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Low-moisture foods contaminated with *Salmonella* spp. have been implicated in several foodborne illness outbreaks in the United States. The food industry has to incorporate preventive control in their process and validate thermal processes to assure food safety. The thermal destruction of *Enterococcus faecium* ATTC 8459 and *Salmonella* spp. was determined at 3 water activity levels (0.11, 0.18 and 0.33) at 80ºC, 85ºC and 90ºC. Aerobic plates counts petrifilms were used as non-selective medium for both bacteria, Enterobacteriaceae petrifilms were used as selective medium for *Salmonella* spp., and peptone water modified with sodium azide as a selective medium for *Enterococcus faecium*. Significant differences were observed for both organisms between the two media with higher D-values on the non-selective medium ($p < 0.05$). Lower D-values were observed for both organisms at higher water activities ($p < 0.05$). The D-values of *Salmonella* spp. were 112.87 min, 61.01 min and 32.36 min at 80ºC, 85ºC and 90ºC, respectively in wheat flour at water activity of 0.11. At water activity of 0.18, the D-values of *Salmonella* spp. were 59.05 min, 30.90 min and 18.78 min at 80ºC, 85ºC and 90ºC, respectively. The D-values of *Salmonella* spp. were 25.10 min, 13.25 min

and 6.22 min at 80ºC, 85ºC and 90ºC, respectively at aw 0.33. Higher D-values were observed for *Enterococcus faecium* ATTC 8459 compared to *Salmonella* spp., at all the conditions of the study, with no significant differences ($p \geq 0.05$), except at the water activity of 0.18 at 85ºC ($p < 0.05$). The D-values of *Enterococcus faecium* ATTC 8459 were 117.97 min, 64.31 min and 38.24 min at 80ºC, 85ºC and 90ºC, respectively in wheat flour at water activity of 0.11. At water activity of 0.18, the D-values of *Enterococcus faecium* ATTC 8459 were 65.26 min, 50.49 min and 19.17 min at 80ºC, 85ºC and 90ºC, respectively. The D-values of *Enterococcus faecium* ATTC 8459 were 29.01 min, 15.09 min and 9.71 min at 80ºC, 85ºC and 90ºC, respectively at water activity of 0.33. The z-values were determined at each water activity, and there were no significant differences between the three (3) water activities ($p \geq 0.05$). *Salmonella* spp. and *Enterococcus faecium* had approximately the same *z*-values. The *z*-values of *Salmonella* spp. ranged from 16.53 to 18.50ºC, while the *z*-values of *Enterococcus faecium* ranged from 18.80 to 21.61ºC. These results suggest that *Enterococcus faecium* ATTC 8459 can be used as a surrogate for *Salmonella* spp. in wheat flour at the three levels of water activity used in the study.

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Chapter 1

INTRODUCTION

Foodborne illness is an important public health issue in the United States and in the world. The Centers for Disease Control and Prevention (Centers for Diseases Control and Prevention, 2011) estimated that approximately 48 million illnesses, 128,000 hospitalizations and 3,000 deaths occur from foodborne illnesses (Centers for Diseases Control and Prevention, 2011). Of the bacterial causes of foodborne illnesses, *Salmonella* is estimated to cause 1,632 foodborne outbreaks, 29,112 illnesses, 1,750 hospitalizations, and 68 deaths (Centers for Diseases Control and Prevention, 2014). *Salmonella* non-thyphoidal is the second of the five top pathogens, after Norovirus, causing the most domestically acquired foodborne illnesses with 1,027,561 cases. It is the first of the five top pathogens causing the most domestically acquired foodborne illnesses resulting in hospitalization with 19,336 cases require hospitalization, and also the first of the five top pathogens causing the most domestically acquired foodborne illnesses resulting in death with 378 cases (Centers for Diseases Control and Prevention, 2011).

Although growth of *Salmonella* is inhibited in food product with low water activity, cells can remain viable in flour and other low water activity food products. Recent investigations have implicated these products as potential sources of foodborne illnesses. In 2008, homemade play dough, raw cake and batter mixes prepared from unheated wheat flour were implicated in a *Salmonella* spp. in New Zealand (Eglezos, 2010). In 2009, flour used to produce ready-to-bake cookie dough was responsible for an *Escherichia coli* O157:H7 outbreak in which 77 consumers were ill, 35 of them were hospitalized and 10 developed hemolytic-uremic syndrome (Neil et. al., 2011). Subsequent to these outbreaks, the Centers for Disease Control and Prevention published

a report demanding that "*foods containing raw flour should be considered as possible vehicles of infection of future outbreaks of STEC (Shiga toxin-producing E. coli) or Salmonella infections…"* (Neil et. al., 2011). Therefore, flour should undergo heat treatment before being used in the confectionery of ready-to-bake cookie dough and raw cake.

Recent *Salmonella* outbreaks (Neil et al., 2009) have forced the food industry to evaluate the safety of wheat flour and wheat flour-based food products (Neil et al., 2009). To avoid the introduction of pathogenic bacteria in the areas of food production, it is recommended that food processors use a surrogate microorganism to determine the conditions to destroy pathogens in raw food products. Surrogate organisms are typically non-pathogenic organisms, having similar characteristics as the target. Researchers have used *Enterococcus faecium* ATTC 8459 as a surrogate for *Salmonella* spp. in thermal destruction, and others have used this microorganism as surrogate for pathogens in different liquid products (Bianchini et al., 2013). The objective of this study was to determine the D-value of *Salmonella* spp. and *Enterococcus faecium* in wheat flour, and the potential use of *Enterococcus faecium* as a surrogate for *Salmonella* spp. in wheat flour for thermal processing.

Chapter 2

LITTERATURE REVIEW

2.1 *Salmonella* spp.

2.1.1 General characteristic

Salmonella spp. are Gram negative, rod-shaped bacteria and belonging to the family of Enterobacteriaceae. They are non-spore forming, facultative anaerobes capable of metabolizing nutrients by both oxidative and fermentative pathways. Most *Salmonella* serotypes are motile via peritrichous flagella, while others are non-motile with dysfunctional flagella. They utilize a wide range of organic substrates: they produce acid and gas from D-glucose and other carbohydrates; produce hydrogen sulfide, decarboxylate lysine, reduce nitrate to nitrite and do not hydrolyze urea. They are oxidase negative and catalase negative and utilize citrate as the sole source of carbon (Montville and Matthews, 2005).

The genus *Salmonella* consists of two species: *Salmonella enterica,* the type species, which is divided into six subspecies, and *Salmonella bongori.* Each one of these species is subdivided into multiple serovars presented in Table 2.1.1.

Table 2.1.1 Salmonella spp. classification according to Kauffmann-White scheme

Genus	Species	Subspecies	Number of serovars
		enterica (I)	1,454
		salamae (II)	489
	enterica	*arizonae* (IIIa)	94
Salmonella		*diarizonae* (IIIb)	324
		houtenae (IV)	70
		indica (VI)	12
	bongori	(V)	20
Total			2463

Popoff, et al. (2000)

The names of the serovars of the subspecies *S. enterica* subsp. *enterica* are based on the associated diseases, their geographic origins, or their usual habitats, while the names of the serovars of the other subspecies as well as those of *Salmonella bongori* itself are based on their antigenic formulae determined according to the Kauffmann-White scheme (Patrick & François, 2007).

Salmonella spp. can survive and adapt to extreme environmental conditions such as, lack or low levels of nutrients, and a wide spectrum of temperatures and pH values (D'Aoust, 2001). They grow in a wide range of temperature with an optimal growth at 37°C. Some strains are able to grow at elevated temperatures (54°C), and other strains have psychrotrophic characteristics enabling them to survive for extended periods in refrigerated foods at 2 to 4°C (Montville and Matthews, 2005). The heat resistance of *Salmonella* spp. can be affected by many factors such as water activity of the food matrix, the types of solutes used to reduce the water activity, the nutritional composition of the media, the growth phase, exposure to sublethal temperatures, high fat content (Larry, 2009). *Salmonella* spp. can grow in a broad pH range of 4.5 to 9.5 with an optimum for growth of 6.5 to 7.5. Many factors such as temperature, presence of salt and nitrite, and the type of acids, determine the minimum pH at which *Salmonella* spp. can survive. Organic acids such as lactic, citric and acetic acids have less bactericidal effect on *Salmonella* spp. than volatile fatty acids (Bell and Kyriakides, 2002). Certain serotypes can develop acid resistance after exposure to mild acid environment of pH 5.5 to 6.0 followed by exposure to a pH ≤ 4.5 (Montville and Matthews, 2005). Water activity can exert a significant effect on *Salmonella* spp. growth with the optimum growth at 0.99 and the lower limit at 0.93. However, some studies showed that *Salmonella* spp.

can survive for extended periods in low-moisture food products such as peanut butter, infant formula, chocolate, cereal products, and dried milk (Podolak et al., 2010).

Salmonella spp. are distributed worldwide and are associated with a wide range of animal sources including livestock, wildlife, poultry, and companion animals. Water and foods of animal origin are known for disseminating *Salmonella* spp. in the environment. Fresh products exposed to contaminated water, farm equipment, fecal contamination from livestock, wild animals, and human carriers can represent an important source of propagation of these bacteria to human populations (Gorski et al., 2010).

2.1.2 *Salmonella* outbreaks

Salmonella outbreaks are mostly associated with the consumption of poultry, beef, pork, eggs, milk, seafood and fresh produce (Gomez et al., 1997). However other low moisture food products such as peanut butter, almond, chocolate, potato chips and snack foods are implicated in the transmission of the bacteria in recent outbreaks (Beuchat et al., 2013). Among the serotypes of *Salmonella* causing most foodborne outbreaks from 2006 to 2013, the top serotypes detected were: Enteritidis, 1,237 (19%); Typhimurium, 917 (14%); and Newport, 674 (10%) (Centers for Diseases Control and Prevention, 2014).

One of the largest outbreaks of foodborne salmonellosis in the US occurred in 1974 where the consumption of egg-containing potato salad stored for up to 16 h at improper temperatures resulted in an estimated 3,400 human cases of *S. enterica* serovar Newport infection (Horwitz et al., 1977). In 1977, the consumption of mayonnaise-containing dressing salad was linked to an outbreak of serovar Enteritidis phage type 4 in a school cafeteria in Sweden (Smittle, 1977). In 1984, the consumption of Cheddar cheese resulting in more than 2,700 confirmed cases involving the serovar Typhimurium was

known as the largest salmonellosis outbreak in Canada (D'Aoust et al., 1984). In 1985, the United States knew another large salmonellosis outbreak involving 16,284 confirmed cases of illness where cross-contamination of pasteurized milk was suspected, and the serovar Typhimurium was the etiological agent (Ryan et al., 1987). The largest salmonellosis outbreak in the US occurred in 1994 causing about 224,000 cases of illness. It was attributed to ice cream produced from milk transported in tanker trucks that had previously carried liquid egg (Jay et al., 2005). Table 1.2 presents some recent salmonellosis outbreaks in the United States.

Table 2.1.2 Examples of recent salmonellosis outbreaks in the United States from 2010 to 2014

Year	Vehicles	Strains	No. of cases/Locations
	Alfalfa Sprouts	*S.* serotype I 4, [5], 12:i:-	140 cases in 26 states
	Shell Eggs	*S.* Enteritidis	1939 cases in 29 states
2010	Cheesy Chicken Rice Frozen Entrée	*S.* Chester	44 cases in 18 states
	Frozen Rodents	*S.* serotype I 4, [5], 12:i:-	34 cases in 17 states
	Red and Black Pepper/Italian-Style Meats	*S.* Montevideo	272 cases in 44 states + DC
	Kosher Broiled Chicken Livers	*S.* Heidelberg	190 cases in 6 states
2011	Turkish Pine Nuts	*S.* Enteritidis	43 cases in 5 states
	Ground Turkey	*S.* Heidelberg	136 cases in 34 states
	Whole Fresh Papayas	*S.* Agora	106 cases in 25 states
	African Dwarf Frogs	*S.* Typhimurium	241 cases in 42 states
	Peanut butter	*S.* Bredeney	42 cases in 20 states
	Mangoes	*S.* Braenderup	127 cases in 15 states
2012	Cantaloupe	*S.* Typhimurium, *S.* Newport	261 cases in 24 states

	Gound beef	*S.* Enteritidis	46 cases in 9 states
	Raw Scraped Ground Tuna	*S.* Bareilly, *S.* Nchanga	425 cases in 28 states
	Foster Farm Chicken	*S.* Heidelberg	634 cases in 29 states + Puerto Rico
2013	Tahiti Sesame Paste	*S.* Montevideo, *S.* Mbandaka	16 cases in 9 stated
	Cucumbers	*S.* Saintpaul	84 cases in 18 states
	Ground Beef	*S.*Typhimurium	22 cases in 6 states
	Live poultry	*S.* Typhimurium	356 cases in 39 states
	Bean sprouts	*S.* Enteritidis	115 cases in 12 states
	Nut butter	*S.* Braenderup	6 cases in 5 states
2014	Organic sprouted Chia powder	*S.* Newport, S. Harford, S. Oranienburg	31 cases in 16 stated
	Live poultry	*S.infantis, S.* Newport, *S.*Hadar	363 cases in 43 states
	Raw cashew cheese	*S.* Stanley	17 cases in 3 states

Centers for Diseases Control and Prevention (2010, 2011, 2012, 2013, 2014)

2.1.3 *Salmonella* pathogenesis

Among the two species of *Salmonella*, the strains belonging to the subspecies *Salmonella enterica* subsp. *enterica* are mostly responsible for diseases in humans and warm-blooded animals, causing up to 99% of the infections (McClelland et al., 2001), and some of those strains are also known for plant contamination (Pezzoli et al., 2007). Based on the host species, *Salmonella* species can be classified in three groups of serotypes; first, host restricted serotypes (HR) that exclusively affect one particular host species, for example *Salmonella* Typhi which causes septicemic typhoid syndrome in humans, and *Salmonella* Gallinarum which causes typhoid in birds; second, host adapted serotypes (HA) that are

prevalent in one particular host species, but can also cause diseases in other species, for example *Salmonella* Dublin in cattle, but can also rarely infect human and sheep, and *Salmonella* Choleraesuis in pigs which can also cause disease in human; third, unrestricted serotypes (UR) that are ubiquitous serotypes able of causing diseases in a wide range of host species, for example *Salmonella* Typhimurium and *Salmonella* Enteritidis that cause enterocolitis in humans and cattle, and intestinal infection in poultry (Uzzau et al., 2000).

Salmonellosis is normally initiated by ingestion of contaminated food or water, and sometimes after contact with another person with the infection. After ingestion, the bacteria enter the intestine from the stomach, and adhere to the cells lining the intestinal epithelium. *Salmonella enterica* can cause three main different clinical conditions: enteric fever, gastroenteritis, and bacteremia. Enteric fever is caused by the typhoidal serotypes 7 to 72 h of after ingestion of the bacteria, and is characterized by fever, headache, abdominal, diarrhea (mostly in children), and constipation (mostly in the adults). Complications can lead to myocarditis, urinary tract infection, and metastatic lesions in bone, joints, liver, and meninges, and haemorrhage (Public Health Agency of Canada, 2010). Supportive therapy and/or the use of proper antimicrobials such as chloramphenicol, ampicilin, or trimethoprim-sufamethoxazole are the best way to eliminate the infection. Gastroenteritis, also called food poisoning, is more commonly caused by the non typhoidal serotype Typhimurium. The disease occurs 8 to 72 h after ingestion of the pathogen, and is characterized by nausea, vomiting, abdominal cramps, diarrhea, and headache. Complications can lead to systemic infections and various chronic conditions (Montville and Matthews, 2005). Bacteremia occurs mostly in

immunosuppressed individuals and patients with comorbid medical conditions such as HIV-AIDS, diabetes, sickle cell disease. Bacteremia can cause septic shock; endocarditis, infection of the aorta, urinary tract infection, pneumonia; pulmonary abscess ((Public Health Agency of Canada, 2010).

The infectious dose depends on the serotype, the health condition of the patients, and the level of acidity in the patient's stomach; approximately 10^3 cells are necessary for non typhoidal serotypes to cause illnesses, while 10^5 cells can cause diseases in the case of typhoidal serotypes. However, a lower dose of these serotypes can cause infection in patients suffering from achlorhydria (characterized by an absence of hydrochloric acid in gastric secretion), compromised immunity, newborns, infants, and elderly (Public Health Agency of Canada, 2010; Montville and Matthews, 2005). A lower dose can also cause disease depending on the chemical composition of the food, which can play an important role in protecting the bacteria. For example, food with high fat content can form hydrophobic lipid micelles that entrap the bacteria and protect them against the bactericidal effect of the gastric acidity (Montville and Matthews, 2005).

2.1.4 *Salmonella* in low-moisture foods

Many vegetative pathogens, including *Salmonella* spp. do not grow in foods with low water activity. Although they do not support the growth of *Salmonella* spp., low-water-activity food products such as powdered milk, chocolate, peanut butter, infant foods, cereal, and bakery products (Beuchat et al., 2013), have been implicated in salmonellosis outbreaks. Food powders are not a cause of foodborne illness when used as additives in products that undergo heat treatment. However, when they are added to ready-to-eat foods, they can be implicated in outbreaks if contaminated with pathogens.

Some studies have shown that, although *Salmonella* spp. cannot proliferate when the substrate water activity is below 0.94, it can survive extended period of time in low-moisture environments (Hiramatsu et al., 2005; Podolak et al., 2010). Janning et al. (1994) who tested 18 bacterial strains, of which *Salmonella* spp. to study their survival in low water activity environment, observed that 248 to 1351 days were necessary to achieve 1 log reduction of *Salmonella* strains, and that *Salmonella* spp. was more resistant to desiccation than the other bacteria used in the study (Janning, et al., 1994).

Burnett et al. (2000) investigated the reduction of *Salmonella* spp. in peanut butter and peanut butter spreads during 24 weeks, and found out that the populations only decreased 2.86 to 4.82 logs at 5ºC. Similarly, Park et al. (2008) observed a reduction of *Salmonella* spp. from 0.34 to 1.29 log in five commercial peanut butters incubated for14 days at 22ºC.

Table 2.1.4 Selected Salmonella outbreaks associated with low-moisture products

Year	Product implicated	Etiologic Agent	Country	Reference
1970	Chocolate	*S.* Durham	Sweden	Gastrin et al., 1972
1972	Fishmeal	*S.* Agona	US	Clark et al.,1973
1973	Milk powder	*S.* Derby	Trinidad	D'Aoust and Maurer, 2007
1982-83	Chocolate	*S.* Napoli	UK	Greenwood and Hooper, 1983
1985-86	Chocolate	*S.* Nima	Canada, US	Hockin et al., 1989
1987	Chocolate	*S.* Typhimurium	Norway, Finland	Kapperud et al., 1990
1993	Paprika-seasoned potato chips	*S.* Saintpaul, *S.* Javiana, *S.* Rubislaw	Germany	Lehmacher et al., 1995
1993	Powdered infant formula	*S.* Tennessee	Canada, US	CDC, 1993
1995	Infant cereals	*S.* Senftenberg	UK	Rushdy et al., 1998
1996	Peanut butter	*S.* Mbandaka	Australia	Ng et al., 1996
1996	Peanut-flavored maize snack	*S.* Agona	Multiple countries	Killalea et al., 1996; Shohat et

				al., 1996
1998	Toasted oats cereals	*S.* Agona	US	CDC, 1998
2000-01	Raw almonds	*S.* Enteritidis	US, Canada	CDC, 2004
2001	Peanuts	*S.* Stanley, *S.* Newport	Multiple countries	Little, 2001
2001	Chocolate	*S.* Oranienburg	Multiple countries	Werber et al., 2002; Ethelberg, 2002; Fisher et al., 2002; Gill et al., 2008
2002	Tahini and Halva	*S.* Montevideo	Australia	Tauxe et al., 2008
2003-04	Raw almonds	*S.* Enteritidis	US, Canada	CDC, 2004
2006	Chocolate	*S.* Montevideo	UK	FSA, 2006
2006-07	Peanut butter	*S.* Tennessee	US	CDC, 2007
2007	Children's snack	*S.* Wandsworth, *S.* Typhimurium	US	CDC, 2007
2008	Puffed cereals	*S.* Agona	US	CDC, 2008a
2008	Powdered infant formula	*S.* Give	France	Jourdan et al., 2008
2008-09	Peanut butter, peanut buttercontaining products	*S.* Typhimurium	US, Canada	CDC, 2009
2009	Red and black pepper	*S.* Montevideo	US	Julian et al., 2010
2011	Turkish pine nuts	*S.* Enteritidis	US	CDC, 2011
2012	Dry dog food	*S.* infantis	US	CDC, 2012
2012	Peanut butter	*S.* Bredeney	US	CDC, 2012
2013	Tahini past	*S.* Montevideo/Mbandaka	US	CDC, 2013

The Association of Food, Beverage and Consumer Product Companies (2009); Sarah et al. (2013)

2.1.5 Mechanisms for *Salmonella* survival in low-moisture foods

When *Salmonella* and other non-sporulating bacteria are in a hostile environment, they develop several survival strategies. They may enter in a dormant state called viable but nonculturable state (VBNC) that enables the bacteria to remain viable in unfavorable conditions, and start growing when the conditions become favorable. In this state, the bacteria cannot grow using traditional laboratory techniques. (Gupte et al., 2003; Oliver, 2010). While some research studies have confirmed that some pathogenic bacteria, such

as *E. coli, Vibrio vulnificus*, and *Edwardsiella tarda* retain their pathogenicity in VBNC state (Du et al., 2007; Olive and Bockian, 1995; Pommepuy et al., 1996*)*, it is difficult to know whether *Salmonella* maintains its virulent characteristics in such a condition (Lesne et al., 2000). Recent investigations advance the possibility that pathogens cannot initiate disease in the VBNC state, but remain virulent, and can cause infection upon resuscitation to the actively metabolizing state (Oliver, 2000).

Filament formation is another strategy *Salmonella* spp. uses to face inimical conditions such as lower water activity, high or low temperatures, and high or low pH values (Mattick et al., 2003). A study carried out by De Rezende et al. (2001) showed extensive formation of filaments by *Salmonella* Typhimurium DT104 cells after exposure to low water activity (De Rezende et al., 2001). Similarly, Mattick et al. (2000) hypothesized that filamentation may improve survival after observing the presence of *Salmonella* filaments after 144 h of incubation in a broth medium with an approximate water activity of 0.95 supplemented with 8% NaCl (Mattick, et al., 2000). This hypothesis is corroborated by Kieboom et al. (2006) who found that *Salmonella* Enteritidis cells exposed at aw of 0.94 to 0.95 at 25ºC for 6 days, elongated, and formed filaments (Kieboom et al., 2006).

Osmoregulation is another important mechanism used by *Salmonella* to limit the loss of water. This survival strategy enables the bacteria to equilibrate its internal cell composition to that of the external environment when exposed to low-moisture conditions. It can be explained by the accumulation of osmoprotectants, such as proline, glycine-betaine, and ectoine. Osmoprotectant can be defined as electrically neutral, low molecular weight compatible solutes used by the cell to limit water loss. Trehalose is also

an important compatible solute in the osmoadaptation of *Salmonella* (Csonka and Hanson, 1991).

Table 2.1.5 Examples of Salmonella survival in foods with low water activity

Food	*Salmonella* serotype(s)	Inoculum (log CFU/g)	Aw/ moisture content	Length of survival	Reference
Dried milk products	Contaminated naturally with three serotypes			≤ 10 mo	Ray, B., et al., 1971
Pasta	Infantis, Typhimurium		12% moisture	≤ 12 mo	Rayman, M. K., et al., 1979
Milk chocolate	Eastbourne	8.0	0.41	> 9 mo at 20ºC	Tamminga, S. K., et al., 1976
		5.0	0.38	≤ 9 mo at 20ºC	
Bitter chocolate	Eastbourne	7.0	0.51	≤ 9 mo at 20ºC	Tamminga, S. K., et al., 1976
Halva	Enteritidis	7.0	0.18	> 8 mo at refrigeration temp	Kotzekidou, P., 1998
Peanut butter	A composite of Agona, Enteritidis, Michigan, Montevideo, Typhimurium	5.7	0.20–0.33	≤ 24 wk held at 5 or 21ºC	Burnett, S. L., et al., 2000
		1.5	0.20–0.33	≤ 24 wk held at 5 ºC	
Paprika powder	Multiple serotypes			<8 mo	Lehmacher, A., et al., 1995

Podolak et al., 2010

2.1.6 Phenotypes associated with *Salmonella* survival in low-moisture environments

Low infectious dose, increased thermal resistance, and cross-tolerance to other stressors are among the most important phenotypes associated with *Salmonella* isolated from low-moisture environments.

2.1.6.1 Low infectious dose

Contrary to other food contaminated with *Salmonella* in which more than 10^5 CFU are necessary to cause infection (Todd et al., 2008), a dose as low as 10–100 CFU of *Salmonella* is sufficient to cause an infection from the ingestion of a low-water activity food. One of the possible explanations of this observation is that the low-water activity product may provide protective properties which allow the bacteria to transit safely through the gastro-intestinal tract (D'Aoust, 1977; Todd et al., 2008). For instance, Aviles et al. (2013) were able to prove that high fat and low-aw combined in peanut butter matrix provided protection to *Salmonella* Tennessee transiting through a simulated GI tract (Aviles et al., 2013). Another explanation is provided by Stackhouse et al. (2012) who proposed that filament formation in low-moisture environments as a response to hostile conditions that may allow the bacteria to achieve high bacterial loads in very short time upon rehydration. The true population of the bacteria may be underestimated since filamentous cells cannot be detected with precision (Stackhouse et al., 2012). This same study advanced that filamentous cells have the ability to survive at low pH and in the presence of 10% bile salts after a 24-h period of exposure, which may give the bacteria an advantage during the transit in the gastrointestinal tract. Another possible explanation is that the entry in the viable but non culturable (VBNC) state allows the detection of a low number of cells, and more importantly some bacteria in this state can conserve their pathogenicity, and cause infection when exposed to favorable conditions (Oliver, 2010; Lesne et al., 2000).

2.1.6.2 Thermal resistance

Thermal resistance is one of the most important phenotypes associated with *Salmonella* survival in low-moisture environments. Previously exposed to moderately low water activity conditions, *Salmonella* has shown increased thermal resistance in subsequent heat treatment (Mattick et al., 2000). The enhanced thermal resistance is dependent upon the food matrices, and also on the humectants used to reduce the water activity. Results from diverse investigations of *Salmonella* heat resistance in low moisture environments suggested that heat resistance augmented in low and intermediate moisture foods, and that was also a function of the intrinsic and extrinsic properties of the food (Sarah et al., 2013). Therefore, it is extremely important that food processors determine the heat resistance of *Salmonella* in their specific food products instead of directly applying D-values and *z*-values from the literature, since those results may not be applicable to the products being tested (Podolak et al., 2010). Those results also showed non-linear survival curves, often showing a concave-upward curvature, which can be explained by a rapid decline in numbers of survivors during the first few minutes due possibly to the death of cells injured during the heat process (Goepfert and Biggie, 1968).

A study conducted by Sumner et al. (1991) to compare the effect of different a_w on thermal resistance of *Salmonella* and *Listeria monocytogenes* in sucrose at 65.6ºC showed that the D-value at water activity of 0.98 was 0.29 min, while it was 40.2 min at an water activity of 0.83 (Sumner et al., 1991). Similarly, heat resistance of *Salmonella* is increased in milk powder at low moisture level. For example, a 2-h heat treatment at 85ºC was not adequate to destroy *Salmonella* in 4 and 7% moisture powders, while 30 min was sufficient at the 25% moisture level (McDonough and Hargrove, 1968).

Archer et al. (1998) studied the heat resistance of *Salmonella* Weltevreden inoculated into flour heated in hot air at an initial water activity range of 0.20 to 0.60 prior to heating. They reported that the $D_{60\text{-}62^{o}C}$ was 875 min at an initial a_w of 0.4, and the $D_{63\text{-}65^{o}C}$ was 29 min at an initial a_w of 0.5. These observations suggested that reducing the water activity of a sample prior to heat treatment caused an increase of the thermal resistance of the bacteria, and that the initial a_w value before heating had a more significant effect on the heat resistance of *Salmonella* Weltevreden in flour than the water activity value during heating of the inoculated product (Archer et al., 1998).

Table 2.1.6 Thermal resistance of Salmonella in food matrices as influenced by water activity

***Salmonella* serotype**	**Food matrice**	**A_w**	**Temp (ºC)**	**D-value (min)**	***z*-value (ºC)**	**Reference**
Anatum	Milk chocolate	Not reported	90	11	24.2	Barrile and Cone, 1970
Enteritidis PT 30	Almonds (oil-roasted)	Not reported	121	0.85	27	Harris, 2008
Typhimurium	Milk chocolate	Not reported	70	816	19	Goepfert and Biggie, 1968
			80	222		
			90	75		
		0.50–0.60	69–71	80	30.3	
			72–74	45		
			75–77	40-45		
		0.45–0.50	69–71	55	53.9	
			72–74	55		
			75–77	40–45		
		0.40–0.45	69–71	55	19.6	
			72–74			
			75–77			
		0.35–0.40	69–71		15.2	
			72–74	75		
Weltevreden	Wheat flour		75–77	80		Archer, J., et al.,1998.
		0.30–0.35	69–71	345	29.2	
			72–74			
			75–77	85		

0.25–0.30	69–71	165	34.7
	72–74	240	
	75–77	150	
0.4	60–62	875	15.2
	63–65, 66–68	80–100	
0.5	63–65	29	53.9

Source: Chen et al. (2009)

2.1.6.3 Cross-tolerance to other stressors

Bacteria isolated from low-moisture food products have been shown to display a higher resistance to lethal conditions. It has been verified that some pathogenic bacteria, of which *Salmonella* spp. and *Escherichia coli*, become more resistant to disinfectants usually used in food production facilities (Kieboom et al., 2006; Stackhouse et al., 2012). Stackhouse et al. (2012) also found that filamentous cells presented an enhanced resistance when exposed to pH as low as 2.0 between 5 and 10 min. Gruzdev et al. (2011) studied the effects of desiccation on the tolerance of *Salmonella enterica* serotype Typhimurium to multiple stresses. The results showed that the desiccated cells became more resistant than non-desiccated cells to many stressors, such as ethanol, sodium hypochlorite, didecyl dimethyl ammonium chloride, hydrogen peroxide, NaCl, bile salts, dry heat, and UV irradiation. These results indicate the limitations of the use of these chemicals and treatments to control the spread of *Salmonella* in low-moisture food production environments.

2.1.7 Sources and risk factors for contamination by *Salmonella* in low-moisture products

Epidemiological and environmental investigations on outbreaks involving *Salmonella* in low-water-activity food products determined that cross-contamination is the major cause of these issues (Centers for Diseases Control and Prevention, 1993, 1998, 2007). Cross-contamination becomes a more crucial food safety issue when it occurs in ready-to-eat (RTE) foods, where there is no further lethal steps to inactivate the pathogenic bacteria. The results of an investigation led by the World Health Organization indicated that a great percentage of foodborne outbreaks in Europe were linked to cross-contamination. The main contributing factors were identified as insufficient hygiene (1.6%), cross-contamination (3.6%), processing or storage in inadequate rooms (4.2%), contaminated equipment (5.7%), and contamination by personnel (9.2%) (Reij et al., 2004). Therefore, the best way to minimize *Salmonella* occurrences in low-moisture foods is to control the risk factors that lead to cross-contamination.

The following risk factors associated with cross-contamination have been identified as the most frequent causes during outbreak investigations: poor sanitation practices, substandard facility and equipment design, improper maintenance, poor operational practices and good manufacturing practices (GMPs), inadequate ingredient control (Podolak et al., 2010).

Salmonella can persist in dry conditions on surfaces for long periods of time, which enhances the ability of the bacteria to be transferred to food during poor sanitation practices (Podolak et al., 2010). Kusumaningrum et al. (2003) suggested that *Salmonella* Enteritidis can survive for extended period of time on dry stainless steel surfaces, and

remain a threat for a long time. The author found that *Salmonella* Enteritidis could be transferred from dry stainless steel surfaces to foods, with transfer rates of 20 to 100% (Kusumaningrum et al., 2003). In 1998, Centers for Diseases Control and Prevention investigated an outbreak of *Salmonella* Agona linked to toasted oat cereal, which led to the conclusion that the unsanitary condition of the equipment, poor employee practices, and poor control of the vitamin spray mixing, and holding process were the most important risk factors associated with that outbreak (Breuer, 1999). Several other studies suggested that dust is an important environmental vector contributing to the spread of *Salmonella* in food production facilities (Craven et al., 1975; Morita et al., 2006).

Cross-contamination in low-moisture food production facilities can also be caused by poor sanitary design, and improper equipment installation and maintenance. An international outbreak of *Salmonella* Eastbourne, where 200 people were affected by contaminated chocolates produced at a Canadian factory, was partly caused by inadequate separation between clean and unclean zones (Craven et al., 1975). Among other flaws in production facility design, flooring materials (rough concrete), leaking pipe, leaky roof, faulty sprinklers were pointed out as causative risk factor for *Salmonella* propagation (Craven et al., 1975; Morita et al., 2006).

Poor choice and control of raw materials and ingredients constitute an important hazard for food contamination, since food ingredients that do not undergo killing steps can carry significant load of pathogens to the finished products. For instance, paprika-powdered potato chips confectioned with paprika powder contaminated with multiple serovars of *Salmonella* was reported to cause an estimated 1,000 cases of salmonellosis (Lehmacher et al., 1995). The report of an investigation led by FDA in 2007 following a *Salmonella*

Wandsworth outbreak identified the seasoning mix used in the snack as the possible culprit. As a result, FDA issued a report where it recommended consumers not to consume any snack food that contained that seasoning mix (U.S. Food and Drug Administration, 2007).

2.1.8 Control of *Salmonella* in low-moisture food production

Salmonella has been implicated in many outbreaks linked to low-moisture food products. Therefore it is crucial that the food industry develop strategies to cope with this issue. Contamination of food with low water activity occurs more often when there is not a killing step in the production process, or when contamination happens after the inactivation step. To mitigate the risks of Salmonella contamination in low-moisture food production plants, the Grocery Manufacturers Association (GMA) has developed guidance with seven elements to control the propagation of *Salmonella*:

- Preventing ingress or spread of *Salmonella* in the facility
- Controlling raw materials and ingredients
- Adhering to stringent hygiene practices in the Primary *Salmonella* Control Area
- Following hygienic design principles
- Preventing growth in the facility by control of moisture
- Validate control measures to inactivate Salmonella
- Establish procedures for verification of Salmonella controls and corrective actions.

The prevention of *Salmonella* entry and spread in the food facility relies primarily on a good facility maintenance, hygiene and pest control. Good facility maintenance can be achieved by ensuring the integrity and design, such as the absence of leak from roof,

crevices in machinery, walls and flooring, adequate separation of pre- and post-processing areas. *Salmonella* propagation can also be avoided by providing the personnel with training in good manufacturing process (GMP), and also making them aware of the negative consequences that a non-adherence to the established guidelines can have on the public health (Beuchat et al., 2013). Another efficient approach to limit *Salmonella* spread is to apply further kill step to control the microbiological quality of the ingredients (for example, spices, raw cocoa beans, raw nuts, raw peanuts, flour and cereal grains) used in the manufacture of the products. For instance, Sperber et al. (2007) reported that the incidence of Salmonella in wheat flour ranged from 0.14% to 1.32%; Pafumi (1986) found *Salmonella* in 1.5% to 8.2% of untreated spice samples; Sagoo et al. (2009) reported the presence of *Salmonella* in 1.5% of production samples and 1.1% of retail samples of dried spices and herbs in the UK.

2.2 Wheat flour

2.2.1 Microbiological safety of wheat flour

Wheat flour is generally considered a microbiologically safe product because of its low water activity (ICMSF, 1998). However, recent outbreaks implicating wheat flour have forced the food industry to take another look at the safety of this food product. As wheat is an agricultural product, it is exposed to diverse microbiological threats, including pathogens. Those pathogens can pass through the whole production chain, from the wheat harvest to the milling process, and finally end up being in the flour-based products, if the flour is not properly treated. Wheat contains a very diverse microflora coming from different sources such as dust, water, plants, insects, soil, fertilizers, and animal feces.

That microflora is mainly consisted of bacteria from the families of *Micrococcaceae*, *Pseudomonadaceae*, *Enterobacteriaceae,Lactobacillaceae* and *Bacillaceae;* yeasts, and molds from the genera of *Alternaria*, *Cladosporium*, *Fusarium*, *Helminthosporium*, *Aspergillus*, *Penicillium*, and *Eurotium (*Lacab et al., 2006*)*. The pathogenic microorganisms mostly encountered in wheat grains can be *Bacillus cereus*, *Clostridium botulinum*, *Clostridium perfringens*, *Escherichia coli*, *Salmonella*, *Staphylococcus aureus;* mycotoxin-producing molds (Berghofer, 2003; Eyles, 1989; Richter et al., 1993). Though low-water-activity environments are unfavorable to pathogens, they can remain in a latent state under such conditions for extended periods of times, and emerge from dormancy when the conditions become favorable to their growth, such as in batter or mixes, and thus cause diseases (Eglezos, 2010). Species of enteric bacteria that normally live on dead organic matter may also be present in grain and milled products, and subsequently in wheat flour, which can be a sign of unhygienic processing or handling.

2.2.2 Foodborne illness outbreaks involving flour

One of the causes of foodborne illness outbreaks involving wheat flour is the fact that many consumers do not follow the directions to bake and cook flour-based products, and some of them even eat those products (refrigerated cookie or biscuit dough, frozen pizzas or pies) without completely cooking them (ConAgra Mills, 2001). In 2008, flour from retail shelf in New Zealand was suspected in an outbreak of *Salmonella* causing sixty-six (66) cases of illness. Although there was no conclusive evidence of the implication of flour in that outbreak, result of the investigation suggested that the victims of the outbreak seemed to have eaten uncooked flour in homemade play dough, and raw cake. In 2009, an investigation of a multistate outbreak of *E. coli* O157 infections identified

ready-to-bake commercial prepackaged cookie dough as a novel vehicle for foodborne transmission of STEC to humans. This outbreak highlighted the health risks associated with the consumption of unbaked products, and the FDA recommended that cookie dough manufacturers use heat-treated flour in the production of such products (Neil et al., 2009). Following a *Salmonella* outbreak involving raw flour, McCallum et al. (2013) investigated the association between *Salmonella* Typhimurium phage type 42 (STM42) and the consumption of raw flour. *Salmonella* Typhimurium phage type 42 was recovered from flour taken from unopened packs purchased from retail stores and packs from three batches of recalled product (McCallum et al., 2013). A US study using 4,796 flour samples from various wheat types found *E.coli* in 12.8% and *Salmonella* in 1.32% of the samples (Richter et al., 1993).

Other pathogenic bacteria can be found in wheat flour, and cause diseases. Frequent flour contamination by *Bacillus cereus* can occur during or after processing. Laurence et al. (2011) conducted an investigation about two outbreaks involving *Bacillus cereus* emetic strains, and found out cereulide production and growth in penne pasta at 4, 8 and 25ºC during seven day storage (Laurence et al., 2011). Another lethal intoxication case involving *Bacillus cereus* occurred in Brussels after consumption of leftovers of spaghetti with tomato sauce. Laboratory analysis of the meal indicated that the bacteria was present in the pasta, and was absent in the tomato sauce (María et al., 2011).

The safety of wheat flour can be affected not only by pathogenic bacteria, but also by fungi producing mycotoxin which not only have detrimental effects on the quality of flour products, but also can cause illnesses in humans (Hussein and Brasel, 2001). The most significant mycotoxins in wheat grains are deoxynivalenol (DON) produced pre-

harvest by *Fusarium graminearum*, zearalenone (ZEA) produced post-harvest by *Fusarium culmorum*, and ochratoxin (OTA) produced post-harvest by *Penicillium verrucosum* and *Aspergillus ochraceus* (Magan et al., 2010). Research has shown that those mycotoxins have detrimental effects on human and animal health. Zearalenone has immunotoxic effect characterized by inhibition of T and B lymphocyte proliferation, and the apoptosis of immune cells in different organs, while deoxynivalenol, also called vomitoxin, possesses gastrointestinal toxicity characterized by vomiting, acute temporary nausea, diarrhea, abdominal pain, headache, dizziness, and fever (Ren et al., 2014; Kazemi et al., 2015).

The US Food and Drug Administration (FDA) has recommended that deoxynivalenol levels in wheat-based foods and feeds should not be higher than 1,000 μg/kg in finished human foods, 10,000 μg/kg in poultry and ruminant feed, and 5,000 μg/kg in other animal feeds (*http://www.cfsan.fda.gov/_dms/ graingui.html*). Ochratoxin A (OTA) is a potent nephrotoxic mycotoxin linked to kidney problems in both livestock and human populations. A study carried out in Spain confirmed the potential presence of OTA produced by *Penicillium verrucosum* in retail wheat flours from the Spanish market (Cabañas et al., 2008).

2.3. *Enterococcus faecium*

2.3.1 General characteristics

Enterococcus faecium is a Gram-positive bacterium belonging to the genus of Enterococcus. Enterococci are members of the lactic acid bacteria (LAB), and are catalase negative, and produce lactic acid from the fermentation of carbohydrates. They

are spherical cells that can occur in pairs or chains, and the colonies they form are 1-2 mm in length and appear wet (Health Protection Agency, 2007). *Enterococcus faecium* is a commensal organism that normally lives in mammalian gastrointestinal tract, but can also be found in the oral cavity and vaginal tract (Huycke et al., 1998). The bacterium can live for extended period of time in various environments such as soil, sewage, and inside hospitals on a variety of surface (Van Wamel et al., 2007). *Enterococcus faecium* is a facultative anaerobic bacterium capable of cellular respiration in both oxygen-rich and oxygen-poor environments (Hancock et al., 2000). It can survive in different harsh environments such extreme temperature (10-45°C), pH (4.5-10.0) and high sodium chloride concentrations (Fisher and Phillips, 2009).

2.3.2 Safety of Enterococcus faecium

Although certain strains of *Enterococcus faecium* are important in the food industry, such as in the production of fermented food products, including cheese and sausages, and are shown to be beneficial to animal and human health (Franz et al., 2011), some strains have been implicated in nosocomial infections (Arias and Murray, 2012). Among the infections caused by *Enterococcus faecium* are urinary infections, bacteremia, bacterial endocarditis, diverticulitis, and meningitis (Fisher and Phillips, 2009). These infections mostly occur in elderly patients with underlying disease, and immunocompromised patients treated with invasive devices, such as urethral or intravascular catheters (Teixeira et al., 2007). One of the most important concerns about this organism is its high level of antibiotic resistance in medical environments. Some strains are intrinsically resistant to β-lactams, aminoglycosides and vancomycin, and some others carry genetic elements responsible for resistance to chloramphenicol, tetracyclines, macrolides, lincosamides,

quinolones, and streptogramins (Teixeira, et al., 2007). The major concern is the emergence of vancomycin-resistant enterococci (VRE). Because of their importance, many discussions have focused on whether these strains can cause foodborne diseases or not. Some researchers believe that VRE originate from the hospital environment, and spread in the community, while others believe they can originate from farm animals that constitute a reservoir, and be transmitted to hospital environments via contaminated meat (Devriese et al., 1996). An investigation conducted by Chadwick et al. (1996) reported that VRE were isolated from chicken, pork and beef samples from retail markets in the UK, and concluded that the gene responsible for vancomycin resistance could be transmitted to the community via the food chain (Chadwick et al., 1996). However, Klein et al. (1998) found that VRE isolates from minced beef and pork were different from clinical isolates (Klein et al., 1998).

2.3.3 Use of *Enterococcus faecium* ATTC 8459 as a surrogate in the food industry

A surrogate is defined as a non-pathogenic organism, or innocuous particle or substance used to study the fate of a pathogen in a specific environment. The two major reasons to use a surrogate are to ensure safety of the products and the workers, and the ability to easily cultivate the surrogate organism (Sinclair et al., 2012). Surrogate organisms can be used to predict inactivation characteristics of target pathogens, and to verify thermal process critical control points in the hazard analysis critical control point plan (Erdogan and Derrick, 2014). It is recommended that an organism used as a surrogate in food processing and plant facilities should be a non-pathogenic organism that behaves similarly to the target pathogen under the same conditions or reduction treatment (Liu and Schaffner, 2007).

Although some strains of *Enterococcus faecium* are associated with nosocomial infections and antibiotic resistance, *Enterococcus faecium* ATTC 8459 has been shown to lack antibiotic resistance genes, and be sensitive to antibiotics. Comparison with the clinical strains showed that this strain is more resistant to low pH (2.4), high temperatures (60°C) and high alcohol concentration (8% ethanol) (Kopit et al., 2014). Previously designated *Micrococcus freudenreichii* ATCC 8459 (Bergan et al., 1970), *Pediococcus* sp. NRRL B-2354, *E. faecium* NRRL B-2354, *Enterococcus faecium* ATTC 8459 has been used in the food industry as a test organism for many decades. A study conducted by the Almond Board of California in which the genomic and characteristics of *Enterococcus faecium* ATTC 8459 was examined showed that this organism was safe and appropriate to be used in process validation (Kopit et al., 2014).

Enterococcus faecium ATTC 8459 has been widely used as a surrogate organism in the validation of thermal treatments because pathogens cannot be used in food production facilities. For instance, researchers have demonstrated the possibility of using *Enterococcus faecium* ATTC8459 as a surrogate for *Salmonella* Enteritidis PT 30 to test the efficacy of pasteurization of almonds using infrared and hot air heating (Yang et al., 2010). In another research study, *Enterococcus faecium* ATTC8459 was used as a substitute for *Escherichia coli* in apple cider during high-temperature, short-time pasteurization (Piyasena et al., 2003). Similarly, Borowski et al. (2009) found that this organism was a suitable substitute for five strains of *Salmonella* in beef jerky (Borowski et al., 2009). Jeong et al. (2011) found that *Enterococcus faecium* ATTC8459 could be used as an acceptable surrogate for *Salmonella* Enteritidis PT30 during moist-air heating of almonds (Jeong et al., 2010). Smith et al. (2014) also used *Enterococcus faecium* as a

surrogate for *Salmonella* to test the efficacy of oven, microwave, and combination of both on peanuts at 163 to 204°C, and the results showed a minimum of 3 log reduction of the surrogate (Smith et al., 2014).

Bianchini et al. (2013) studied the possibility of using *Enterococcus faecium* ATTC8459 as a surrogate for *Salmonella enterica* during extrusion of a balanced carbohydrate-protein meal. Results from this study revealed that the minimum temperature necessary to achieve a 5-log reduction of *Enterococcus faecium* ATTC8459 was higher than that needed for a 5-log reduction of *Salmonella,* 73.7ºC and 60.6ºC respectively (Bianchini et al., 2013). Ma et al. (2007) developed thermal surrogate microorganisms in ground beef for in-plant critical control point validation studies. They compared the rates of thermal inactivation of three bacteria, of which *Enterococcus faecium* ATTC8459, to those of *Listeria monocytogenes* or *Salmonella* Senftenberg 775W at four different temperatures, 58, 62, 65, and 68ºC. They found that *Enterococcus faecium* ATTC8459 had a decimal reduction 4.4 to 17.7 times greater than that of *L. monocytogenes*, and 3.6 to 14.6 times greater than that of *Salmonella* Senftenberg 775W (Ma et al., 2007). Enache et al (2015) compared the thermal resistance of *Salmonella* Tennessee and *Enterococcus faecium* at 85ºC inoculated onto talc powder with an adjusted water activity. The result of this study suggested that *Enterococcus faecium* had a greater heat resistance than *Salmonella* Tennessee. Very recently, Elizabeth et al. (2015) conducted some work on the validation of baking to control *Salmonella* serovars in hamburger bun manufacturing. They compared the thermal resistance of 3 *Salmonella* serotypes (Typhimurium, Newport, and Senftenberg), *Enterococcus faecium* ATCC 8459, and *Saccharomyces cerevisiae* for 9, 11, and 13 min at 280ºC oven temperature. They found that all the organisms had more

than 6 log reduction, and that *Enterococcus faecium* ATCC 8459 showed a greater thermal resistance than *Salmonella* and *Saccharomyces cerevisiae,* which makes *Enterococcus faecium* a suitable surrogate for *Salmonella* for validation of commercial baking operations (Elizabeth et al., 2015).

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Chapter 3

3- Comparative study of the D-values of *Salmonella* spp. and *Enterococcus faecium* in wheat flour

3.1 Abstract

Low-moisture foods contaminated with *Salmonella* spp. have been implicated in several foodborne illness outbreaks in the United States. The food industry has to incorporate preventive control in their process and validate thermal processes to assure food safety. The thermal destruction of *Enterococcus faecium* ATTC 8459 and *Salmonella* spp. was determined at 3 water activity levels (0.11, 0.18 and 0.33) at 80ºC, 85ºC and 90ºC. Aerobic plates counts petrifilms were used as non-selective medium for both bacteria, Enterobacteriaceae petrifilms were used as selective medium for *Salmonella* spp., and peptone water modified with sodium azide as a selective medium for *Enterococcus faecium*. Significant differences were observed for both organisms between the two media with higher D-values on the non-selective medium ($p < 0.05$). Lower D-values were observed for both organisms at higher water activities ($p < 0.05$). The D-values of *Salmonella* spp. were 112.87 min, 61.01 min and 32.36 min at 80ºC, 85ºC and 90ºC, respectively in wheat flour at water activity of 0.11. At water activity of 0.18, the D-values of *Salmonella* spp. were 59.05 min, 30.90 min and 18.78 min at 80ºC, 85ºC and 90ºC, respectively. The D-values of *Salmonella* spp. were 25.10 min, 13.25 min and 6.22 min at 80ºC, 85ºC and 90ºC, respectively at aw 0.33. Higher D-values were observed for *Enterococcus faecium* ATTC 8459 compared to *Salmonella* spp., at all the conditions of the study, with no significant differences ($p \geq 0.05$), except at the water activity of 0.18 at 85ºC ($p < 0.05$). The D-values of *Enterococcus faecium* ATTC 8459 were 117.97 min, 64.31 min and 38.24 min at

80ºC, 85ºC and 90ºC, respectively in wheat flour at water activity of 0.11. At water activity of 0.18, the D-values of *Enterococcus faecium* ATTC 8459 were 65.26 min, 50.49 min and 19.17 min at 80ºC, 85ºC and 90ºC, respectively. The D-values of *Enterococcus faecium* ATTC 8459 were 29.01 min, 15.09 min and 9.71 min at 80ºC, 85ºC and 90ºC, respectively at water activity of 0.33. The *z*-values were determined at each water activity, and there were no significant differences between the three (3) water activities ($p \geq 0.05$). *Salmonella* spp. and *Enterococcus faecium* had approximately the same *z*-values. The *z*-values of *Salmonella* spp. ranged from 16.53 to 18.50ºC, while the *z*-values of *Enterococcus faecium* ranged from 18.80 to 21.61ºC. These results suggest that *Enterococcus faecium* ATTC 8459 can be used as a surrogate for *Salmonella* spp. in wheat flour at the three levels of water activity used in the study.

3.2 Introduction

Salmonella spp. has been well recognized as a foodborne pathogen for its implication in many foodborne illness outbreaks. According to the U.S. Centers for Disease Control and Prevention, *Salmonella* spp. is one of the five top pathogens mostly involved in domestically acquired foodborne illnesses causing 19,336 cases of hospitalization and 378 deaths (Centers for Disease Control and Prevention, 2011). *Salmonella* spp. outbreaks are mostly associated with high water activity food products (0.94). However, many other food products with low water activity level have been implicated in various *Salmonella* outbreaks. While *Salmonella* spp. do not grow in low moisture environments, the organism can survive in such environments for prolonged periods because of its ability to adapt to extremely dried conditions (Janning et al., 1994; Hiramatsu 2005; Piyasena 2003).

Wheat flour has been considered a microbiologically safe product because of its low water activity. However, recent *Salmonella* outbreaks (McCallum et al., 2001; Neil et al., 2009) have forced the food industry to evaluate the safety of wheat flour and wheat flour-based food products (Neil et al., 2009). *Enterococcus faecium* ATTC 8459 has been used as a surrogate organism for *Salmonella* spp. for validation of thermal treatments (Kopit et al., 2014). For instance, *Enterococcus faecium* ATTC 8459 has been used as a surrogate for *Salmonella* Enteritidis PT 30 to test the efficacy of pasteurization of almonds using infrared and hot air heating (Yang et al., 2009). Borowski et al. (2009) stated that *Enterococcus faecium* ATTC 8459 was a surrogate for five strains of *Salmonella* in beef jerky (Borowski et al., 2009). Liu et al. (2015) also demonstrated that *Enterococcus faecium* NRRL B2354 was a valid surrogate for *Salmonella* spp. for

thermal treatment of wheat flour at different water activity levels (Liu et al., 2015). The objective of this study is to determine thermal destruction parameters of *Enterococcus faecium* ATTC 8459 and *Salmonella* spp. in wheat flour at different water activity levels (0.11, 0.18 and 0.33).

3.3. Materials and methods

Wheat flour

Soft wheat flour from was obtained from ConAgra Mills (ConAgra Mills, Safeguard, ready-to-eat flour), and was stored under refrigeration. Microbiological testing was conducted to determine the initial *Salmonella* population in the flour before use in the experiment. Five (5) grams of flours were taken from each lot, diluted in 25 mL of peptone water 0.1%, and plated Enterobacteriaceae petrifilms. The plates were then incubated for 24 h at 37ºC. The results showed no presence of *Salmonella* in the samples.

Bacterial strains and inoculation

Five *Salmonella* spp. serotypes/strains obtained from low-water activity products were used in the study: *Salmonella* Schwarzengrund, #479818 from extruded dry pet food; *Salmonella* Senftenberg, #447237 from soybean meal; *Salmonella* Agona, #442967 from puffed rice cereal; *Salmonella* Derby, #S260 from rice flour; and *Salmonella* Typhimurium, S544. The cultures were maintained as glycerol stocks at -80ºC.

The frozen stock culture of 5 different strains of *Salmonella* were streaked onto tryptic soy agar for isolation, and then incubated at 37ºC for 24 h. One colony was then streaked onto xylose lysine deoxycholate agar (XLD agar) for confirmation and incubated for 24 h. The bacteria were then transferred into TSB tubes and incubated for 24 h. One hundred

(100) µL from the TSB tubes was spread onto TSA plates to create lawns. The cells were harvested by adding 1.5 mL of 0.1% peptone water to each plate and loosening the lawn with a sterile spreader. The suspensions from each strain were combined in equal volume and vortexed for 30 s to ensure uniform distribution of the cells. The same procedures were followed to harvest *Enterococcus faecium* ATTC8459 cells with the exception that a single colony was streaked onto m-Enterococcus agar for confirmation.

Wheat flour (300 g) was spread on an aluminum foil inside a biosafety cabinet, and 30 mL of the inoculum was sprayed onto the flour. A food grade dye from McCORNICK&CO.,INC. was also used to color the inoculum in order to ensure that it was evenly sprayed on the flour. The inoculated sample was dried in the hood for 1 h, and then mixed in a blender (Waring Commercial, Serial N.5011S) for 10 min to ensure an even distribution of the inoculum in the flour and also to break the chumps.

Sample preparation

The flour was placed overnight in an air-tight oven (BINDER, Serial N.09-14813) containing a desiccant at 38°C to lower the water activity. The samples were then transferred to plastic bags, sealed, placed in Ziploc bags to maintain the low water activity, and then stored in a refrigerator. The water activity was adjusted to the target level 15-16 h prior to submitting the samples to the thermal treatment, and the inoculated samples were always used within one week. The water activity of the samples was measured using a water activity meter (Aqualab, Model Series 3 TE, Serial # 09048826B). When the water activity was lower than the target level, the sample flour was placed in the hood, and the water activity was measured until it reached the target level. The corresponding moisture contents (%) measured through hot air oven method at

105ºC for 24 h were 6.03±0.09, 7.18±0.15 and 10.22±0.09 for water activity of 0.11, 0.18 and 0.33, respectively.

Determination of the D-values

The samples were treated at three different temperatures for each water activity, 80ºC, 85ºC, and 90ºC. Prior to processing the samples, the water activity was measured, and samples with water activity ± 0.005 from the target were used for the experiment. Three (3) g of inoculated samples were place inside thermal-death-time (TDT) obtained from Millard Manufacturing Corp. The disks were then immersed in water baths (Model: A&C Series) at the different temperatures of the study. Come-up times were determined using 3 grams of non-inoculated flour sample inside a disk with a K-type thermocouple fixed at the center of the sample in the disk. The come-up times to reach the target temperatures were used as the time zero (60 seconds at 80ºC, 40 seconds at 85ºC, and 30 seconds at 90ºC). Samples with water activity of 0.11 were treated for 840 min at 80ºC; 480 min 85ºC and 300 min at 90ºC; samples with water activity of 0.18 for 440 min at 80ºC, 330 minutes at 85ºC, and 130 min at 90ºC; and samples with water activity of 0.33 for 200 min at 80ºC, 100 min at 85ºC, and 60 min at 90ºC. 10 disks were used for each temperature, and were removed from the water baths at equally spaced time intervals, and immediately immersed in cold water bath to stop the thermal treatment.

Recovery and enumeration

Wheat flour samples inoculated with *Salmonella* were placed in sterile stomacher filter bags with 10 mL of peptone water 0.1%, and homogenized for 1 min in a stomacher. 1 mL was then transferred to dilution test tubes to make serial dilutions. 1 mL from the dilution tubes was plated in duplicate on aerobic count plate petrifilm (3M™ Petrifilm™

Aerobic Count Plates), and on Enterobacteriaceae count plates (3M™ Petrifilm™ Enterobacteriaceae Count Plates). The petrifilm plates were then incubated for 24 h at 37ºC.

Wheat flour samples inoculated with *Enterococcus faecium* ATTC8459 were placed in sterile stomacher filter bags with 10 mL of peptone water 0.1%, and homogenized for 1 min in a stomacher. 1 mL of the suspension was transferred to two set of tubes: one set of tubes containing modified peptone water prepared by adding 0.4 g of sodium azide, and 4 g of dipotassium phosphate per liter of peptone water, and the other set containing regular peptone water (0.1%). Sodium azide is the selective agent to suppress the growth of Gram-negative organisms, and dipotassium phosphate acts as a buffer for the medium. 1 mL from the dilution tubes was plated in duplicate on aerobic count plate petrifilm (3M™ Petrifilm™ Aerobic Count Plates). The petrifilm plates were incubated for 48 h at 37ºC. The colonies of *Salmonella* spp. and *Enterococcus faecium* ATTC8459 were reported as log CFU/g.

Statistical design and analysis

The experimental design was a split-split-split plot design with:

1- Organisms as the plot factor in a completely randomized design,
2- Water activity as the split plot factor,
3- Temperatures as split split plot factor, and
4- Media as the split split split plot factor which was nested in organisms

Analysis of variance, with 5% level of significance, was used to test the effects of organisms, water activity, temperatures and media interaction on the D-values. Multiple comparisons were used to separate means when effects were significant.

Analysis of variance (ANOVA) was used to determine the effects of media, water activity and heat treatment on the D-values of *Salmonella* spp. and *Enterococcus faecium*. Multiple comparisons allowed evaluating the significance of the differences between the two organisms at the 3 water activity levels and the 3 temperatures on the non-selective medium APC. Statistical analysis was carried out using SAS 9.2, and all tests were conducted at the 5% level of significance.

3.4. Results and discussion

Effect of plating media on D-values. Both *Salmonella* spp. and *Enterococcus faecium* had higher D-values on the non-selective medium than on the selective media (Fig.3.1). This result was expected since the injured cells cannot grow on the selective media, while the non-selective medium allows not only the recovery of the injured cells, but also the growth of other bacteria present in the flour that might survive the heat treatment. There was a significant difference between the selective medium and the non-selective for *Enterococcus faecium* ($p \leq 0.05$), while the difference was not significant for *Salmonella* spp. ($p > 0.05$). When the enriched peptone water was used to prepare the serial dilutions for *Enterococcus faecium*, it was not possible to see the presence of the colonies on the petrifilm at the dilution zero, which made it difficult to determine the real D-values of the bacterium. Therefore, only the non-selective medium was used to compare the D-values of the two organisms.

Aw and D-values: The mean water activity levels were 0.11±0.005, 0.18±0.005 and 0.33±0.005. The D-values (min) for the water activity level of 0.11 at 80ºC ranged from 112.87 to 117.97, from 61.01 to 64.31, and from 32.36 to 38.24 at, 85ºC and 90ºC respectively (Table 3.1). *Enterococcus faecium* showed higher D-values than *Salmonella* spp. at all three temperatures with no significant differences ($p \geq 0.05$) (Fig. 3.2).

The D-values (min) for water activity 0.18 ranged from 59.05 to 65.26, from 30.90 to 50.49, and from 18.78 to 19.17 at 80ºC, 85ºC and 90ºC respectively (Table 3.1). There were no significant differences between *Salmonella* spp. and *Enterococcus faecium* at 80ºC and 90ºC ($p \geq 0.05$), and *Salmonella* spp. had a lower D-value than *Enterococcus faecium* at 85ºC ($p < 0.05$).

At the water activity of 0.33, the D-values (min) ranged from 25.10 to 29.01, from 13.25 to 15.09, and from 6.22 to 9.71 at 80ºC, 85ºC and 90ºC respectively (Table 3.1). *Enterococcus faecium* had a higher D-value than the *Salmonella* cocktail at 80ºC ($p < 0.05$), whereas there were no significant differences between the two organisms at 85^0C and 90^0C ($p \geq 0.05$). The D-values found in this study for *Salmonella* spp. for the water activity of 0.33 at 80ºC and 85ºC were much higher than those found by Smith (2014) at water activity of 0.31 at the same temperatures (Table 3.2). She reported that *Salmonella* Enteritidis PT30 had D-values 10.27 and 5.05 min at 80ºC and 85ºC respectively (Smith, 2014). Some laboratory testing was conducted in order to determine whether the differences were due to the strains used in the two studies. Results showed no difference between the single strain *Salmonella* Enteritidis PT30 and the cocktail at water activity of 0.33 at 80ºC and 85ºC (Table 3.2). These differences may be due to the methods used to adjust the water activities in the two studies.

Previous studies found that there were significant differences when comparing the heat resistance of *Salmonella* spp. and *Enterococcus faecium* in several food matrices, with the latter showing higher heat resistance (Bianchini et al, 2013; Borowski et al., 2009; Jeong et al., 2010; Smith et al., 2015). In this present study, in most of the cases, there were not significant differences between the D-values of the two organisms, although *Enterococcus faecium* always displayed higher D-values. Other studies have also found no significant differences between the two organisms. For instance, Rachon and Gibbs (2015) found in paprika powder (with a water activity of 0.45) that *Salmonella* Enteritidis PT30 had a D-value of 2.82 min at 80ºC, while *Enterococcus faecium* showed a D-value of 2.67 min. They also compared the D-value of the two organisms in rice flour (aw of 0.2) at 80ºC, and the results showed a D-value of 11.35 min for *Salmonella* Enteritidis PT30, and 11.79 min for *Enterococcus faecium.* Liu et al. (2015) also observed no significant differences between the D-values of *Salmonella* Enteritidis PT30 and *Enterococcus faecium* in wheat flour at water activity of 0.44 treated at 85ºC (2.11 min and 2.26 min, respectively).

Smith et al. (2015) determined the thermal resistance of *Salmonella* Enteritidis PT30 and *Enterococcus faecium* B2354 in wheat flour at different water activity levels and temperatures. Some of the results indicated minor differences between the D-values of the two organisms for the water activity of 0.44. The D-values (min) of *Enterococcus faecium* B2354 were 7.29 and 2.26 at 80ºC and 85ºC, respectively, while the D-values (min) of *Salmonella* Enteritidis PT30 were 5.51 and 2.11. The z-values ranged from 10.2ºC to 14.83ºC at the water activity of 0.44, while they ranged from 16.53ºC to 21.6ºC in this present study.

When comparing the D-values of both *Salmonella* spp. and *Enterococcus faecium* at the three water activity levels, the D-values were higher at lower water activities ($p<0.05$). The D-values (min) ranged from 32.36 to 117.97, 18.78 to 65.26 and 6.22 to 29.01 for the water activity 0.11, 0.18 and 0.33, respectively. These results are in accordance with previous research studies that suggested that heat resistance of microorganisms augmented in low and intermediate moisture foods (Mattick et al., 2000; Sumner et al., 1991; McDonough, F. E., and R. E. Hargrove, 1968; Archer, J., et al., 1998; Chick, 2011).

Z-values. The D-values of each organism was converted into log in order to determine the *z*-values at each water activity level. The *z*-value indicates that an increase of the temperature will cause 1 log change of the D-value. There was not a significant difference between the *z*-values at the three (3) water activity levels for both organisms ($p\geq0.05$). The mean *z*-values (ºC) for *Salmonella* spp. ranged from 16.53 to 18.50, whereas the *z*-values for *Enterococcus faecium* ranged from 18.80 to 21.61°C (Table 3.3). *Enterococcus faecium* had higher *z*-values than *Salmonella* spp. although the differences were not significant ($p\geq0.05$)), except at the water activity of 0.33 ($p<0.05$). Liu et al. (2015) observed higher *z*-values for *Salmonella* EPT30 than *Enterococcus faecium* in wheat flour at water activity of 0.44, 14.83°C and 10.12°C, respectively. However, a study conducted by Rachon and Gibbs (2015) have found no significant differences between the *z*-values of the two organisms in rice flour at water activity of 0.55: 11.80ºC for S*almonella* SPT30 and 12.80ºC for *Enterococcus faecium.*

3.5 Conclusion

Though low water activity environments are unfavorable to most pathogens, they can remain in a latent state under such conditions for extended periods of times, and emerge from dormancy when the conditions become favorable to their growth, such as in batter or mixes, and thus cause diseases (Eglezos, 2010).. Thermal resistance is one of the most important phenotypes associated with *Salmonella* survival in low-moisture environments. Previously exposed to moderately low-aw conditions, *Salmonella* has shown increased thermal resistance in subsequent heat treatment (Mattick et al., 2000). This study examined the potential use of *Enterococcus faecium* as a surrogate for *Salmonella* spp. by comparing their D-values in wheat flour at the water activity levels. Several studies have used *Enterococcus faecium* as a surrogate for pathogens in different liquid food products (Annous and Kozempel, 1998; Piyasena et al., 2003). *Enterococcus faecium* has also been used as a surrogate for *Salmonella* in low moisture food products (Almond Board of California, 2007; Borowski et al., 2009; Jeong et al., 2011), and in cereal flours such as rice flour, wheat (Rachon and Gibbs, 2015; Smith et al., 2015). In this study, *Enterococcus faecium* displayed higher D-values than *Salmonella* spp. at all the water activities tested, which corroborated the findings from the aforementioned studies that stated *Enterococcus faecium* was a suitable surrogate for *Salmonella* spp. in wheat flour.

3.6 References

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3.7- List of Tables

Table 3.1 D-values of *Salmonella* spp. and *Enterococcus faecium* on the non-selective media

Aw	Temp (ºC)	Organisms	Rep 1	Rep 2	rep 3	Average	Stdv
0.11	80	*E. faecium*	108.70	113.64	131.58	117.97	12.04
		Salmonella spp.	105.26	109.89	123.46	112.87	9.46
	85	*E. faecium*	62.50	60.98	69.44	64.31	4.51
		Salmonella spp.	56.18	60.61	66.23	61.01	5.04
	90	*E. faecium*	35.71	36.1	42.92	38.24	4.05
		Salmonella spp.	30.21	34.6	32.26	32.36	2.20
0.18	80	*E. faecium*	63.69	64.52	67.57	65.26	2.04
		Salmonella spp.	55.87	59.17	62.11	59.05	3.12
	85	*E. faecium*	49.75	48.54	53.19	50.49	2.41
		Salmonella spp.	28.74	30.3	33.67	30.90	2.52
	90	*E. faecium*	18.66	19.61	19.23	19.17	0.48
		Salmonella spp.	18.05	19.05	19.23	18.78	0.64
0.33	80	*E. faecium*	25.19	30.58	31.25	29.01	3.32
		Salmonella spp.	22.27	25.77	27.25	25.10	2.56
	85	*E. faecium*	14.71	14.93	15.63	15.09	0.48
		Salmonella spp.	13.7	13.00	13.05	13.25	0.39
	90	*E. faecium*	10.52	9.9	8.71	9.71	0.92
		Salmonella spp.	5.62	6.49	6.56	6.22	0.52

Table 3.2: D-values of the cocktail of 5 *Salmonella* strains compared to the D-values of the single strain used by Smith D. (2014) and in this study

Strains	Water activities	Temp. (°C)	D-values
Cocktail of 5 *Salmonella* strains	0.33	80	25.10 ±2.56
		85	13.25 ±0.39
Salmonella Enteritidis PT30 (Smith D., 2014)	0.31	80	10.27 ±0.65
		85	5.05 ± 0.18
Salmonella Enteritidis PT30 (This study)	0.33	80	19.31 ± 0.81
		85	12.94± 1.04

Table 3.3 *z*-values of *Salmonella* spp. and *Enterococcus faecium* on the non-selective media

Aw	Rep 1	Rep 2	Rep 3	Average	Stdv
			Enterococcus faecium		
0.11	20.7	20.08	20.53	20.44	0.32
0.18	18.76	19.34	18.32	18.81	0.51
0.33	26.39	20.41	18.03	21.61	4.31
			Salmonella spp.		
0.11	18.45	19.92	17.15	18.51	1.39
0.18	17.86	18.87	19.61	19.24	0.52
0.33	16.72	16.69	16.18	16.53	0.30

3.8 List of figures

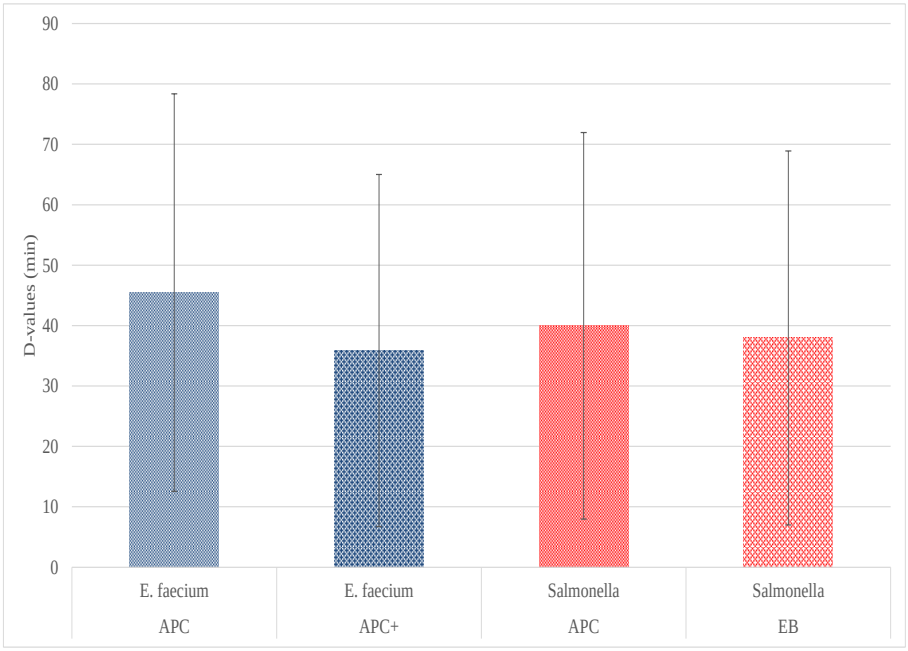


Figure 3.1 Effects of media (EB: selective medium for *Salmonella* spp., APC+: selective medium for *Enterococcus faecium*, and APC: non-selective medium

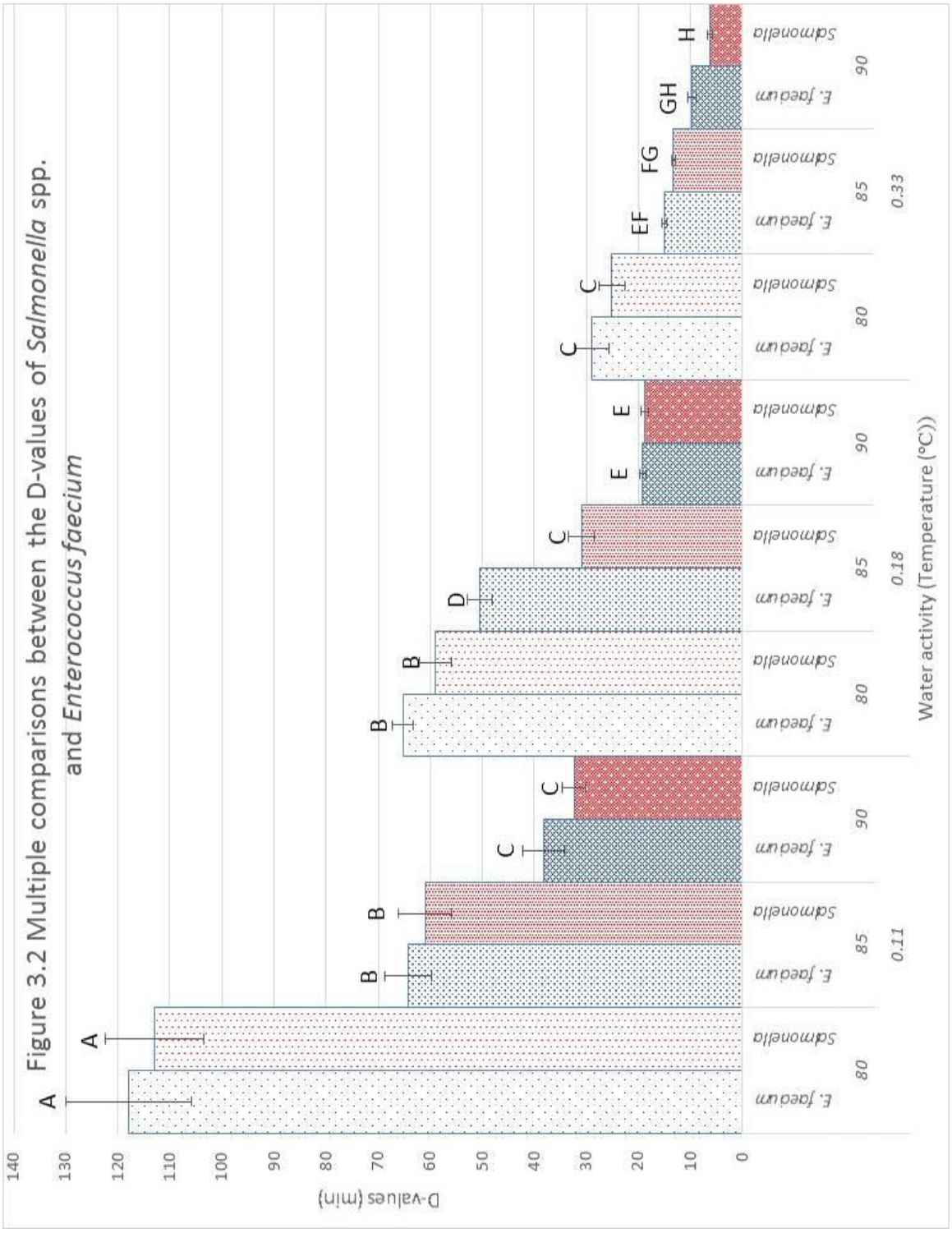


Figure 3.2 Multiple comparisons between the D-values of Salmonella spp. and Enterococcus faecium
D-values (min)
Water activity (Temperature (°C))
E. faecium
Salmonella
80
85
90
0.11
0.18
0.33
A
A
B
B
B
B
C
C
C
D
E
E
C
C
EF
FG
GH
H

CHAPTER 4

Recommendations for future research

This study evaluated the potential use of *Enterococcus faecium* ATTC 8459 as a surrogate for *Salmonella* spp. in wheat flour using small disks and water baths. Future research should be conducted to clarify some important aspects of this study:

1- In this study, small disks containing 3g of sample wheat flour were used to study the D-values of *Salmonella* spp. and *Enterococcus faecium* ATTC 8459. It would be very important to study these D-values with higher volume of samples using radio frequency as the heat treatment method.

2- There are conflicting data regarding the relationship between *z*-value and water activity. In this study, there were no significant differences between the three water activity levels. In another study comparing the *z*-values of *Salmonella* spp. and *Enterococcus faecium* in wheat flour at water activity of 0.25, 0.44 and 0.65, the authors found the lowest z-values at the water activity of 0.44. Future research should be conducted to elucidate the relationship between water activity and *z*-value.

3- The D-values obtained for *Salmonella* spp. at the water activity of 0.33 were much higher than those found in the study conducted by Smith et al. (2014) at the water activity of 0.31. These differences were not due to the strains used in the two studies since the D-values obtained for both the cocktail of *Salmonella* spp. and the single strain of *Salmonella* EPT30 were approximately the same, following the same procedures used in this study to adjust the water activity.

Therefore, it would be important to study the effect of different methods to adjust the water activity on the heat resistance of *Salmonella*.

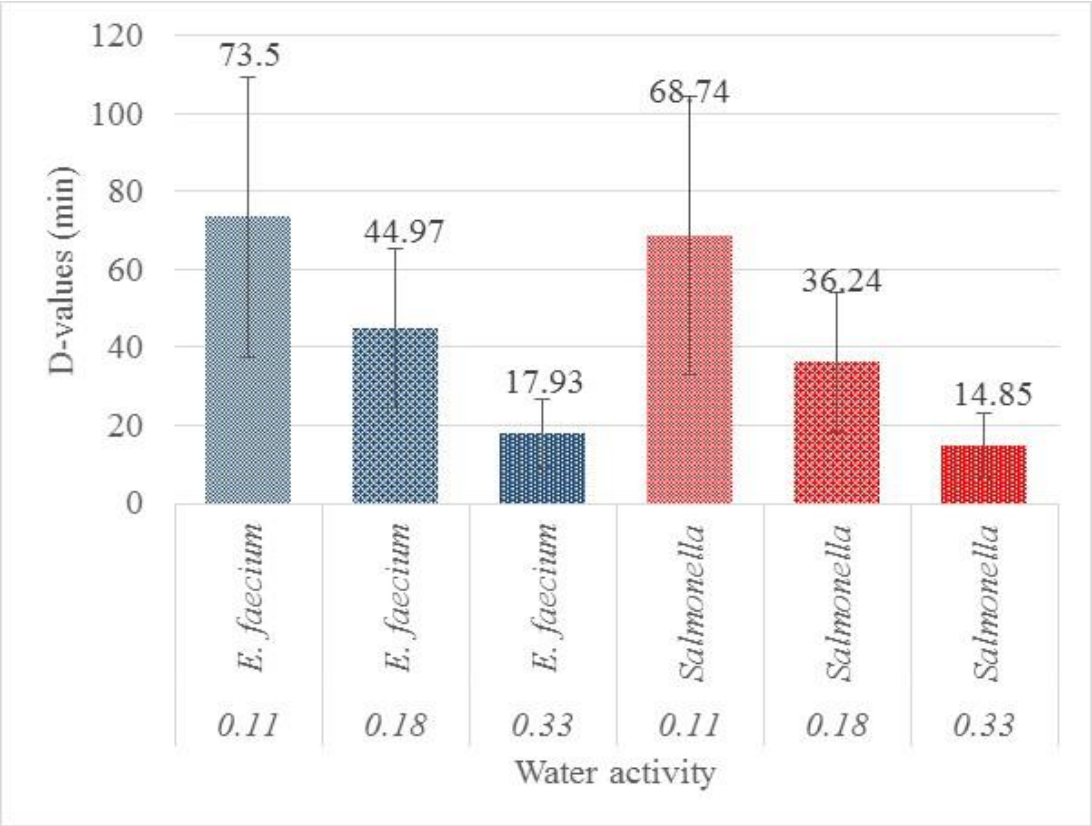
APPENDICES

A.1. D-values of *Salmonella* spp. and *Enterococcus faecium* on the selective media

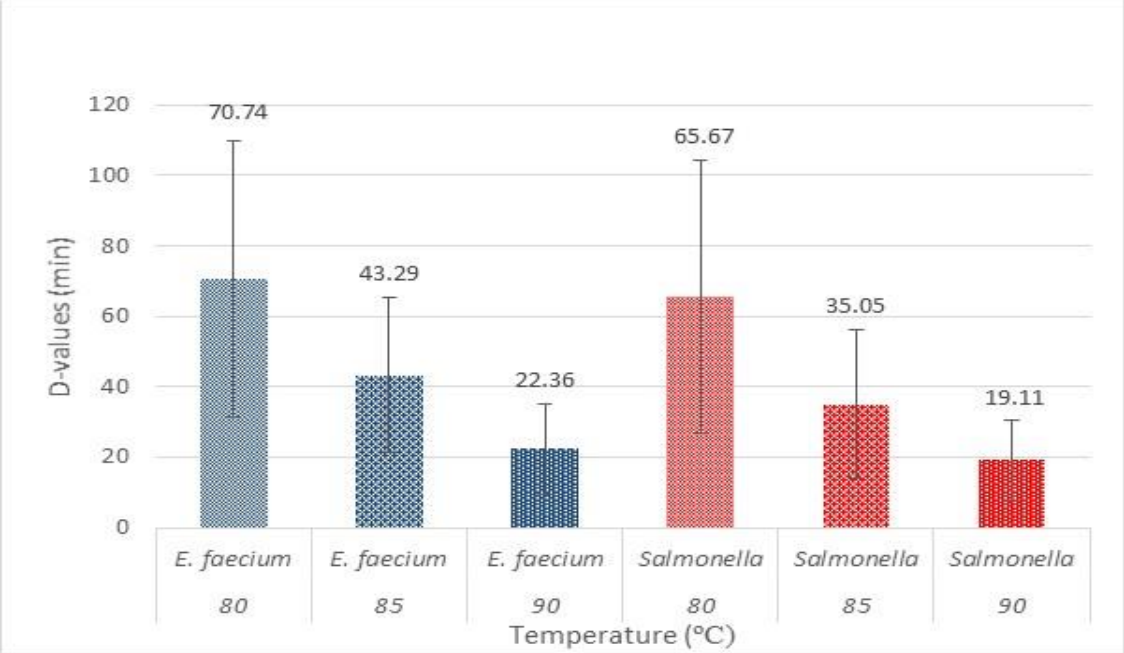
Aw	Temp (ºC)	Organisms	Rep 1	Rep 2	rep 3	Average	Stdv
0.11	80	*E. faecium*	96.15	104.17	108.70	103.01	6.35
		Salmonella spp.	105.26	109.89	114.94	110.03	4.84
	85	*E. faecium*	52.63	49.02	60.24	53.96	5.73
		Salmonella spp.	55.56	58.82	58.82	57.73	1.89
	90	*E. faecium*	22.62	24.88	25.13	24.21	1.38
		Salmonella spp.	28.82	31.95	31.25	30.67	1.64
0.18	80	*E. faecium*	49.02	51.02	56.5	52.18	3.87
		Salmonella spp.	54.64	54.35	56.5	55.16	1.17
	85	*E. faecium*	33.67	32.26	34.48	33.47	1.12
		Salmonella spp.	27.03	29.5	33.56	30.03	3.30
	90	*E. faecium*	16.21	15.38	16.29	15.96	0.50
		Salmonella spp.	16.98	18.18	17.86	17.67	0.62
0.33	80	*E. faecium*	25	19.08	23.15	22.41	3.03
		Salmonella spp.	21.05	23.2	23.81	22.69	1.45
	85	*E. faecium*	11.11	10.53	11.24	10.96	0.38
		Salmonella spp.	12.99	12.56	12.5	12.68	0.27
	90	*E. faecium*	6.9	6.29	6.49	6.56	0.31
		Salmonella spp.	5.56	6.02	6.06	5.88	0.28

A.2. *z*-values of *Salmonella* spp. and *Enterococcus faecium*

Aw	Rep 1	Rep 2	Rep 3	Average	Stdv
		Enterococcus faecium			
0.11	20.7	20.08	20.53	20.44	0.32
0.18	18.76	19.34	18.32	18.81	0.51
0.33	26.39	20.41	18.03	21.61	4.31
		Salmonella spp.			
0.11	18.45	19.92	17.15	18.51	1.39
0.18	17.86	18.87	19.61	19.24	0.52
0.33	16.72	16.69	16.18	16.53	0.30



A.3: Effects of water activities



A.4 Effects of temperatures

A.4: SAS Program

A.4.1 D-values codes

```
data D-values;
TITLE1 'Thermal treatment';
TITLE2 'Thermal treatment Pathogen-Surrogate Comparison';
input REP MED$ AW$ TEMPERATURE$ ORG$ DVALUES;
cards;
1       mENT 0.11    80C    ENT 96.15
1       mENT 0.11    85C    ENT 52.63
1       mENT 0.11    90C    ENT 22.62
1       EntAPC       0.11   80C    ENT 108.70
1       EntAPC       0.11   85C    ENT 62.50
1       EntAPC       0.11   90C    ENT 35.71
1       SalEB  0.11   80C    SAL 105.26
1       SalEB  0.11   85C    SAL 52.56
1       SalEB  0.11   90C    SAL 28.82
1  SalAPC  0.11   80C SAL 105.26
1  SalAPC  0.11   85C SAL 56.18
1  SalAPC  0.11   90C SAL 30.21
1       mENT 0.18    80C    ENT 49.02
1       mENT 0.18    85C    ENT 33.67
```

1	mENT	0.18	90C	ENT	16.21
1	EntAPC	0.18	80C	ENT	63.69
1	EntAPC	0.18	85C	ENT	49.75
1	EntAPC	0.18	90C	ENT	18.66
1	SalEB	0.18	80C	SAL	54.64
1	SalEB	0.18	85C	SAL	27.03
1	SalEB	0.18	90C	SAL	16.98
1	SalAPC	0.18	80C	SAL	55.87
1	SalAPC	0.18	85C	SAL	28.74
1	SalAPC	0.18	90C	SAL	18.05
1	mENT	0.33	80C	ENT	25
1	mENT	0.33	85C	ENT	11.11
1	mENT	0.33	90C	ENT	6.9
1	EntAPC	0.33	80C	ENT	25.19
1	EntAPC	0.33	85C	ENT	14.71
1	EntAPC	0.33	90C	ENT	10.52
1	SalEB	0.33	80C	SAL	21.05
1	SalEB	0.33	85C	SAL	12.99
1	SalEB	0.33	90C	SAL	5.56
1	SalAPC	0.33	80C	SAL	22.27
1	SalAPC	0.33	85C	SAL	13.7
1	SalAPC	0.33	90C	SAL	5.62
2	mENT	0.11	80C	ENT	104.17
2	mENT	0.11	85C	ENT	49.02
2	mENT	0.11	90C	ENT	24.88
2	EntAPC	0.11	80C	ENT	113.64
2	EntAPC	0.11	85C	ENT	60.98
2	EntAPC	0.11	90C	ENT	36.01
2	SalEB	0.11	80C	SAL	109.89
2	SalEB	0.11	85C	SAL	58.82
2	SalEB	0.11	90C	SAL	31.95
2	SalAPC	0.11	80C	SAL	109.89
2	SalAPC	0.11	85C	SAL	60.61
2	SalAPC	0.11	90C	SAL	34.6
2	mENT	0.18	80C	ENT	51.02
2	mENT	0.18	85C	ENT	32.26
2	mENT	0.18	90C	ENT	15.38
2	EntAPC	0.18	80C	ENT	64.52
2	EntAPC	0.18	85C	ENT	48.54
2	EntAPC	0.18	90C	ENT	19.61
2	SalEB	0.18	80C	SAL	54.35
2	SalEB	0.18	85C	SAL	29.5
2	SalEB	0.18	90C	SAL	18.18
2	SalAPC	0.18	80C	SAL	59.17
2	SalAPC	0.18	85C	SAL	30.3
2	SalAPC	0.18	90C	SAL	19.05

2 mENT 0.33 80C ENT 19.08
2 mENT 0.33 85C ENT 10.53
2 mENT 0.33 90C ENT 6.29
2 EntAPC 0.33 80C ENT 30.58
2 EntAPC 0.33 85C ENT 14.93
2 EntAPC 0.33 90C ENT 9.9
2 SalEB 0.33 80C SAL 23.2
2 SalEB 0.33 85C SAL 12.56
2 SalEB 0.33 90C SAL 6.02
2 SalAPC 0.33 80C SAL 25.77
2 SalAPC 0.33 85C SAL 13
2 SalAPC 0.33 90C SAL 6.49
3 mENT 0.11 80C ENT 108.7
3 mENT 0.11 85C ENT 60.24
3 mENT 0.11 90C ENT 25.13
3 EntAPC 0.11 80C ENT 131.58
3 EntAPC 0.11 85C ENT 69.44
3 EntAPC 0.11 90C ENT 42.92
3 SalEB 0.11 80C SAL 114.94
3 SalEB 0.11 85C SAL 58.82
3 SalEB 0.11 90C SAL 31.25
3 SalAPC 0.11 80C SAL 123.46
3 SalAPC 0.11 85C SAL 66.23
3 SalAPC 0.11 90C SAL 32.26
3 mENT 0.18 80C ENT 56.5
3 mENT 0.18 85C ENT 34.48
3 mENT 0.18 90C ENT 16.29
3 EntAPC 0.18 80C ENT 67.57
3 EntAPC 0.18 85C ENT 53.19
3 EntAPC 0.18 90C ENT 19.23
3 SalEB 0.18 80C SAL 56.5
3 SalEB 0.18 85C SAL 33.56
3 SalEB 0.18 90C SAL 17.86
3 SalAPC 0.18 80C SAL 62.11
3 SalAPC 0.18 85C SAL 33.67
3 SalAPC 0.18 90C SAL 19.23
3 mENT 0.33 80C ENT 23.15
3 mENT 0.33 85C ENT 11.24
3 mENT 0.33 90C ENT 6.49
3 EntAPC 0.33 80C ENT 31.25
3 EntAPC 0.33 85C ENT 15.63
3 EntAPC 0.33 90C ENT 8.71
3 SalEB 0.33 80C SAL 23.81
3 SalEB 0.33 85C SAL 12.5
3 SalEB 0.33 90C SAL 6.06
3 SalAPC 0.33 80C SAL 27.25

```
3   SalAPC  0.33    85C SAL 13.05
3   SalAPC  0.33    90C SAL 6.56
;
proc glm;
classes REP MED AW TEMPERATURE ORG;
model DVALUES = ORG|MED(ORG)|AW|TEMPERATURE rep(org) aw*rep(org)
temperature*rep(org aw);
random rep(org) aw*rep(org) temperature*rep(org aw) /test;
lsmeans ORG|MED(ORG)|AW|TEMPERATURE /;
means ORG|MED(ORG)|AW|TEMPERATURE /;
run;
```

A.4.2 *z*-value codes

```
data Didier1;
TITLE1 'Thermal treatment';
TITLE2 'Thermal treatment Pathogen-Surrogate Comparison';
input REP $ AW$ ORG$ ZVALUES;
cards;
1               0.11            ENT             20.7
1               0.11            SAL              18.45
1               0.18            ENT             18.76
1               0.18            SAL              17.86
1               0.33            ENT             26.39
1               0.33            SAL              16.72
2               0.11            ENT             20.08
2               0.11            SAL              19.92
2               0.18            ENT             19.34
2               0.18            SAL              18.87
2               0.33            ENT             20.41
2               0.33            SAL              16.69
3               0.11            ENT             20.53
3               0.11            SAL              17.15
3               0.18            ENT             18.32
3               0.18            SAL              19.61
3               0.33            ENT             18.03
3               0.33            SAL              16.18
;
proc glm;
classes ORG AW TEMPERATURE ;
model ZVALUES = AW|ORG rep(org);
random rep(org) /test;
lsmeans AW|ORG /;
means A|ORG /;
run;
```

```
proc mixed;
classes ORG AW rep  ;
model ZVALUES = AW|ORG;
random rep(org);
lsmeans AW*ORG / diff;
*means A|ORG /;
run;
```