RADIO FREQUENCY DIELECTRIC HEATING AND HYPERSPECTRAL IMAGING OF
COMMON FOODBORNE PATHOGENS

by

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Abstract

Intervention techniques to control foodborne pathogens, and rapid identification of pathogens in food are of vital importance to ensure food safety. Therefore, the first objective of this research was to study the efficacy of radio frequency dielectric heating (RFDH) against *C. sakazakii* and *Salmonella* spp. in nonfat dry milk (NDM) at 75, 80, 85, or 90°C. Using thermal-death-time (TDT) disks, D-values of *C. sakazakii* in high heat (HH)- and low heat (LH)-NDM were 24.86 and 23.0 min at 75°C, 13.75 and 7.52 min at 80°C, 8.0 and 6.03 min at 85°C, and 5.57 and 5.37 min at 90°C, respectively. D-values of *Salmonella* spp. in HH- and LH-NDM were 23.02 and 24.94 min at 75°C, 10.45 and 12.54 min at 80°C, 8.63 and 8.68 min at 85°C, and 5.82 and 4.55 min at 90°C, respectively. The predicted (TDT) and observed (RFDH) destruction of *C. sakazakii* and *Salmonella* spp. were in agreement, indicating that the organisms' behavior was similar regardless of the heating system (conventional *vs.* RFDH). However, RFDH can be used as a faster and more uniform heating method for NDM to achieve the target temperatures. The second objective of this research was to study if hyperspectral imaging can be used for the rapid identification and differentiation of various foodborne pathogens. Four strains of *C. sakazakii*, 5 strains of *Salmonella* spp., 8 strains of *E. coli*, and 1 strain each of *L. monocytogenes* and *S. aureus* were used in the study. Principal component analysis and *k*NN (*k*-nearest neighbor) were used to develop classification models, which were then validated using a cross-validation technique. Classification accuracy of various strains within genera including *C. sakazakii*, *Salmonella* spp. and *E. coli*, respectively was 100%; except within *C. sakazakii,* strain BAA-894, and within *E. coli,* strains O26, O45 and O121 had 66.67% accuracy. When all strains were studied together (irrespective of their genera) for the classification, only *C. sakazakii* P1, *E. coli*

O104, O111 and O145, *S.* Montevideo, and *L. monocytogenes* had 100% classification accuracy; whereas, *E. coli* O45 and *S.* Tennessee were not classified (classification accuracy of 0%).

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Dedication

I would like to dedicate this dissertation to my mother, Late Mrs. Martha Michael, who is not here to share the most prestigious and important part of my life, but will always be in my heart as a loving memory for the rest of my life.

Chapter 1 - Introduction

Novel and faster process intervention techniques to control foodborne pathogens, and rapid detection and identification of these pathogens, are vital to ensure food safety. Although traditional intervention processes (such as pasteurization, retorting, and low pH and water activity) are widely used throughout the food industry, researchers are continuously exploring new methods, which along with being faster, are still safe and efficient to control/inactivate pathogens in food. Many antimicrobial interventions are limited in their applications due to inherent food matrix influences (such as low-moisture foods) and resultant negative food quality impacts. Similarly, traditional cultural methods used for the detection and/or identification of foodborne pathogens are considered to be the gold standards; however, supply chain pressures dictate use of rapid methods, at least at the presumptive screening level, so that manufacturers can quickly screen component materials, meet manufacturing food safety objectives, and release safe food inventories into wholesale, retail distribution and food services.

Despite being one of the best food safety systems in the world, foodborne illnesses, hospitalizations and deaths occur frequently and are seen across a broad range of food products in the U.S. Bacterial contaminants result in most of the foodborne illnesses (Loir et al., 2003). *Salmonella* spp. contamination results in the most foodborne illnesses (1,027,561) and deaths (378) in the U.S. annually (CDC, 2012e). *Listeria monocytogenes* ranks second as a foodborne bacterium resulting in most of the deaths (255; CDC, 2012e). Due to the health risks associated with *L. monocytogenes*, the United States Department of Agriculture's Food Safety and Inspection Service (USDA-FSIS) maintains a "zero tolerance" policy in ready-to-eat foods (FSIS, 2012a).

As a result of the large and widely publicized "Jack in the Box" fast-food restaurant outbreak in 1993 associated with *Escherichia coli* O157:H7 (which resulted in 623 illnesses and deaths of 4 children), the FSIS declared *E. coli* O157:H7 as an adulterant in non-intact raw beef products in 1994 (FSIS, 2012b). Due to the immergence of new foodborne non-O157 Shiga toxin producing *E. coli* (STEC) strains associated with certain raw beef products, in June 2012, the FSIS also declared six non-O157 STEC serotypes (namely, O26, O45, O103, O111, O121 and O145) as adulterants in raw non-intact beef products (FSIS, 2012c). *Staphylococcus aureus* is not only one of the top five illness causing bacteria in the U.S., but also is responsible for many human diseases all over the world (Kadariya et al., 2014). On the other hand, opportunistic pathogens like *Cronobacter sakazakii* generally do not cause illnesses in healthy adults, but can cause severe illnesses, sometimes life threatening, in newborns and infants (Fiore et al., 2008).

Radio frequency dielectric heating (RFDH) is a fast and effective thermal intervention technique that can be implemented in certain food processing operations to inactivate foodborne pathogens. During RFDH, electric energy is first converted into radio waves, and these radio waves generate heat within the food (Rowley, 2001). At present, RFDH is used for post-baking drying of biscuits and bread, drying of herbs, spices and vegetables, and defrosting of frozen meat and fish (Rowley, 2001); however, validated applications of radio frequency dielectric heating as a thermal inactivation technique are rather scarce. Process specific RFDH protocols as antimicrobial intervention techniques may hold promise for low-moisture foods because of the better uniformity of heat distribution that can be achieved.

Hyperspectral imaging is immerging as a new technology in the field of rapid detection and identification of foodborne pathogens. Hyperspectral imaging combines imaging with spectroscopy by collecting both spatial and spectral information of a sample (Gowen et al.,

2007). Currently, hyperspectral imaging is being used in astronomy, agriculture, pharmaceutical and medicine. However, numerous food scientists are conducting research to develop protocols and models to study applications of hyperspectral imaging in the field of food safety and quality. Using hyperspectral imaging, researchers have studied peach and tomatoes ripeness, beef tenderness and pork quality (Polder et al., 2002; Lu and Peng, 2006; Qiao et al., 2007; Naganathan et al., 2008). Researchers have also studied the detection and classification of bacterial colonies (grown on cultural media) of various pathogens such STEC and *Campylobacter* spp. using hyperspectral imaging (Yoon et al., 2010, 2013a, b).

It is important to scientifically validate the thermal effects of RFDH against pathogens in dry food matrices, and investigate the efficacy of hyperspectral imaging as a technique of rapid detection of pathogens. Therefore, this research was divided into two major objectives. First, RFDH was evaluated as a method to shorten the processing time to achieve target end-point temperatures and validate that RFDH provides a similar microbial lethality compared to a conventional dry heating method. Nonfat dry milk is a widely utilized ingredient in many foods, including baby formula, and has been associated with significant *Salmonella* spp. and *C. sakazakii* outbreaks; therefore, a RFDH processing protocol for nonfat dry milk was investigated to determine its effectiveness for eliminating high levels of artificially contaminated *Salmonella* spp. and *C. sakazakii*.

The second major objective of this research was to utilize the CytoViva® hyperspectral imaging system (Auburn, AL) to generate pure culture hyperspectral graphs of important foodborne pathogens to create a hyperspectral library that might ultimately be utilized to rapidly and directly identify presumptive pathogens isolated from food samples. These hyperspectral graphs were then compared and grouped using principal component analysis (PCA) and *k*NN (*k*

nearest neighbor) modeling, and validated using a cross-validation technique. The sub-objective of this hyperspectral imaging research was to determine if culture age and previous exposure to an antimicrobial treatment (lauric arginate) affects the hyperspectral graphs of selected pathogens.

Chapter 2 - Literature Review

2.1 Foodborne Illnesses

Foodborne illness is defined as the development of a sickness or health problem, sometimes chronic or life threatening, due to consumption of contaminated food or beverage (CDC, 2012e). These contaminants present in the foods or beverages could be either biological pathogens (such as bacteria, fungi, viruses or parasites) or toxic chemicals (such as pesticides or exotoxins produced by pathogens). All humans are at risk of getting foodborne illness; however, infants, young children, pregnant women, elderly and immunocompromised people are at a greater risk of encountering serious health problems (FSIS, 2013a).

As foodborne illness occurs due to ingestion and passage of contaminated food through the gastrointestinal tract, the most common symptoms (but not limited to) include nausea, vomiting, diarrhea, abdominal cramps and fever (CDC, 2012e). Depending upon the type and amount of contaminant consumed, and consumer's age and health condition, onset of symptoms of foodborne illness may occur within minutes to weeks (FSIS, 2013a). People at greater risk of acquiring foodborne illnesses can get sick even by consuming contaminants at a very low levels; whereas, some healthy people could show no symptoms of illness but could still carry and even spread these contaminants.

While the safety of the U.S. food supply is among the best in the world; according to the Centers for Disease Control and Prevention (CDC), it is estimated that foodborne infections and poisonings result in 48 million illnesses (i.e. 1 in every 6 Americans), 128,000 hospitalizations and 3,000 deaths in the U.S. annually (CDC, 2012e). The North Carolina Department of Health and Human Services (NCDHHS, 2013) reported that every year over 1,000 foodborne disease outbreaks are recorded in the U.S. It is also estimated that foodborne disease associated health

care activities, productivity losses and decreased health quality costs the U.S. $50 to 80 billion annually (Kadariya et al., 2014). According to the CDC's "2011 Estimates for Foodborne Illness", the eight most common foodborne pathogens resulting in a majority of illnesses, hospitalizations and deaths include: Norovirus, non-typhoidal *Salmonella*, *Clostridium perfringens*, *Campylobacter* spp., *Staphylococcus aureus*, *Toxoplasma gondii*, *Escherichia coli* and *Listeria monocytogenes*. Table 2.1 presents the top five pathogens estimated to be responsible for foodborne illnesses, hospitalizations and deaths in the U.S. annually.

Table 2.1 Top five pathogens estimated to be responsible for domestically acquired foodborne illnesses, hospitalizations and deaths, respectively, in the U.S. annually*

Pathogen	Estimated number of		
	Illnesses	Hospitalizations	Deaths
Norovirus	5,461,731	14,663	149
Non-typhoidal *Salmonella*	1,027,561	19,336	378
Clostridium perfringens	965,958	-	-
Campylobacter spp.	845,024	8,463	76
Staphylococcus aureus	241,148	-	-
Toxoplasma gondii	-	4,428	327
Listeria monocytogenes	-	-	255
Escherichia coli O157	-	2,138	-

* Adapted from CDC (2012e)

The terms "food poisoning" and "food infection" are sometimes misused or used as synonyms; but in reality food poisoning and infection are two different types of foodborne

illness/disease. Further sub-classifications of foodborne illness are presented in Figure 2.1. More than 250 foodborne illnesses exist but most of these diseases are infections rather than poisoning, and two-thirds of foodborne outbreaks are caused by bacteria (Loir et al., 2003; CDC, 2012e).

Food poisoning results from the ingestion of a toxin present in a food. Food poisoning can occur because of naturally present toxins (such as poisonous mushrooms), exotoxins produced by microorganisms present in the food (such as mycotoxins and botulinum toxin), food contaminated with pesticides or heavy metals (such as lead and mercury), water used for irrigation or cleaning, and cross contamination (Frazier and Westhoff, 2005b; NCDHHS, 2013). Generally, symptoms of food poisoning occur soon after consuming contaminated food, and may result in sudden vomiting or diarrhea (NCDHHS, 2013).

On the other hand, food infection is a result of consuming food contaminated with viable pathogens (such as bacteria, viruses, fungi or parasites). In case of bacterial infection, cells enter the gastrointestinal tract, grow and release enterotoxins and metabolites during lysis (Frazier and Westhoff, 2005a). Compared to food poisoning, symptoms of food infections may take from days to weeks to appear (NCDHHS, 2013). Food infection can occur due to consumption of undercooked food, unpasteurized milk, food cross-contaminated during or after processing or cooking, temperature abused food during storage, and usage of contaminated water for cleaning.
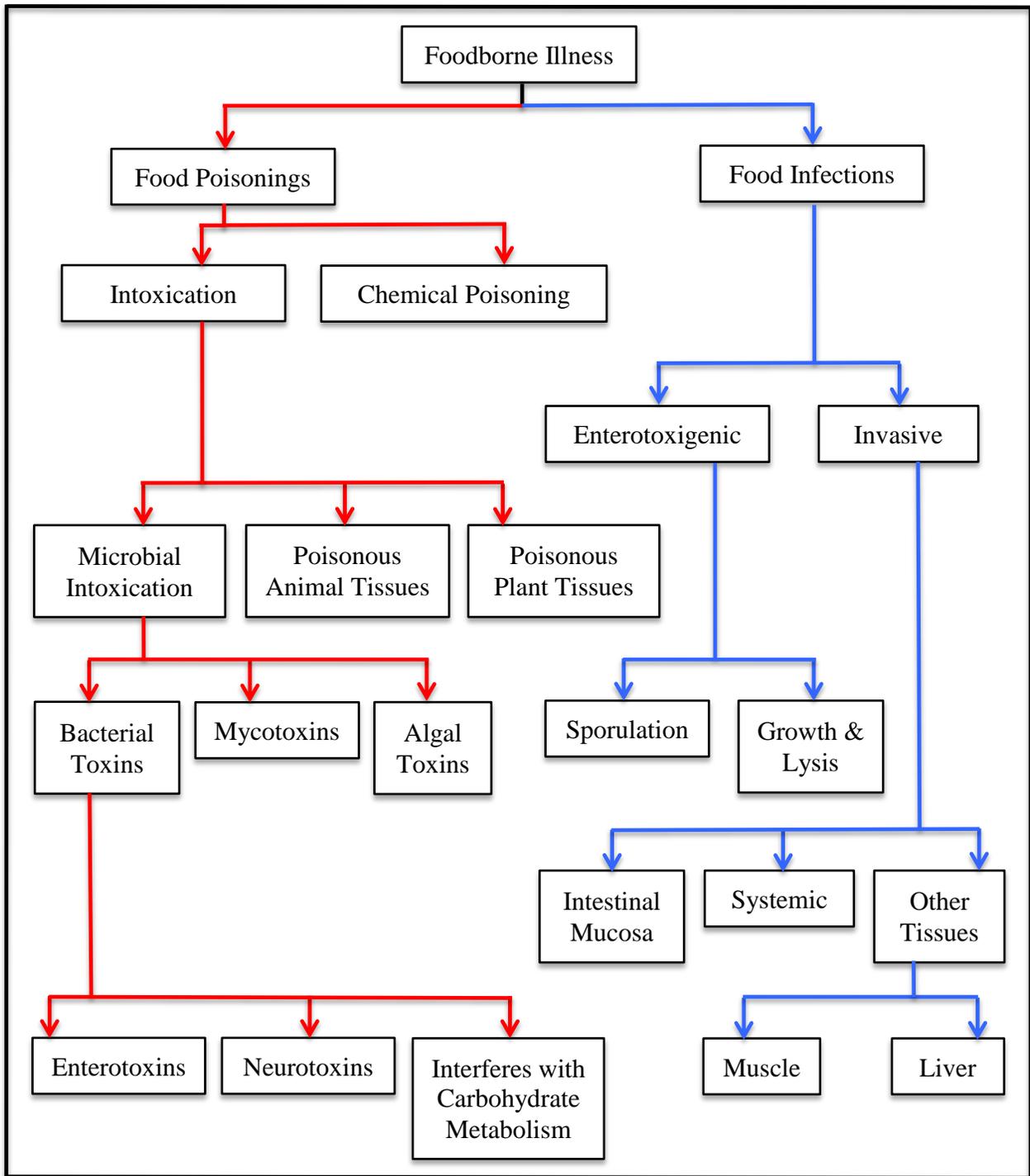
Figure 2.1 Classification of foodborne illness (adapted from Frazier and Westhoff, 2005a)

Despite great efforts by food manufacturers and government agencies, foodborne

outbreaks can occur locally, regionally or nationally. A foodborne outbreak is defined as an

incident when two or more individuals get a similar sickness after consuming a food or beverage from the same source (CDC, 2014b). In many cases, a foodborne outbreak can spread from person-to-person making people sick that have not consumed contaminated food. A local foodborne outbreak can occur when a group of people consumes a contaminated food from a common local source such as food served in restaurants, schools or social events. Whereas, an interstate outbreak can occur due to an incident where processed food (such as juices, ready-to-eat food or uncooked packaged meat) manufactured in a plant is distributed and consumed in multiple states. Some of the recent foodborne outbreaks reported in the U.S. are presented in Table 2.2.

2.2 Common Foodborne Bacteria

2.2.1 Introduction

Bacteria belonging to both Gram-positive and Gram-negative groups have been reported to cause either foodborne infections or poisonings in humans. Moreover, advancements in science and technology are helping to discover new bacteria, which could possibly be human pathogens. As a result of the extensive use of food safety intervention techniques such as chemical antimicrobials and heat, some bacteria can evolve to develop resistances and adapt to various food system stresses. The misuse of antibiotics in the past to treat sickness and enhance animal productivity has also led to develop of resistance against various antibiotics. These antibiotic resistant bacteria are of great food safety concern because traditional antibiotic treatments become less effective for infected patients, making treatment and recovery complicated (Kaźmierczak et al., 2014). Although the list of foodborne pathogenic bacteria is quite long, this section of literature review will discuss five common foodborne bacteria used in the various studies of this dissertation.

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Table 2.2 Recent foodborne illness outbreaks reported in the U.S.*

Timeline	Contaminant	Source	Location	Illnesses	Hospitalizations	Deaths
May-Aug, 2014	*Cyclospora cayetanensis*	Cilantro from Puebla, Mexico	Texas	126	0	0
Jan-July, 2014	*Salmonella* Braenderup	Almond & peanut butter by nSpired Natural Foods Inc., Oregon	4 States	4	1	0
Jan-July, 2014	*Salmonella* Newport, Hartford & Oranienburg	Organic chia powder	16 States	31	5	0
Apr-May, 2014	*Escherichia coli* O121	Raw clover sprouts by Evergreen Fresh Sprouts, Idaho	6 States	19	7	0
Apr-May, 2014	*Escherichia coli* O157:H7	Ground beef by Wolverine Packaging Company, Michigan	4 States	12	7	0
Aug-Dec, 2013	*Listeria monocytogenes*	Cheese products by Roos Foods, Delaware	2 States	8	7	1
Nov, 2014	*Salmonella* Heidelberg	Mechanically separated chicken by Tyson Foods, Inc., Tennessee	Tennessee	9	2	0
Nov, 2013-Jan, 2014	*Salmonella* Stanley	Raw cashew Cheese by The cultured Kitchen, California	3 States	17	3	0

* Data collected from CDC (2014a, c, d, e, f, g, h and i)

2.2.2 Cronobacter sakazakii

Cronobacter sakazakii is a Gram negative, facultative anaerobe, peritrichous, motile, non-spore former, oxidase negative, catalase positive and rod shaped (3 × 1 μm size) opportunistic pathogen (Iversen et al., 2007). *Cronobacter sakazakii* belongs to the family *Enterobacteriaceae*. *Cronobacter sakazakii* was previously referred to as 'yellow-pigmented *Enterobacter cloacae*' before it was classified as a new species and named as *Enterobacter sakazakii* in 1980 (Iversen et al., 2007). The name *E. sakazakii* was officially changed to *Cronobacter sakazakii* after Iversen et al. (2007) proposed (on the basis of DNA profiling of 210 *E. sakazakii* strains) that *C. sakazakii* should be separated from the *Enterobacter* genus and reclassified as a new species. Therefore, in 2008 *E. sakazakii* was reclassified into a separate genus called *Cronobacter* containing 7 species: *C. sakazakii*, *C. malonaticus*, *C. turicensis*, *C. dublinensis*, *C. muytjensii*, *C. condimenti* and *C. universalis* (Strydom et al., 2012; Yan et al., 2012). C*ronobacter sakazakii* was named after a Japanese microbiologist, Riichi Sakazakii, because of his research contributions related to the *Enterobacteriaceae* family (Fiore et al., 2008).

Cronobacter sakazakii can cause bloodstream and central nervous system infections (Lehner, 2010). Humans of all age groups are susceptible to *C. sakazakii* infections but infants <12 months of age are most prone. Among infants, neonates (<28 days olds), underweight infants, or infants born with immunodeficient or HIV positive mothers are at the highest risk of acquiring *C. sakazakii* infections (Fiore et al., 2008). *Cronobacter sakazakii* can cause life-threatening forms of meningitis, bacteraemia, septicemia, necrotizing enterocolitis and necrotizing meningoencephalitis in infants after ingestion (Iversen and Forsythe, 2003; Drudy et al., 2006). However, no healthy adult has ever been reported to suffer meningitis or any serious

illness from *C. sakazakii* infection (Drudy et al., 2006). Despite a low frequency of reported infections, the mortality rates of *C. sakazakii* infections have been reported to be as high as 50%; however, this number has decreased to <20% in recent years (FAO/WHO, 2004a). It is estimated that in the U.S., the annual rate of *C. sakazakii* infections in infants (<12 months old) is 1 per 100,000; whereas, in underweight neonates it is 8.7 per 100,000 (FAO/WHO, 2004b).

Pathogenicity studies of *C. sakazakii* have shown that the organism persists and replicates in macrophages, and shows moderate attachment and invasion of human endothelial cells (FAO/WHO, 2008). At present, the minimum infectious dose and virulence factors of *C. sakazakii* are unknown; however, it is believed that *C. sakazakii* colonizes in the intestines, brain and/or liver, and produces enterotoxins (Erickson and Kornacki, n.d.; Richardson et al., 2009). *Cronobacter sakazakii* infections are generally treated with the antibiotics ampicillin, gentamicin and/or chloramphenicol (Erickson and Kornacki, n.d.; Drudy et al., 2006). Symptoms of *C. sakazakii* infections in infants include irritability, poor appetite, jaundice, grunting respirations and unstable body temperature (Erickson and Kornacki, n.d.).

In recent years, many sporadic *C. sakazakii* outbreaks in infants have been associated with the consumption of rehydrated powdered infant formula (PIF). The exact cause of the contamination has never been confirmed but contaminated PIF was always suggested as the vehicle of transmission (Biering et al., 1989; FAO/WHO, 2004a). The first two known cases of *C. sakazakii* neonatal infection resulting in death were reported in England in 1958 (Drudy et al., 2006; Weisbecker, 2009). Recent outbreaks related to *C. sakazakii* are presented in Table 2.11. *Cronobacter sakazakii* has been isolated from a wide range of environmental sources, including water, soil, fruits, vegetables, herbs, cereals, grains, rice, wheat, production facilities and

hospitals; however, PIF products have always been the food source related to *C. sakazakii* infections in neonates and infants (Fiore et al., 2008; Lehner, 2010).

Cronobacter sakazakii can grow at temperatures ranging from 5.5 to 45°C (with optimum growth temperature of 39.4°C) and pH ranging from 5 to 9. It can survive at water activity (a_w) as low as 0.2 (ESR, 2010). *Cronobacter sakazakii* can survive harsh conditions by forming capsules and biofilms; and production of yellow pigment in most of the strains protects *C. sakazakii* against ultra violet light and oxygen radicals (Lehner, 2010). *Cronobacter sakazakii* can survive spray-drying processes, thus, exposing *C. sakazakii* to sublethal heat-shock. Chang et al. (2010) reported that heat-shocked *C. sakazakii* BCRC 13988 [at 47°C for 15 min in tryptic soy broth (TSB)] had greater tolerance to 15% ethanol when exposed for 15 min (752× survival compared to control), greater tolerance to freezing when stored at -20°C for 7 days (322× survival compared to control), greater tolerance to dry stress when exposed to dry air at 37% humidity for 6 h (48× survival compared to compared), greater tolerance to osmotic stress when exposed to 75% sorbitol in TSB for 36 h (119× survival compared to control), and greater tolerance to low pH when exposed to pH 3.3 for 60 min (72× survival compared to control).

Some strains of *C. sakazakii* have been known to form biofilms on various surfaces [such as stainless steel (SS), glass and polyvinyl chloride (PVC)], which can result in cross contamination of food being processed in a manufacturing facility. *Cronobacter sakazakii* strains were able to form biofilms on silicon, latex and polycarbonate cuts obtained from infant feeding bottles; and capsulated strains formed thicker layers of biofilms compared to non-capsulated strains. Park and Kang (2014) studied the effect of modified atmosphere packaging (100% nitrogen or carbon dioxide) on the biofilm cells of a 3-strain cocktail of *C. sakazakii* (ATCC 12868, ATCC 29004 and FSM 30) grown on SS and PVC coupons. They were able to grow *C.*

sakazakii biofilm on SS and PVC coupons (up to 7.0 to 7.67 cfu/cm^2 levels) in 6 days; however, they concluded that during storage of 5, 10, 15 and 20 days, log reductions of *C. sakazakii* in modified atmosphere storage were ~1 to 2.5 log cfu/cm^2 greater compared to air storage. They also concluded that *C. sakazakii* biofilm on PVC was more resistant to sanitizers compared to SS.

2.2.3 Escherichia coli

Escherichia coli is the most extensively studied and researched organism in the history of microbiology. *Escherichia coli* is an important part of naturally occurring microflora in the intestines of humans and other warm-blooded animals. Numerous strains of *E. coli* present in intestines help mammals to digest food and absorb important nutrients (Hayhurst, 2004a). Most *E. coli* strains are harmless or even beneficial to health; however, some strains are pathogenic and can induce illnesses ranging from mild infections to life-threatening conditions. As *E. coli* is a natural habitant of the intestinal microflora of humans and animals, it can be easily found in the environment, water, food, milk and produce (Smith and Fratamico, 2005).

Escherichia coli is a member of the *Enterobacteriaceae* family characterized as Gram negative, non-spore forming, rod-shaped, chemoorganotrophic, peritrichous, motile and facultative anaerobic bacteria (Smith and Fratamico, 2005). *Escherichia coli* can grow at a temperature range from 7 to 45°C, with optimum temperature conditions of 35 to 42°C. *Escherichia coli* can grow at the pH range of 4 to 10, and a$_w$ ≥0.95 (Beauchamp and Sofos, 2010).

Escherichia coli can be categorized into several specific groups based on their virulence factors, pathogenicity mechanisms, clinical syndromes and distinct O:H serotypes. Isolates are commonly differentiated by serotyping three main surface antigens: 'O' (somatic), 'H' (flagella)

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and 'K' (capsule) antigens (Montville and Matthews, 2005e). Currently, 167 'O' antigens, 53 'H' antigens and 74 'K' antigens have been identified; however, only 'O' and 'H' antigens are determined when serotyping *E. coli* strains associated with diarrheal disease (Montville and Matthews, 2005e). The 'O' antigen identifies the serogroup of an *E. coli* strain; whereas, 'H' antigens identifies the serotype.

As summarized by Montville and Matthews (2005e), the various categories of diarrheagenic *E. coli* are briefly discussed as follows:

1. Enteropathogenic *Escherichia coli* (EPEC):

Humans are the major reservoir of EPEC. Enteropathogenic *E. coli* can cause severe diarrhea, especially in infants; and EPEC have been shown to induce lesions in the adhered cells. *Escherichia coli* O55, O86, O111ab, O119, O125ac, O126, O127, O128ab and O142 are the major EPEC 'O' serotypes associated with human illnesses.

2. Enterotoxigenic *Escherichia coli* (ETEC):

Like EPEC, humans are the primary reservoir of ETEC. Enterotoxigenic *E. coli* causes infantile diarrhea and is frequently associated with traveler's diarrhea. Enterotoxigenic *E. coli* attach and colonize small intestines using fimbriae, and produce heat-stable and -labile enterotoxins causing diarrhea. Major ETEC 'O' serotypes associated with illnesses are O6, O8, O15, O20, O25, O27, O63, O78, O85, O114, O128ac, O148, O159 and O167.

3. Enteroinvasive *Escherichia coli* (EIEC):

Enteroinvasive *E. coli* causes bloody (dysentery) and non-bloody diarrhea by invading and multiplying within intestinal epithelial cells (primarily in the colon), causing cell death. Humans are the major reservoir of EIEC, and 'O' serotypes generally associated with illnesses are

O28ac, O29, O112, O124, O136, O143, O144, O152, O164 and O167 (O124 being the most frequently encountered).

4. Diffusely Adhering *Escherichia coli* (DAEC):

Diffusely adhering *E. coli* are generally associated with mild, non-bloody diarrhea in children (older than infants). The risk of getting DAEC diarrhea increases in children from age 1 to 5 years (reasons are not yet understood). Diffusely adhering *E. coli* generally do not produce heat-stable or-labile enterotoxins, or higher amounts of Shiga toxins. Illnesses related to DAEC are mostly associated with O1, O2, O21 and O75 serogroups.

5. Enteroaggregative *Escherichia coli* (EAEC):

Enteroaggregative *E. coli* causes persistent diarrhea in infants and children. Enteroaggregative *E. coli* adheres to HEp-2 cells in a characteristic pattern that looks like a stack of bricks. Common 'O' stereotypes associated with EAEC infections include O3, O15, O44, O77, O86, O93, O111 and O127.

6. Enterohemorrhagic *Escherichia coli* (EHEC):

Although >200 serotypes of EHEC have been isolated from humans, only serotypes capable of inducing diarrhea are considered as true EHEC. Enterohemorrhagic *E. coli* produce Shiga toxins (also called Verotoxins; Stxs) similar to toxins-produced by *Shigella dysenteriae*; therefore, EHEC are also known as Shiga/Vero toxin producing *E. coli* (STEC or VTEC; CDC, 2012h). *Escherichia coli* O157:H7 is the most commonly encountered STEC and is the major cause of EHEC associated illnesses in the U.S. Other major non-O157:H7 STECs include O26:H11, O111:H8 and O157:NM.

Historically, the worst foodborne disease outbreak associated with *E. coli* O157:H7 took place from July through August 1996 in Sakai City, Japan. In total, 9,578 cases of *E. coli*

O157:H7 infection along with 11 deaths were reported (WHO, 1996). Most of infected patients were school children and teachers, or the family members of infected children. The source of the outbreak was never positively identified; however, lunches served in various schools of Sakai City were associated with the outbreak. Radish seeds and/or uncooked sprouts were considered as the primary suspects for the contamination (Anderson, 2011).

The incidence that drastically changed the perspective of U.S. government agencies, manufacturers and restaurants regarding *E. coli* contamination of foods was the "Jack in the Box" outbreak in 1993 (Benedict, 2013). The outbreak was related to undercooked hamburgers served in Jack in the Box fast-food restaurants. This outbreak resulted in 623 illnesses and the death of 4 children in Washington, Idaho, California and Nevada (Golan et al., 2004; Frame, 2013). As a result of this outbreak, in September 1994, the Food Safety and Inspection Service (FSIS) declared *E. coli* O157:H7 as an adulterant in raw non-intact beef products (FSIS, 2012b). Usually, chemicals or physical elements are considered as food adulterants; however, it was for the first time that the term "adulterant" was used for a microorganism (*E. coli* O157:H7) in the U.S. by the FSIS (Andrews, 2013). In continuing the efforts to minimize food safety risk due to *E. coli* O157:H7, FSIS required raw beef manufacturers to reassess their Hazardous and Critical Control Points (HACCP) plans considering *E. coli* O157:H7 to be a major microbial risk, and include intervention/control steps if required.

Several non-O157 Shiga toxigenic *E. coli* (STEC) have also surfaced as serious foodborne pathogens. Although there are several non-O157 STECs, which are capable of causing human illnesses, *E. coli* serotypes O26, O45, O103, O111, O121 and O145 (known as the "Big Six" non-O157 STECs) are responsible for 75 to 80% of illnesses attributed to non-O157 STEC (FSIS, 2012b). According to the CDC's estimate, STECs cause more than 265,000 illnesses,

3,600 hospitalizations and 30 deaths; whereas, non-O157 STECs are responsible for 36,700 illnesses, 1,100 hospitalizations and 30 deaths in the U.S. annually (Bottemiller, 2010; CDC, 2012h). The CDC also estimates that about a third of STEC illnesses in the U.S. are associated with non-O157 serotypes (Kaspar et al., 2010).

Among Big Six STECs, serotypes O26 and O111 are the most common non-O157 STECs isolated from specimens submitted to CDC (Wang et al., 2012a). In June 2012, Big Six STECs were also declared as adulterants in non-intact raw beef products and FSIS requires routine testing of non-intact beef products for their presence (along with *E. coli* O157:H7; FSIS, 2012c). Since 2006, in addition to beef-associated outbreaks, various foods have been associated with STEC-related illnesses including leafy greens, cookie dough, nuts, cheese and dry fermented meats (CDC, 2013f). Some of the recent STEC related outbreaks are presented in Table 2.3. In 2011, a very large foodborne outbreak in Germany traced back to fenugreek seed sprouts contaminated with a new pathogenic serotype, *E. coli* O104:H4, raised concerns about the emerging *E. coli* serotypes as foodborne pathogens; and should foods other than beef products be also regulated for *E. coli* (Shaw, 2012).

Table 2.3 Recent foodborne illness outbreaks associated with Shiga Toxin-Producing
Escherichia coli **reported in the U.S.***

Contaminant	Year	Source	Location	Illnesses	Deaths
E. coli O121	April-May, 2014	Clover sprouts	6 States	19	0
E. coli O157:H7	April-May, 2014	Ground beef	4 States	12	0
E. coli O157:H7	Oct.-Nov., 2013	Ready-to-eat salads	4 States	33	0
E. coli O121	Dec., 2012-April, 2013	Frozen food products	19 States	35	0
E. coli O157:H7	Oct.-Nov., 2012	Organic spinach	5 States	33	0
E. coli O145	April-June, 2012	Unidentified	9 States	18	1
E. coli O26	Dec., 2011-Jan., 2012	Clover sprouts	11 States	29	0

* Data collected from CDC (2012i, j, k; 2013g, h; 2014 n, o)

Shiga toxin producing *E. coli* are divided into the following 5 (**A** through **E**) seropathotypes (Kasper et al, 2010; FSIS, 2012b):

A: Serotypes commonly causing outbreaks and severe disease such as hemolytic uremic syndromes (HUS). This includes *E. coli* O157:H7 and O157:NM.

B: Serotypes occasionally involved in outbreaks but causing severe disease. This group consists of the following 13 STEC serotypes: O26:H11, O26:NM, O45:H2, O45:NM, O103:H2, O103:H11, O103:H25, O103:NM, O111:H8, O111:NM, O121:H19, O121:H7 and O145:NM.

C: Serotypes rarely associated with outbreaks and resulting in low incidences of human illnesses. This group includes STECs O91:H21, O104:H21 and O113:H21.

D: Serotypes involved in non-severe illnesses.

E: Serotypes not associated with human illnesses.

The incubation period for development of STEC-related illness can vary from 1 to 8 days; however, healthy adults infected by STECs generally recover within 4 to 10 days without any treatment (Smith and Fratamico, 2005). Patients with mild STEC infections are advised to not take antidiarrheal drugs in order to allow *E. coli* to escape intestines rather than staying trapped in the body, and drink lots of fluids (such as juices and energy drinks) to avoid dehydration (Hayhrust, 2004b). Symptoms of mild STEC infection include watery diarrhea, abdominal cramps, mild fever, headache and nausea. Generally, in healthy adults STEC infections are mild and self-limiting; but children and elderly (with underdeveloped or compromised immune system) can have life threatening severe STEC complications. During severe STEC infections medical assistance must be used as it can result in bloody diarrhea (hemorrhagic colitis), kidney failure, lysis of red blood cells, or a combination of these three manifestations (called hemolytic uremic syndrome) [Beauchamp and Sofos, 2010]. Although minimum infectious dose depends on the heath and age of the host, and type and amount of infecting serotype, cell numbers as low as <50 can cause illness (Tilden et al., 1996). Antibiotic treatments are generally not recommended during STEC infections as it is believed that this can increase stx production or release preformed stx in the body (Smith and Fratamico, 2005). However, other treatment options against severe STEC infections are being researched such as use of a synthetic analog of stx receptors, humanized stx neutralizing antibodies and bacteriophages.

Although there are variations in the virulence factors among pathogenic STECs, they all produce Shiga toxin 1 (stx1), Shiga toxin 2 (stx2), or both. It has been reported that production of

stx2 during *E. coli* infection can produce more severe illnesses (such hemolytic colitis or HUS) compared to the production of stx1 alone or both stxs (Ostroff et al., 1989). One subunit of stx attaches to the host cell and transfers stx into the cell while the other stx subunit interferes with protein synthesis in the cell resulting in inflammatory reactions. Virulent *E. coli* also contains a locus of enterocyte effacement (LEE) that attaches and produces effacing lesions on the intestinal tract of the host. Locus of enterocyte effacement also delivers virulence factors inside the host enterocytes through a type III secretion mechanism, and many of the virulent STECs are capable of producing enterohemolysin (Kaspar et al., 2010). An increased concern in the medical field and the food industry regarding STECs is the development of antibiotic resistance and transfer of virulent genes from more virulent STECs to non-virulent or less virulent STECs. The most common antibiotic resistances encountered in *E. coli* O157:H7 are against the commonly used antibiotics tetracycline, streptomycin and ampicillin. As bacteria continuously exchange their genetic materials (sometime even with bacteria belonging to different genera), it is highly likely that antibiotic resistant and extremely virulent STECs can transfer their characteristics to other *E. coli* (Smith and Fratamico, 2005; Kaspar et al., 2010).

  Escherichia coli O157:H7 and the Big Six STECs can also form biofilms on food processing and contact surfaces, posing a great food safety risk due to cross contamination by dispersing and/or shedding loosely bound bacterial cells into the product during processing (Fouladkhah et al., 2013). These pathogens can form biofilms on various surfaces (such as stainless steel and polystyrene) due to poor or incomplete sanitization practices in food processing facilities, and their biofilm formation is enhanced with increase in temperature and time (Wang et al., 2012a; Fouladkhah et al., 2013). *Escherichia coli* can form biofilms to survive against adverse conditions; moreover, bacterial cells in biofilms have greater resistance to

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various antimicrobial treatments such as antibiotics, heat and disinfectants (Őner and Őlmez, 2011; Wang et al., 2012b).

2.2.4 Listeria monocytogenes

Listeria monocytogenes is a Gram positive, non-spore forming, facultative anaerobic, catalase positive, oxidase negative, motile (through few peritrichous at temperatures <30°C) and rod-shaped foodborne pathogen (Paoli et al., 2005; Porto-Fett et al., 2010). *Listeria monocytogenes*, like other *Listeria* spp., produces acid from D-xylose, L-rhamnose, α-methyl-D-mannoside and D-mannitol; however, the characteristic of *L. monocytogenes* that separates it from other nonpathogenic species in the genus *Listeria* is the ability to lyse red blood cells through β-hemolysis (Montville and Matthews, 2005d; Porto-Fett et al., 2010). *Listeria monocytogenes* is ubiquitous and can be found in soil, water, animals (such as poultry and cattle), seafood, raw milk, dairy products manufactured using raw milk, and the environments of food processing facilities (Buchholz and Mascola, 2001; Food Safety, 2014). Moreover, *L. monocytogenes* can virtually be isolated from any damp place in the environment (Barry, 2007).

The genus *Listeria*, belonging to the family *Listeriaceae*, is further divided into six species: *L. monocytogenes, L. innocua, L. seeligeri, L. welshimeri, L. ivanovii* and *L. grayi*. Among these *Listeria* species, *L. monocytogenes* is a human pathogen; whereas, *L. ivanovii* is an animal pathogen (Montville and Mathews, 2005d; Porto-Fett, 2010). *Listeria monocytogenes* consists of 13 serotypes including: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e and 7 (Porto-Fett, 2010). Among these serotypes, 1/2a, 1/2b and 4b account for 95% of isolates obtained from the patients suffering from *L. monocytogenes* infections (Farber and Peterkin, 1991; Buchholz and Mascola, 2001).

Infection caused by consuming food contaminated with *L. monocytogenes* is called

listeriosis. Although anyone can become infected with *L. monocytogenes*, newborns, infants,

elderly, pregnant women, and immunocompromised people are at the greatest risk of acquiring

listeriosis (CDC, 2014l). Symptoms of non-invasive listeriosis can vary with the infected

individual: a) pregnant women (perinatal): fever, fatigue and body aches; b): others (non-

perinatal): headache, stiff neck, confusion, convulsions, fever and muscle aches (Buchholz and

Mascola, 2001; CDC, 2114l). Invasive listeriosis occurs due to intracellular growth of *L.*

monocytogenes. The pathogen enters into mammalian cells through phagocytosis, and then is

released from the membrane bound vacuole, where it multiplies. *Listeria monocytogenes* uses an

actin polymerization process for intracellular and cell-to-cell movement. It can spread to a vast

variety of host tissues, with the liver being the most dominant site of infection (Gandhi and

Chikindas, 2007). The cellular mechanism of *L. monocytogenes* pathogenicity involves five

steps, which are summarized in Table 2.4. Severe cases of invasive listeriosis can lead to

meningitis, septicemia and other central nervous system infections (Gandhi and Chikindas,

2007).

Listeriosis during pregnancy can be very dangerous because it often results in

miscarriage, stillbirth, premature delivery or life-threatening listeriosis infection to the baby

(CDC, 2014l). Reduction in cell-induced immunity during pregnancy to permit the presence and

growth of the fetus (which the mother's body considers a foreign genetic material) increases the

susceptibility for listeriosis (Buchholz and Mascola, 2001). About 14% of listeriosis cases occurs

in pregnant women and 58% of listeriosis cases occur in elderly individuals 65 years or older.

Moreover, pregnant women and elderly are 10 and 4 times, respectively, more susceptible to

acquiring listeriosis compared to the general population (CDC, 2014l). The incubation period of

L. monocytogenes before a patient typically starts showing the symptoms of listeriosis can vary

from 3 to 70 days, and listeriosis can last from days to weeks (Food Safety, 2014).

Table 2.4 Steps involved in the cellular mechanism of pathogenesis of *Listeria*

monocytogenes*

Step	Mechanism	Description
1	Adhesion and invasion	Adhered *L. monocytogenes* cells penetrate the intestinal membrane through enterocytes by a process mediated by internalin, and in defense, host cells trap *L. monocytogenes* cells in phagosomes
2	Lysis of primary vacuoles	Listeriolysin O (LLO) and phosphatidylinositol-specific phospholipase C (PI-PLC) lyse phagosomes, releasing *L. monocytogenes* cells into host cell's cytoplasm
3	Intracellular growth	*L. monocytogenes* propagates in the cytoplasm by scavenging host cells' carbon sources for energy, which is mediated by hexose phosphate transporter (Hpt) and lipoate protein ligase (LpLA1)
4	Cell-to-cell spread	*L. monocytogenes* cells travel across host cells using actin polymerization protein (ActA)
5	Lysis of two-membrane vacuole	Listerial phosphatidylcholine-specific phospholipase C (PC-PLC) and LLO lyses the two-membrane vacuole and releases *L. monocytogenes* cells in the neighboring cells

* Adapted from Paoli et al. (2005)

Listeriosis can be transmitted from ingesting food contaminated with *L. monocytogenes*,

from infected mother to newborn, from animals to humans (especially farm workers and

veterinarians), and through nosocomial infection to newborns (Farber and Peterkin, 1991;

Buchholz and Mascola, 2001). Antibiotics such as ampicillin are usually used to treat listeriosis;

however, addition of gentamicin to the treatment is sometimes recommended (Buchholz and

Mascola, 2001). The infectious dose of *L. monocytogenes* to trigger listeriosis is estimated as 10^9

cells; however, the number of *L. monocytogenes* cells required to cause listeriosis depends up on several factors such as health and age of the host, number of cells ingested, and susceptibility of host to the strain of *L. monocytogenes* ingested (Farber and Peterkin, 1991). Healthy individuals can ingest smaller amount of *L. monocytogenes* cells without showing any symptoms of listeriosis; however, in cases of immunocompromised individuals, smaller doses may cause listeriosis. Some of the recently reported outbreaks are presented in Table 2.5.

Table 2.5 Recent foodborne illness outbreaks associated with *Listeria monocytogenes**

Timeline	Source	Location	Illnesses	Hospitalizations	Deaths
August-December, 2013	Cheese	2 States	8	7	1
May-July, 2013	Cheese	5 States	6	6	1
March-September, 2012	Cheese	14 States	22	20	4
August-November, 2011	Cantaloupe	28 States	147	143	33

* Data collected from CDC (2012f, g; 2013e; CDC, 2014m)

The optimum growth temperature for *L. monocytogenes* ranges from 30 to 37°C; however, it can grow at temperatures ranging from -4 to 45°C, and can survive freezing for an extended period of time. *Listeria monocytogenes* has been reported to grow across a pH range of 4.4 to 9.6, but the optimum growth pH is from 6 to 8. *Listeria monocytogenes* can grow at an a_w as low as 0.91 (Porto-Fett, 2010). Because of these characteristics (ability to grow at refrigerated temperatures, low pH and a_w, and without oxygen), strategies such as refrigeration, vacuum

packaging and acidification used to ensure food safety for other pathogens will not ensure safety against *L. monocytogenes* (Barry, 2007; Porto-Fett, 2010).

Being one of the leading causes of deaths related to bacterial foodborne illnesses, *L. monocytogenes* has become a serious concern regarding food safety (Table 2.1). Since 1990, the U.S. has exerted a "zero-tolerance" policy for the presence of *L. monocytogenes* in foods, especially post-lethality exposed ready-to-eat (RTE) foods; and RTE foods positive for the presence of *L. monocytogenes* are considered adulterated (Buchholz and Mascola, 2001; Montville and Matthews, 2005d; FSIS, 2012a). In order to control *L. monocytogenes* in post-lethality exposed RTE foods, the FSIS requires that manufacturers comply with one of three alternatives provided in 9 C.F.R. 430. A brief description of these processing alternatives is presented in Table2.6. However, several institutes and associations related to the food industry filed a petition asking the FSIS to replace the "zero-tolerance" policy in post-lethality RTE foods to <100 cfu/g *L. monocytogenes* in RTE foods, which do not support the growth of *L. monocytogenes* (FSIS, 2005). However, no changes have been made by the FSIS to the "zero tolerance" policy for *L. monocytogenes* in RTE foods in the U.S.

Table 2.6 *Listeria* control alternatives approved by the Food Safety and Inspection Service

(FSIS) for manufacture of post-lethality exposed ready-to-eat meat products*

Alternative	Description
1	The manufacturer uses a post-lethality treatment to reduce or eliminate *L. monocytogenes* in the product **<u>and</u>** an antimicrobial agent or antimicrobial process to limit or suppress growth of *L. monocytogenes* in the product
2	The manufacturer uses a post-lethality treatment, antimicrobial agent **<u>or</u>** antimicrobial process to limit, suppress or eliminate *L. monocytogenes* in the product
3	The manufacturer relies on sanitation alone to control *L. monocytogenes* in the processing environment and on the product

* Adapted from FSIS (2012a)

Listeria monocytogenes can form strong biofilms on/in the food processing equipment

and render a great challenge for thoroughly cleaning and sanitizing food processing facilities and

equipment. Moreover, cells shed from biofilms are more resistant to disinfectants and sanitizers

compared to planktonic cells. Presence of *L. monocytogenes* biofilms in food processing

facilities poses a great threat of processed/cooked food products (especially RTE) getting

contaminated and resulting in outbreaks and recalls. Beresford et al. (2001) studied the ability of

L. monocytogenes 10403S to form biofilms on 17 different coupon surfaces relevant to food

processing facilities (such as stainless steel, aluminum, polycarbonate, polyvinylchloride, nitryl

rubber, silicon rubber, and natural white rubber). Tryptic soy agar with ~8 log cfu/ml was used to

create biofilms on various surfaces by: a) short contact time (by submerging without contact

time); and b): 2 h contact time. Beresford et al. (2001) reported that biofilms developed on all

surfaces irrespective of contact time; however, *L. monocytogenes* cells that adhered during the

short contact time varied from 3.09 to 5.04 cfu/coupon; whereas, cells that adhered during 2 h contact time varied from 4.97 to 6.37 log cfu/coupon.

2.2.5 Salmonella **spp.**

Salmonella spp. are characterized as Gram negative, rod shaped, facultative anaerobic, catalase positive, oxidase and urease negative, and non-spore forming pathogenic bacteria. Most salmonellae are peritrichously flagellated and motile; however, few non-flagellated or having non-functional flagella strains do occur (Montville and Matthews, 2005a). *Salmonella* spp. catabolize D-glucose to produce acid and gas, produce hydrogen sulfide, decarboxylate lysine and ornithine, reduce nitrate, and utilize citrate (Andrew and Bäumler, 2005; Montville and Matthews, 2005a). *Salmonella* spp. cannot catabolize lactose or sucrose; however, some strains can acquire the ability to utilize lactose and/or sucrose due to mutation (Montville and Matthews, 2005a). These mutated strains are of great concerns because they can result in false presumptive tests based on biochemical reactions and can escape detection. Occurrence of some strains that cannot decarboxylate lysine or that possess urease activity is also a matter of concern in presumptive biochemical confirmation of *Salmonella* spp.

Salmonella spp. belong to the family Enterobacteriaceae. The genus *Salmonella* was named after an American veterinarian, Daniel E. Salmon, who first isolated and described a strain of *Salmonella* (now know as *Salmonella enterica* serotype Choleraesuis) in 1885 (Andrew and Bäumler, 2005). According to the World Health Organization (WHO) Collaborating Center for Reference and Research at the Pasteur Institute (Paris, France), the genus *Salmonella* is divided into two species: *Salmonella enterica* (contains six different subspecies) and *Salmonella bongori* (contains only one subspecies). These *Salmonella* subspecies are further divided into various serotypes/serovars (Brenner et al., 2000; Montville and Matthews, 2005a). As presented

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in Table 2.7, various subspecies of *Salmonella* are referred to by a Roman numeral from I to VI. *Salmonella* spp. is classified into various serotypes on bases of antigenic formulas created by confirming the presence of somatic (O) and flagellar (H) antigens (Andrew and Bäumler, 2005; Montville and Matthews, 2005a).

Table 2.7 Classification of genus *Salmonella**

Species	Subspecies	No. of Serotypes
S. enterica	*S. enterica* subsp. *enterica* (I)	1,454
	S. enterica subsp. *salamae* (II)	489
	S. enterica subsp. *arizonae* (IIIa)	94
	S. enterica subsp. *diarizonae* (IIIb)	324
	S. enterica subsp. *houtenae* (IV)	70
	S. enterica subsp. *indica* (VI)	12
S. bongori (V)	-	20
Total	-	2,463

* Adapted from Brenner et al. (2000)

Infection caused by *Salmonella* spp. is called salmonellosis; and 99% of human salmonellosis is caused by *S. enterica* subsp. *enterica* (Bailey et al., 2010; Andrew and Bäumler, 2005). *Salmonella* spp. is the leading cause of bacterial foodborne illnesses, with over a million infections and 300 deaths reported annually in the U.S. (Table 2.1). It is estimated that in the U.S., 139.4 cases of salmonellosis are reported per 100,000 infants (FAO/WHO, 2004b). Salmonellosis can lead to two types of clinical conditions: typhoidal fever and non-typhoidal enterocolitis or bacteremia. In some cases, salmonellosis can induce chronic conditions such as reactive arthritis, Reiter's syndrome and ankylosing spondylitis, lasting for a few months to years (Montville and Matthews, 2005a). All population groups can get salmonellosis; however,

children, elderly and people with weak immune systems are at greater risk. According to the FDA (2012a), depending upon the type of infecting *Salmonella* serotype, and age and health of the host, infectious dose for non-typhoidal salmonellosis can be as low as one cell; whereas, for typhoidal salmonellosis it can be <1000 cells.

Typhoid fever is caused by *S. enterica* subsp. *enterica* serotypes Typhi, Paratyphi A, Paratyphi B and Paratyphi C. Symptoms of typhoid fever may appear after 7 to 28 days of incubation and include diarrhea, high fever, abdominal pain and headache, followed by an acute phase of typhoid fever. Generally, typhoid infection is treated using the antibiotics chloramphenicol, ampicillin or trimethoprim-sulfamethoxazole (Andrew and Bäumler, 2005; Montville and Matthews, 2005a). *Salmonella* serotypes Typhimurium and Enteritidis are frequently related to enterocolitis caused by foodborne infection. Symptoms of enterocolitis (nausea, vomiting, diarrhea, and abdominal pains) generally appear from 12 to 72 h of infection. Generally, enterocolitis infection due to *Salmonella* spp. is self-limiting and does not require any treatment, and illness lasts from 4 to 7 days. However, patients are advised to drink lots of fluids and electrolytes during enterocolitis infection (Montville and Matthews, 2005a; Andrew and Bäumler, 2005; Bailey et al., 2010). Antibiotics are not advised in such cases because it can prolong the duration of infection and also because of the fear of pathogens becoming resistant to various antibiotics.

Salmonellae cells attach to, colonize and invade intestinal cells during salmonellosis. After invading host cells, salmonellae destroy epithelial and phagocytic cells through the process of apoptosis. The *inv* pathogenicity factor, responsible for invasion by *Salmonellae*, consists of 30 genes. During invasion, salmonellae grow and replicate inside endocytotic vacuoles, and are finally released into the tissue. Diarrhea during salmonellosis is believed to be due to this

invasion of intestinal cells by salmonellae cells rather than any enterotoxin (Montville and Matthews, 2005a). Virulence plasmids are only present in *Salmonella* serotypes Typhimurium, Dublin, Gallinarum-Pullorum, Enteritidis, Choleraesuis and Abortusovis; whereas, highly infectious *Salmonella* serotype Typhi does not have a virulence plasmid. These virulence plasmids help the rapid multiplication of salmonellae cells within the host cells and spread infection to tissues other than the intestine (Montville and Matthews, 2005a). Recent foodborne outbreaks related to *Salmonella* spp. (apart from those presented in Table 2.2) are presented in Table 2.8.

The optimum growth temperature for *Salmonella* spp. is 37°C; however, some strains can grow at temperatures as low as 2°C and as high as 54°C. *Salmonella* spp. can grow at pH ranging from 4.5 to 9.5, with optimum growth pH of 6.5 to 7.5. *Salmonella* spp. cannot grow at $a_w < 0.93$ (Bell and Kyriakides, 2002; Montville and Matthews, 2005a). The heat resistance of *Salmonella* spp. increases with a decrease in a_w and/or increase in pH (Bell and Kyriakides, 2002; Riemann, 1968). *S*. Senftenberg 775W is considered to be the most heat resistant serotype and is included in many studies related to thermal parameters of *Salmonella* spp. (Henry et al., 1969). It has been reported that dry-stressed salmonellae are more resistant to some antimicrobial treatments compared to non-stressed salmonellae. Gruzdev et al. (2011) reported that desiccated cells of *Salmonella* Typhimurium, Enteritidis, Newport and Infantis were more resistant to 10 to 30% ethanol solutions, 10 to 100 ppm bleach solutions, 0.5 to 2.0% hydrogen peroxide solutions, 0.1 to 1 M sodium chloride solutions, 1 to 10% bile salts solutions, and 125 $\mu W/cm^2$ UV irradiation; however, dry-stress decreased the resistance of salmonellae against acetic and citric acid treatments.

Salmonella spp. can form biofilms on both biotic and abiotic surfaces. It has been reported that *Salmonella* spp. can form biofilms on various surfaces (such as plastic, rubber, cement, glass and stainless steel) commonly encountered on farms, slaughterhouses and food processing facilities. Salmonellae cells in biofilms are extremely resistant to environmental stresses such as desiccation, heat, low pH, ionizing irradiation, disinfectants and antibiotics (Steenackers et al., 2012). Stepanović et al. (2004) investigated the biofilm formation of 122 *Salmonella* spp. and 48 *L. monocytogenes* strains on a plastic surface in brain heart infusion (BHI), tryptic soy broth (TSB), meat broth (MB), and 20 times diluted TSB (1/20 -TSB). They reported that all strains of *Salmonella* spp. and *L. monocytogenes* formed biofilms on plastic in all types of media; however, the quantity of biofilms formed by *Salmonella* spp. were greater compared to biofilms formed by *L. monocytogenes*. Stepanović et al. (2004) also reported that for *Salmonella* spp., diluted media (1/20-TSB) was most effective in promoting biofilm formation, in contrast to *L. monocytogenes*, where diluted media was least promoting for forming biofilms.

Table 2.8 Recent foodborne illness outbreaks associated with *Salmonella* spp. *

Timeline	Contaminant	Source	Location	Illnesses	Deaths
2013-14	*Salmonella* Heidelberg	Chicken	29 States and Puerto Rico	634	0
2013	*Salmonella* Montevideo and Mbandaka	Tahini Sesame Paste	9 States	16	1
2013	*Salmonella* Saintpaul	Cucumber	18 States	84	0
2013	*Salmonella* Heidelberg	Chicken	13 States	134	0
2013	*Salmonella* Typhimurium	Ground beef	6 States	22	0
2012	*Salmonella* Bredeney	Peanut butter	20 States	42	0
2012	*Salmonella* Braenderup	Mangoes	15 States	127	0
2012	*Salmonella* Typhimurium and Newport	Cantaloupe	24 States	261	3
2012	*Salmonella* Enteritidis	Ground beef	9 States	46	0

* Data collected from CDC (2014k); CDC (2013 a, b, c and d); CDC (2012 a, b, c and d)

2.2.6 Staphylococcus aureus

Staphylococcus aureus is a Gram positive, spherical (~0.5 to 1.5 μm in diameter), catalase positive, non-motile, non-spore forming, and facultative anaerobic pathogenic bacterium (Montville and Matthews, 2005c; Seo and Bohach, 2010). Most *S. aureus* strains can produce four types of hemolysins (alpha, beta, gamma and delta hemolysins), and other enzymes such as coagulase, nucleases, proteases, lipases and collagenases (Dinges et al., 2000; Seo and Bohach, 2010). *Staphylococcus aureus* belongs to the family Micrococcaceae, and the genus *Staphylococcus* is further divided into 53 species and subspecies (Montville and Matthews, 2005c; Seo and Bohach, 2010).

Staphylococcus aureus is a ubiquitous organism. Although humans and animals are the main reservoirs of *S. aureus* (25% of healthy humans and animals carry *S. aureus* on their skin, and hair, as well as in their noses and throats), it is present in air, sewage, water, milk, food processing equipment and environmental surfaces. *Staphylococcus aureus* is frequently associated with foods such as raw and processed meat, poultry, eggs, bakery products, and milk and other dairy products (Stewart, 2005; CDC, 2006; Seo and Bohach, 2010). Humans involved in food preparation and processing are the main source of *S. aureus* contamination (Montville and Matthews, 2005c; CDC, 2006; Kadariya et al., 2014). One percent of human *S. aureus* carriers carry methicillin resistant *S. aureus* (NIAID, 2012). Pathogenic *Staphylococcus* species, other than *S. aureus*, which are commonly encountered include *S. chromogenes*, *S. sciuri*, *S. xylosus*, *S. simulans* (commonly associated with dairy products), *S. intermedius* (commonly associated with canines), *S. epidermidis* and *S. haemolyticus* (commonly associated with hospital infections (Seo and Bohach, 2010).

Staphylococcal foodborne illness is characterized as poisoning/intoxication; therefore, it is not necessary to ingest live *S. aureus* cells to get sick but heat-stable enterotoxins already secreted by *S. aureus* into the food can cause sickness. Symptoms of *S. aureus* foodborne poisoning include nausea, vomiting, diarrhea, headache and abdominal cramping; and can onset within 30 min to 8 h of consumption of contaminated food (Montville and Matthews, 2005c; Stewart, 2005). Staphylococcal food poisoning is generally self-limiting and complete recovery normally occurs within 1 to 2 days. However, rare cases of enterocolitis have been reported due to multiplication of *S. aureus* in the intestinal tracts after ingestion (Kadariya et al., 2014; Stewart, 2005). Staphylococci were first linked with a foodborne illness in 1914 due to the consumption of contaminated milk (Montville and Matthews, 2005c); and recent cases of staphylococcal poisoning are presented in Table 2.9. *Staphylococcus aureus* causes 241,148 foodborne illnesses in the U.S. annually; however, it rarely causes death (~ 6 deaths; FDA, 2012b; Kadariya et al., 2014).

Table 2.9 Recent foodborne illness outbreaks associated with *Staphylococcus aureus**

Timeline	Source	Location	Illnesses	Hospitalizations
April, 2013	Restaurant	Kansas	>20	4
August, 2012	Turkey	Colorado	60	-
July, 2012	Chicken, sausage & rice dish	USA Military Base	22	13
June, 2012	Food handlers	Australia	22	6
July, 2011	Egg benedict	Oregon	7	2
June, 2011	Cake topping	2 States	100	-

* Data collected from Falkenstein (2011); Flynn (2011b); Goetz (2012); Pillsbury et al. (2012); Teague et al. (2012); Dyke (2013)

Staphylococcus aureus is considered to be a primary cause of human diseases and foodborne illnesses throughout the world (Kadariya et al., 2014; MDH, 2010). Other than causing foodborne poisoning, *S. aureus* can cause toxic shock syndrome, pneumonia, wound infections, and nosocomial bacteremia (FDA, 2012b). *Staphylococcus aureus* is also the leading cause of skin and soft tissue diseases (MDH, 2010). Although *S. aureus* food poisoning is self-curing, the development of *S. aureus* infection due to invasion and adherence inside body tissues and skin infections can result in severe conditions.

Staphylococcus aureus can produce multiple types of enterotoxins (called staphylococcal enterotoxins, SE) and possibly result in food poisoning due to the combined effect of multiple SEs. Although these SEs have similar homology, they differ serologically. Some SEs lack emetic properties, so poisoning due to these SEs does not induce vomiting (Seo and Bohach, 2010). On the basis of antigenicity, SEs have been differentiated into various types, and named using various alphabetic and numeric systems (Table 2.10). Staphylococcal enterotoxin A is highly heat resistant and is responsible for most *S. aureus* attributed foodborne poisonings (Kadariya et al., 2014). Three genes (*agr*, *sar* and *sae*) regulate the expression of *S. aureus* virulence factors (Montville and Matthews, 2005c). The infectious dose of SE is <1µg in a food (which happens at a population density of $>10^5$ cfu/g *S. aureus* in food); however, in some cases (infants, elderly and immunocompromised people), this infectious does can be as low as 100 to 200 ng (FDA, 2012b).

Table 2.10 Classification of staphylococcal enterotoxins (SE) and staphylococcal enterotoxins-like (SEl) on the basis of antigenicity*

SE/SEl	Sub-SE/SEl	Emesis
SEA	-	Yes
SEB	-	Yes
SEC	SC1	Yes
	SC2	Yes
	a	Yes
SED	-	Yes
SEE	-	Yes
SEG	-	Yes
SEH	-	ND
SEI	-	Weak
SEl	SElJ	ND
	SElK	ND
	SElL	No
	SElM	ND
	SElN	ND
	SElO	ND
	SElP	ND
	SElQ	No
	SElR	ND
	SElU	ND
TSST1[a]	-	

* Adapted from Seo and Bohach (2010)

ND: Not determined

a: Toxic shock syndrome toxin 1

Staphylococcus aureus is considered as a pathogen of serious concern due to its toxin production, ability to survive gastrointestinal conditions, and resistance to many commonly used antibiotics. Staphylococcal enterotoxins have resistance against gastrointestinal proteases such

trypsin, chymotrypsin, rennin and papain; therefore, can survive in the intestines (Seo and

Bohach, 2010). Staphylococcal toxins are very heat resistant and are not destroyed at cooking

temperatures. Pasteurization has no affect on biological activity of SEs; however, SEs can be

destroyed when heated at 121°C for 8 min (Seo and Bohach, 2010). *Staphylococcus aureus* can

grow over the temperature range of 6 to 48°C (with optimum being 37°C); therefore, it is

believed that refrigeration may not always ensure food safety against *S. aureus* if food is already

contaminated (Seo and Bohach, 2010). The optimum growth pH for *S. aureus* ranges from 7.0 to

7.5 (but can grow over a pH range of 4.5 to 9.3); and it can grow at a_w as low as 0.83, which is

much lower compared to most of the other mesophiles (Seo and Bohach, 2010; FDA, 2012b).

The high osmotolerance of *S. aureus* is a great concern in low a_w products because, with a lack

of competitive microflora, *S. aureus* can grow to large numbers producing greater amounts of

toxins (Seo and Bohach, 2010).

Staphylococcus aureus can adhere to surfaces of food processing equipment and facilities

and form biofilms. These biofilms are very resistant to sanitizers and can contaminate processed

products by shedding *S. aureus* cells. de Souza et al. (2014) assessed the capability of three

strains of *S. aureus* (S3, S28 and S54) to adhere to and form biofilms on polypropylene and

stainless steel coupons in meat-based broth at 7 and 28°C; along with efficacy of sanitizers

[sodium hypochlorite (250 mL/L) and peracetic acid (30 g/L)] against these biofilms. They

reported that all three strains of *S. aureus* were successfully able to form biofilms on

polypropylene and stainless steel at both temperatures; and were able to shed cells from biofilms

during the 15 days of study. de Souza et al. (2014) also reported that sanitizers used in the study

were not able to remove biofilms completely. This suggests that ability of *S. aureus* biofilms to

disperse cells and become resistant against sanitizers can result in the cross contamination of processed food products and can make sanitization difficult.

Increased resistance of *S. aureus* against commonly use antibiotics (including the penicillin group) has tremendously raised the concerns about public food safety (Kluytmans, 2009). Antibiotic resistant *S. aureus* is more of a concern for treating infections rather than foodborne poisonings. The first case of penicillin resistant *S. aureus* was reported in 1941, just two years after the introduction of penicillin for medical purposes; and by the1980's, ~90% of *S. aureus* strains isolated from patients were penicillin resistant (Pesavento et al., 2007). Methicillin resistant *S. aureus* (MRSA) are the most commonly encountered antibiotic resistant strains of *S. aureus*. In the past, MRSA infections were reported in hospitalized patients (nosocomial infections) but rarely in communities (community infections); however, a continuous increase in community *S. aureus* infections has been reported (Pesavento et al., 2007). Reported occurrence of nosocomial MRSA infections in healthy people, having no contact with hospitals, suggests that nosocomial strains are spreading in communities (Davies, 2013). Vancomycin is the most common antibiotic used for treating MRSA infections (Pesavento et al., 2007). Recently, a cephalosporin antibiotic has also been developed against MRSA (Kaźmierczak et al., 2014).

Infections due to consumption of food contaminated with multi-antibiotic resistant *S. aureus* increases the complications regarding the treatment of patients (Kaźmierczak et al., 2014). In such cases, the first step in the treatment of *S. aureus* infections is to determine if the infecting *S. aureus* is resistant to antibiotics or not; if *S. aureus* is resistant, antibiotic resistance evaluations must be conducted to determine the antibiotic/s to which the particular strain is susceptible.

Staphylococcus aureus and MRSA strains have been isolated from meat animals (such as pigs, poultry and cattle) raised in farms and workers working on such farms (Pesavento et al., 2007; Kadariya et al., 2014; Nadimpali et al., 2014). Transfer of MRSA from contaminated food and MRSA carrier workers poses a great risk of spreading *S. aureus* and MRSA infection (Kluytmans, 2009). Nadimpali et al. (2014) conducted a study to determine the presence of *S. aureus* and MRSA in the noses of workers in a hog operation facility in North Carolina. They reported that out of 22 workers examined for 14 days, 86.4% carried *S. aureus*, 4.5% carried MRSA and 45.5% carried multi-drug resistant *S. aureus* strains during at least one sampling point.

Yurdakul et al. (2013) reported that out of 30 raw chicken samples (collected from supermarkets in Adana, Turkey) tested for antibiotic resistant *S. aureus*, 100% were resistant to tetracycline, and 25% were resistant against erythromycin and chloramphenicol. Waters et al. (2011) reported that out of 136 raw meat and poultry samples (chicken, 46; turkey, 26; pork, 26; beef, 38), 77, 42, 41 and 37% were positive for *S. aureus* in turkey, pork, chicken and beef, respectively. They also reported that multiple resistances of 32 isolated *S. aureus* strains against tetracycline, ampicillin, penicillin and erythromycin were highly prevalent.

2.3 *Cronobacter sakazakii* and *Salmonella* spp. in Dry Milk Powder and Powdered Infant Formula

According to American Dairy Products Institute (ADPI), dry milk powder (DMP) should have standard plate count and coliform count $\leq 10,000/g$ and $\leq 10/g$, respectively; whereas, *Salmonella* spp., *Listeria* spp. and coagulase-positive *Staphylococcus* should be negative (ADPI, 2002). According to World Health Organization, *C. sakazakii* and *Salmonella enterica* are considered as 'Category A' organisms (i.e. having a clear evidence of causality) in powdered

infant formula (PIF) (FAO/WHO, 2004b). Selected outbreaks associated with DMP and PIF contaminated with *C. sakazakii* or *Salmonella* spp. are present in Table 2.11. Dry milk powder and PIF are not sterilized products; therefore, if contaminated pathogens can survive during storage and multiply when powders are hydrated, or DMP can carry pathogen contamination into a broad range of food products in which it is utilized as an ingredient. Contamination in these powders can occur during processing (such as drying or packaging), post-pasteurization contamination of milk used, and/or from ingredients used during manufacturing.

To minimize *C. sakazakii* and *Salmonella* infections and outbreaks in infants, it is very important to follow good manufacturing practices at homes and hospitals during preparing and storing PIF (Fiore et al., 2008). In hospitals, dedicated clean areas should be used for the preparation of PIF by specifically trained personnel (Fiore et al., 2008). Aseptic preparation of PIF following label instructions, refrigerating rehydrated PIF within 2 to 4 h of preparation, and not using prepared PIF that has been refrigerated for >24 h reduces the risk of *C. sakazakii* infection in infants (FAO/WHO, 2007; Fiore et al., 2008). If available, ready-to-eat infant food products should be substituted for PIF for reduce food safety risks.

When possible, breast feeding should be encouraged for the neonates as mother's milk contain essential A immunoglobulins and other essential components (ISDI, 2004; FAO/WHO, 2007; Fiore et al., 2008). If that's not possible, mothers should be informed and trained about aseptic preparation and handling of PIF (ISDI, 2004). Preparing PIF using water at ≥70 °C can prevent *C. sakazakii* infection by killing the bacteria if contamination were to exist in the PIF (FAO/WHO, 2007). Decreasing the frequency of feeding PIF and shortening the storage time after PIF preparation can decrease the chances of *C. sakazakii* infection (ISDI, 2004; FAO/WHO, 2007).

Table 2.11 Recent foodborne illness outbreaks associated with dry milk powder and powdered infant formula contaminated with *Cronobacter sakazakii* or *Salmonella* spp. *

Contaminant	Year	Source	Location	Illnesses	Deaths
C. sakazakii	2011	Unknown	Missouri	2	1
C. sakazakii	2008	Unknown	New Mexico	2	1
C. sakazakii	2004	Powdered infant formula	France	4	2
C. sakazakii	2004	Powdered infant formula	New Zealand	5	1
C. sakazakii	2001	Powdered infant formula	Tennessee	11	1
C. sakazakii	1999-2000	Powdered infant formula	Israel	2	0
S. Tennessee	May, 1993	Powdered infant formula	Canada; U.S.A.	3	0
S. Virchow	1996	Powdered infant formula	Spain	48	0
S. Anatum	1996-97	Powdered infant formula	France; U.K.	17	0
S. Agona	2004-05	Powdered infant formula	France	141	0
S. Worthington	Jan-July, 2005	Milk powder	France	49	0

* Data collected from Louie et al. (1998); Himelright et al. (2001); InVs (2005); Drudy et al. (2006); Cahill et al. (2008); Baumbach et al. (2009); Astley (2012);

Cronobacter sakazakii is not known to be isolated from fresh raw milk (Lehner et al., 2010); therefore, presence of *C. sakazakii* in milk is likely due to pre- or post-pasteurization contamination. Lehner et al. (2010) took 100 raw milk, 91 milk concentrate and 172 DMP samples from two different Swiss production facilities, and tested them for the presence of Enterobacteriaceae and *Cronobacter* spp. They reported that *Cronobacter* spp. were only present in milk powders and were absent in raw milk and milk concentrate samples.

The nutrient rich medium of PIF and DMP containing lactose, proteins and milk fat may have a protective affect on pathogens (such as *C. sakazakii* and *Salmonella* spp.) during spray drying and dry storage and help in pathogen growth when these powders are hydrated. *Cronobacter sakazakii* and *Salmonella* spp. can survive the dry stress in powders, and some capsulated strains of *C. sakazakii* have been reported to survive up to 2.5 years in PIF. Barron and Forsythe (2007) used 10 desiccated strains of *C. sakazakii* (6 able to capsulate and 4 not able to capsulate) to study their survival through the dry stress of PIF by individually inoculating PIF (at ~5 to 8 log cfu/g levels) with these strains and storing at room temperature for 2.5 years. They reported that capsulated *C. sakazakii* strains survived longer compared to non-capsulated strains. Barron and Forsythe (2007) also reported that one non-capsulated and 4 capsulated *C. sakazakii* strains were viable up to 2 years; whereas, 2 capsulated *C. sakazakii* stains maintained their viability up to 2.5 years. They suggested that survival of *C. sakazakii* in the dry environment of PIF could be attributed to the protective effect of proteins, lactose and milk fat present in PIF against the osmotic effect; however, survival of *C. sakazakii* in other powders or food products may vary depending on their composition.

McDonough and Hargrove (1968) studied the effect of temperature (ranging from 4.4 to 50°C) and various preservative treatments on the survival of a cocktail of *Salmonella* serotypes

Senftenberg 775W, Typhimurium TM1 and New Brunswick 1608 inoculated (at ~5.2 to 6.3

cfu/g) in DMP during 15 weeks of storage. They reported that salmonellae survival in DMP

decreased as the storage temperature increased. There was only ~1 log reduction at 4.4°C, but no

salmonellae was detected at 43.3 and 50°C at the end of storage. McDonough and Hargrove

(1968) also reported that during storage, counts decreased in both inoculated control DMP and

DMPs containing individual preservatives (0.1% diethylpyrocarbonate, 500 ppm potassium

sorbate or 0.2% sodium benzoate), and preservatives did not have any lethal affect on

salmonellae in DRP, which otherwise are effective against salmonellae in liquid milk.

Although *C. sakazakii* is thought to be more heat tolerant compared to other

Enterobacteriaceae present in dairy products, *C. sakazakii* and *Salmonella* spp. cannot survive

milk pasteurization temperatures (Erickson and Kornacki, n.d.; Read et al., 1968; Drudy et al.,

2006; Osaili et al., 2009). Iversen et al. (2004) reported that the D-values of *C. sakazakii* grown

in infant formula milk were 1.1 to 1.8 min at 60°C and 0.11 to 0.12 min at 62°C. They also

reported that *z*-values of *C. sakazakii* were 5.7 to 5.8°C. However, *C. sakazakii* and *Salmonella*

spp. can survive the spray-drying process; therefore, they can be present in milk powders if post

pasteurization contamination of milk occurs. Arku et al. (2008) reported that four strains of *C.*

sakazakii (ATCC 51329, CFS 155, CFS 237 and CFS 1001) were able to survive a typical spray

drying process (at inlet and outlet temperatures of 160 and 90°C, respectively) using inoculated

(at ~7 and 2 log cfu/mL levels) 10% reconstituted skim milk; and could be detected (ranging

from ~2 to 5 log cfu/g) in the DMP with higher inoculum up to 12 weeks. LiCari and Potter

(1970) reported that four *Salmonella* serotypes (Typhimurium, Thompson, Tennessee and

Kentucky) survived spray drying (at inlet and outlet temperatures of 226.7 to 293.3 and 121.1°C,

respectively) of individually inoculated (at ~ 7.0 to 7.5 log CFU/100g levels) 40% condensed

milk. Although LiCari and Potter (1970) noticed the log reductions ranging from 4.3 to 4.9, all four *Salmonella* spp. were present at levels ranging from 2.2 to 5.6 log cfu/100 g, and *S. Tennessee* was the most heat resistant.

Iversen and Forsythe (2004) conducted a survey to determine the presence of *C. sakazakii* and other *Enterobacteriaceae* in 82 PIF, 49 dried infant food, and 72 DMP samples along with 283 other food products purchased from retail outlets across the U.K., Europe, Asia, Africa and the U.S. They reported that 2 PIF, 5 dried infant foods, and 4 DMP samples were positive for *C. sakazakii*. In another survey conducted by Chao et al. (2009), presence of *C. sakazakii* was determined in 136 infant follow-up-formula (FUF) and 179 infant food (IF) samples purchased and analyzed in 7 different countries: Brazil (31 FUF and 0 IF samples), Korea (24 FUF and 6 IF samples), Malaysia (12 FUF and 18 IF samples), UK (38 FUF and 64 IF samples), Indonesia (0 FUF and 15 IF samples), Portugal (20 FUF and 30 IF samples) and Jordan (11 FUF and 46 IF samples). Chao et al. (2009) reported that *C. sakazakii* was not isolated from any products from Brazil, Korea and Malaysia; and out of 136 FUF samples, only 1 sample (from Jordan) was positive for *C. sakazakii*. They also reported that out 179 IF samples, 6 from U.K., 3 from Portugal, 7 from Jordan and 6 from Indonesia were positive for *C. sakazakii*. They concluded that occurrence of *C. sakazakii* was less frequent in infant FUF compared to PIF and other IF samples.

In January 2011, a 'grade B' dairy plant in Winthrop, MN owned by Dairy Farmers of America was found to be contaminated with *Salmonella* Senftenberg (Flynn, 2011a). Flynn (2011a) reported that out 106 environmental swabs taken by the FDA from various locations in the plant, three were found positive for *S.* Senftenberg. Unsanitary conditions of the plant and

workers were indicated to be the reasons behind the contamination. A warning letter was issued to the plant giving them fifteen days to clean and sanitize the plant.

2.4 Radio Frequency Dielectric Heating as a Thermal Intervention Technique

2.4.1 Radio Frequency Dielectric Heating

Radio frequency dielectric heating (RFDH) is an electro-heating process (similar to microwave heating) in which electric energy is converted into electromagnetic waves that subsequently generate volumetric heat within a product (Rowley, 2001; Piyasena et al., 2003; Marra et al., 2009). Foods consist of dipoles (such as water) and ions (such as salts), which contribute to heat generation within a food product during RFDH (FDA, 2000). During RFDH, a food product is placed between the capacitor plates of a RFDH unit/oven and an alternating electric field is applied. The frictional heat is generated within the food product by the following two phenomena (Figure 2.2; Piyasena et al., 2003; Ramaswamy and Tang, 2008; Marra et al., 2009):

1. Dipole rotation: The dipoles in the food align themselves along the electric field and oscillate continuously along the changing electric field.

2. Ionic depolarization: The ions in the food move toward the opposite charged regions of the electric field and migrate continuously according to the changing electric field.
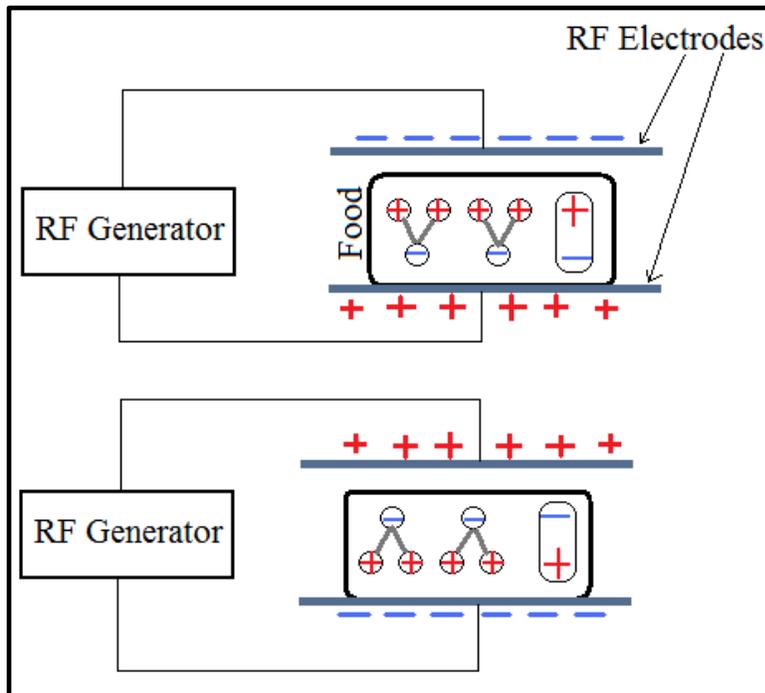
Figure 2.2 Dipolar rotation and ionic depolarization of a food product during radio frequency (RF) heating

Heat generated by the above two mechanisms is then distributed throughout the food product by conduction and/or convection (Fellows, 2000). Therefore, food products with uniformly distributed ingredient/components will have more uniform temperature distribution within the products compared to the products with non-uniform ingredient distribution. During RFDH, ionic depolarization is considered the dominant mechanism contributing towards heat generation within the products. In contrast, during microwave heating, depending upon the food composition, both dipole rotation and ionic depolarization can be dominant mechanisms for the heat generation (Marra et al., 2009).

Compared to microwaves with frequency ranging from 300 to 30,000 MHz, the radio wave region in the electromagnetic spectrum ranges from 1 to 300 MHz. Microwave ovens use

magnetrons or klystrons to generate microwaves. These microwaves are then randomly reflected towards the target food product using metallic plates or chambers, and for uniform heating rotating trays or stirrers are used (Marra et al., 2009). Whereas, radio-wave ovens use parallel plate electrodes to generate radio waves to heat the food product (placed in the oven without touching the electrodes). This mechanism of RFDH is commonly used to heat thick products; however, the other two common methods used to generate radio waves include (Marra et al., 2009):

1. <u>Fringe-Field Applicators</u>: Fringe-field applicators consist of electrodes in shapes of a series of bars, rods or narrow plates. These are most suitable for heating thin products.

2. <u>Staggered Through-Field Applicators</u>: Staggered through-field applicators consist of staggered rod or tube shaped electrodes on either side of a conveyor belt. These are most suitable for products of intermediate thickness.

Because some radio wave frequencies can interfere with radar and communication systems, the frequencies approved by the Electromagnetic Compatibility Regulations and Federal Communications Commission for industrial, scientific and medical applications are presented in Table 2.12**.** Penetration power and heat distribution in a food product by RFDH depends on three factors; permittivity, dielectric constant, and loss factor of the product. These factors further depend on the moisture content and temperature of the food being processed, and the radio frequency being used (Fellows, 2000).

Table 2.12 Microwave and radio wave frequencies (MHz) allowed for use in the U.S. by the Federal Communications Commission*

Radio Frequencies	Microwaves
13.56 ± 0.00668	915 ± 13
27.12 ± 0.16	2450 ± 50
40.68 ± 0.02	5800 ± 75
	24125 ± 125

*: Values collected from FDA (2000); Rowley (2001); Marra et al. (2009)

Some of the advantages and disadvantages of RFDH over microwave and/or conventional heating are as follows (FDA, 2000; Rowley, 2001; Fu, 2004; Ramaswamy and Tang, 2008):

1. Advantages:

    I.   Compared to microwave ovens, radio wave ovens work at much higher power; therefore, have much lower operational/running costs.

    II.   Because radio waves have longer wavelengths, they have greater penetration power and more uniform heating compared to microwaves.

    III.   Time required to achieve target temperatures using RFDH is significantly shorter compared to conventional and microwave heating. Therefore, RFDH can significantly reduce the overall thermal treatment time and maintain the food quality (especially for the sterilization process of foods).

    IV.   Because of faster and more uniform heating during RFDH, there is no surface over heating and fewer cold spots.

V. Radio frequency dielectric heating allows better process control compared to conventional heating.

2. Disadvantages:

I. Unlike microwave ovens, radio wave ovens are only available for industrial use or laboratory research (but not for domestic use) because of the impedance nature of high-power radio frequency coupling. Domestic microwave ovens work at power levels of <75 kW but RFDH ovens work at power of hundreds of kilowatts.

II. Because of uneven heating of irregularly shaped food products, radio waves are only considered suitable for food products with regular shapes (such as rectangular and square).

III. Due to the concentration of radio waves at the edges of food containers, food at the edges can become overheated (this is called fridge or edge effect).

2.4.2 Radio Frequency Dielectric Heating of Various Foods

Currently, RFDH is being used at industrial scale for food quality control such as post-baking drying of biscuits, cereals, pastries and breads; drying of herbs, spices and vegetables; and defrosting of meat and fish (Rowley, 2001). However, researchers are actively studying ways to implement RFDH at industrial scale as a thermal intervention technique to inactivate foodborne pathogens, especially in sterilization processes. Radio frequency dielectric heating has great potential as a sterilization technique because along with being faster it can still maintain food quality. This can be very useful in the manufacturing of products for specific purposes and needs such as: a) nutritious, palatable, shelf-stable and ready-to-eat foods for soldiers at war and astronauts going on space missions; and b) shelf-stable, low acid (>4.5 pH), ready-to-eat foods for emergency responses (Ramaswamy and Tang, 2008).

Orsat et al. (2004) studied the shelf life of hams pasteurized (at 75 and 85°C for 10 min) using radio frequency heating (27.12 MHz), vacuum packaged in polyethylene, nylon or polypropylene, and stored at 4°C for 28 days. They concluded that radio frequency treated hams at 85°C and packaged in all three packaging materials maintained a superior quality compared to the control for 28 days by reducing the standard plate counts and moisture loss, and maintaining greater product sensory and acceptance qualities (color, odor, sliminess and overall acceptance).

Guo et al. (2006) reported that during the shelf life of cooked (to 72°C) inoculated (*E. coli* K12 at 7 log cfu/g) ground beef stored at 4°C for 30 days, no significant differences were found between *E. coli* counts of ground beef cooked using radio frequency heating (at 1.5 kW and 27.12 MHz) and ground beef cooked (rapped in aluminum foil) in a water bath during 30 days of storage (0 colonies detected on the lowest dilution). However, they reported that the cooking time of ground beef using radio frequency heating (4.25 min) was significantly shorter than using a water bath (150.33 min), and final temperature distribution was more uniform in radio frequency cooked ground beef (ranging from 72.39 to 75.34°C) compared to water bath cooked (ranging from 72. 28 to 87.11°C). Guo et al. (2006) suggested that radio frequency heating could replace conventional methods because of shorter cook times and uniform heating. Other than the food industry, RFDH is widely used and being explored in the fields of textiles, pharmaceutical and woodworking, and as environmental disinfectant and disinfestant (Fu, 2004; Lagunas-Solar et al, 2006).

In microbiology, efficacy of a thermal treatment is established by determining the following three parameters (Orsat et al., 2004; Frazier and Westhoff, 2005c):

1. <u>D-value (Decimal Reduction Time)</u>: D-value is defined as the time required to decrease the population a specific microorganism in a specific medium and at a specific temperature by

90% (equivalent to 10-fold or 1 log). D-value is calculated as absolute value of the inverse of the slope of a graph plotted between log count of the viable microorganism *vs.* time.

2. <u>*z*-value (Thermal Resistant Constant)</u>: *z*-value is defined as the change in temperature required to change the log of D-value of a specific microorganism in a specific medium by 90% (equivalent to 10-fold or 1 log). *z*-value is calculated as the absolute value of the inverse of the slope of a graph plotted between log of D-values *vs.* temperature.

3. <u>F-value</u>: F-value is defined as the time required at a specific temperature to decrease the population of a specific microorganism in a specific medium by a specific number. The term "Fo" (Sterilization Value) indicates that the F value of a microorganism was calculated at 121.1°C (or 250°F).

Although RFDH is used in baking, drying, and defrosting, commercial applications for food pasteurization and sterilization are scarce in North America (FDA, 2000; Rowley, 2001; Ramaswamy and Tang, 2008). However, various investigations have been done to study the thermal inactivation of different pathogens in different foods using RFDH. Luechapattanaporn et al. (2004) conducted a study to validate thermal inactivation kinetics of *Clostridium sporogenes* PA 3679 spores in mashed potatoes (6.3 pH and 0.998 a_w) using radio frequency heating (at 27.12 MHz and 6 kW). Using thermal-death-time disks immersed in a hot oil bath, they first determined the D-value of *C. sporogenes* spores as 0.61 min at 121.1°C and *z*-value as 10°C; and then calculated Fo-value as 7.3 min. Luechapattanaporn et al. (2004) showed during the microbial challenge study that radio frequency heating was able to achieve the desired lethality of *C. sporogenes* spores. In a similar study, Luechapattanaporn et al. (2005) also validated thermal inactivation kinetics of *Clostridium sporogenes* PA 3679 spores in scrambled eggs using radio frequency heating.

It is a widely accepted concept in the scientific community that microbial inactivation in foods using RFDH is solely due to the thermal effect; however, some scientists defer and argue that non-thermal effects of RFDH play a vital role in the microbial inactivation (FDA, 2000). These non-thermal effects of RFDH are attributed to selective heating of microbes, electroporation, cell membrane rupture, cell lysis due to electromagnetic energy, and decrease in microbial cell surface hydrophobicity (Geveke et al., 2007; Geveke and Brunkhorst 2008; Ukuku et al., 2012).

2.5 Hyperspectral Imaging as an Immerging Rapid Identification Technique for Foodborne Pathogens

2.5.1 Hyperspectral Imaging

Hyperspectral imaging (HSI) is an emerging technology that has a great potential in rapid detection and identification of foodborne pathogens. The increased food safety concerns due to foodborne pathogens throughout the world have reinforced the need for rapid detection and identification methods. Although, at present, traditional methods are still used for the detection and identification various pathogens, these methods can take from 4 to 7 days to give confirmatory results. Moreover, these methods are cumbersome, labor intensive, expensive and often lack serotype-level discriminatory power. Therefore, rapid detection methods for foodborne pathogens, at least at the presumptive level, are required for the functioning of a safe and fast food supply chain.

Hyperspectral imaging technology was originally designed for remote sensing applications, but now it is been used in astronomy, agriculture, pharmaceuticals and medicine (Smith, 2012; Gowen et al., 2007). Hyperspectral imaging is extensively researched for its

practical applications in the area of food safety and quality. Hyperspectral imaging is a non-destructive method of analyzing and detecting a specimen, and combines imaging with spectroscopy to acquire both spatial and spectral information of a specimen (such as bacterial cells or colonies; Gowen et al., 2007). Hyperspectral imaging utilizes optical characteristics of specimens for the identification; therefore, HSI works on the interactions between light (visible and/or near infrared light) and the molecular structure of the specimen. When a sample is exposed to a light source, it reflects, transmits or absorbs light, or in some cases produces fluorescence. This reflectance, transmittance, absorbance or fluorescence (along with spatial images) can be recorded at specific wavelength intervals to obtain the molecular/chemical information of a sample by generating spectral signatures/graphs of that sample (ElMasry and Sun, 2010; Dale et al., 2013). In food safety and quality analyses, reflectance mode using wavelength ranges from 400 to 700 nm (visible range), 700 to 1000 nm (near infrared range), or a combination of both is most commonly used (Dale et al., 2013).
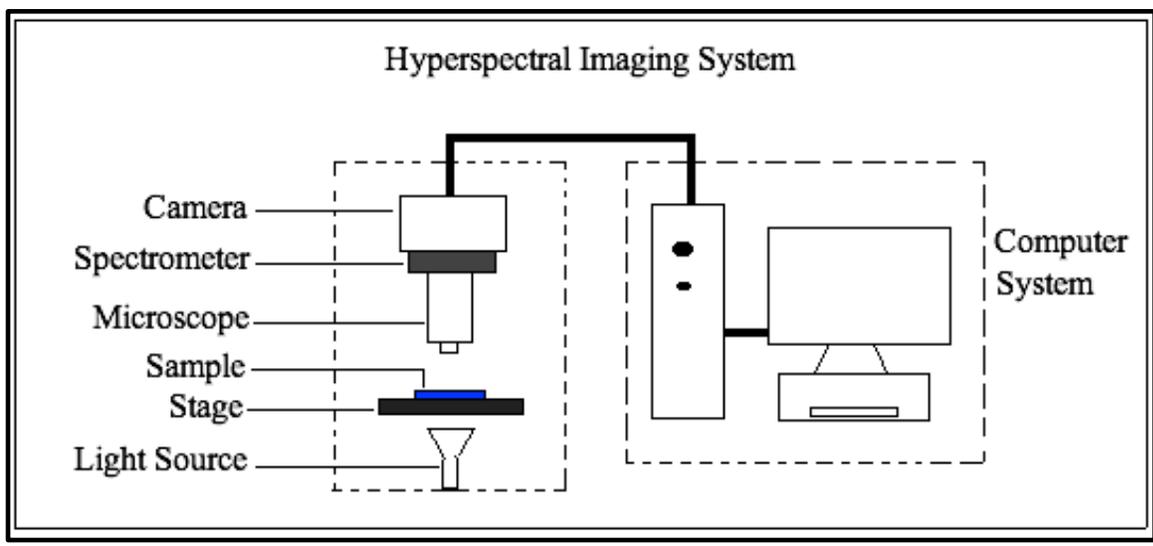
Figure 2.3 Basic components of hyperspectral imaging system

The basic components of a HSI system consist of a light source, stage (for placing samples), microscope (optional), spectrophotometer, camera and a computer (Gowen et al., 2007; Figure 2.3). Electromagnetic radiations of a selected wavelength range (at specific wavelength intervals) are bombarded onto a sample, and 2-dimensional spatial images of the sample are generated at various wavelengths, resulting in a 3-dimensional hypercube (Figure 2.4). The hypercube consists of 2-dimensional images (x and y axis) along with the 3rd-dimension as wavelength (ElMasry and Sun, 2010; Dale et al., 2013). The region of interest (ROI; number of pixels) can be selected from the 2-dimensional image and the spectral graphs/signatures of the ROI can be generated using computer software (such as Environment for Visualizing Images). These spectral signatures provide vital information regarding the biochemical composition of the selected ROI, and can be stored digitally to serve as reference libraries. These libraries can be used for identifying an unknown specimen by matching the spectral signatures of the unknown specimen with the spectral signatures in the reference libraries (Gowen et al., 2007; ElMasry and Sun, 2010; Dale et al., 2013).
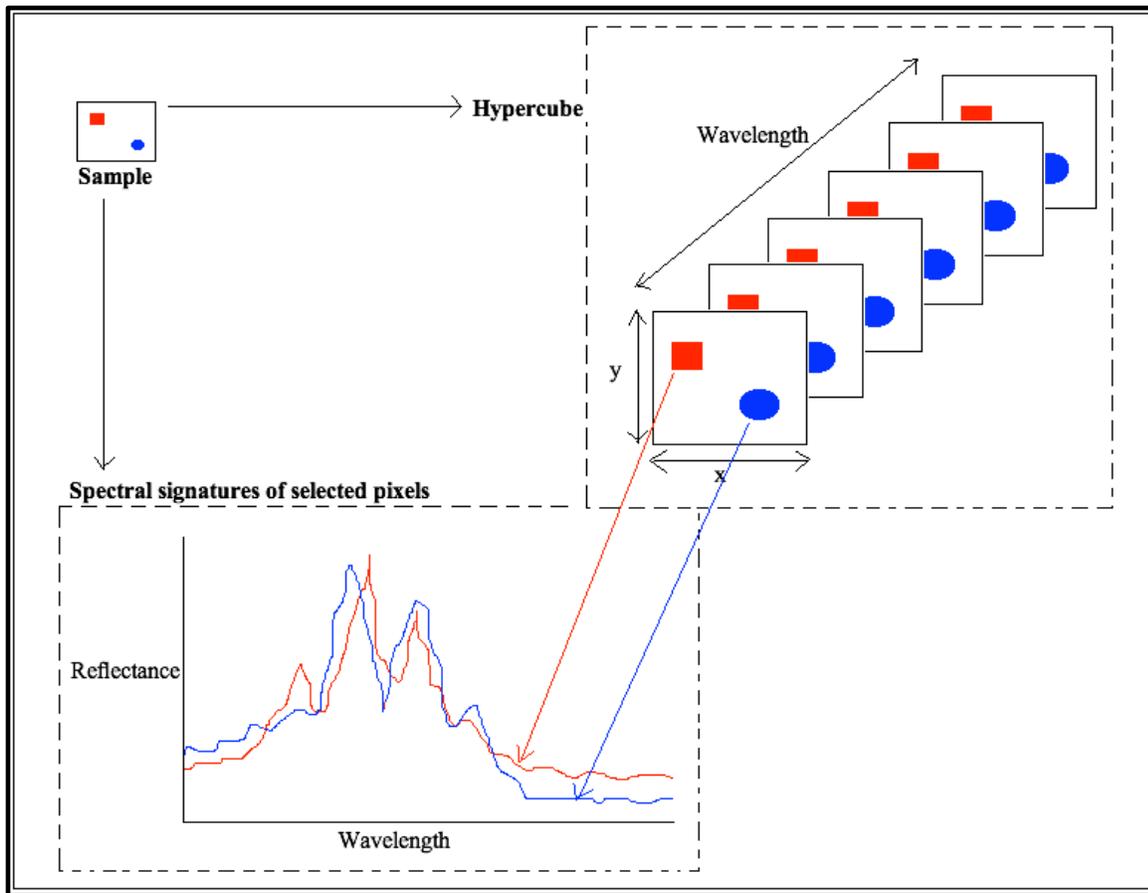
Figure 2.4 Typical output of hyperspectral imaging

2.5.2 Hyperspectral Imaging of Various Pathogens

Yoon et al. (2013b) used visible-near infrared (400 to 1000 nm) HSI to differentiate and classify Big Six STEC culture colonies grown individually on Rainbow Agar plates at 37°C for 24 h. They generated absorbance spectral graphs for each Big Six serotype, and reported that each serotype has distinctive absorbance. They used Principle Component Analysis (PCA) and various models to classify HSI images; and reported that using k-fold cross-validation of models, serotypes O111 and O121 showed >99% classification accuracy; whereas, serotypes O26, O45, O103 and O144 showed 84 to 100% classification accuracy. Yoon et al. (2013a) in a similar study used visible-near infrared HSI to differentiate colonies of Big Six STEC grown on

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Rainbow Agar plates as a mixed culture at 37°C for 24 h. They reported that absorbance spectral graphs for various Big Six STEC were distinguishable from each other in the wavelength range of 400 to 700 nm, but no differences were noticed between 750 to 1000 nm.

Using HSI (400 to 900 nm range), Yoon et al. (2010) were able to differentiate *Campylobacter* spp. colonies from non-*Campylobacter* colonies (such as *Acinetobacter baumannii*, *Flavobacterium odoratum*, *Brevundimonas diminuta* and *Sphingomonas paucimobilis*) grown on Blood Agar and Campy-Cefex Agar at 37°C for 24 or 48 h. They reported that overall % relative reflectance of colonies decreased as age of the cultures increased, and Blood Agar was a better culture medium compared to Campy-Cefex Agar for detection accuracy of *Campylobacter* spp. colonies. Del Fiore et al. (2010) reported that various fungal strains (*Aspergillus niger* 7096, *A. parasiticus* 2999, *A. flavus* 3357, *Fusarium graminearum* 126, *F. verticilloides* 4040, *Penicillium* spp.) grown on Potato Dextrose Agar could be differentiated using HSI on the basis of spectral reflectance between 400 to 1000 nm wavelength range. They also used HSI to differentiate fungal infected maize kernels from healthy maize kernels.

Siripatrawan et al. (2011) used hyperspectral imaging for the rapid detection of *E. coli* K12 on artificially inoculated fresh spinach. They inoculated spinach with *E. coli* at four levels ranging from 5.1 to 7.4 log cfu/g and acquired three-dimensional hypercubes consisting of spectral and spatial information at wavelengths ranging from 400 to 1,000 nm. They used PCA and artificial neural network (ANN) for processing data and developing a prediction algorithm to predict the number of *E. coli* cells on the spatial plane of a spinach hyperspectral image on the basis of different colors. Siripatrawan et al. (2011) were able to successfully predict *E. coli* populations on the spinach samples, as counts predicted through their ANN algorithm were in

agreement with the *E. coli* counts obtained from plating on 3M Pertifilm for *E. coli* and coliforms.

Jun et al. (2010) individually spot-inoculated *E. coli* O157:H7 strain FDA-SEA 13B88 and *Salmonella enterica* serotype Poona (grown in M9 medium) on stainless steel, high-density polyethylene, Formica and two polished granite plates (10×10 cm^2) to form thin and thick biofilms. After 3 days of incubation at 22°C, they used fluorescence HSI (400 to 700 nm wavelength range) to detect biofilms and differentiate inoculated from non-inoculated surfaces. They reported that *E. coli* had a distinctive relative florescence intensity compared to *S.* Poona and both bacterial biofilms had different fluorescence spectra compared with the background surfaces. They also reported that relative fluorescence intensity of thick biofilms were greater than that of thin biofilms, suggesting that the thickness of biofilms can affect associated spectral signatures.

Jun et al. (2009) also studied relative fluorescence intensity spectra (416 to 700 nm wavelength range) of biofilms of *E. coli* O157:H7 strain 3704 and *S.* Typhimurium ATCC 35648 (grown in M9 medium) on stainless steel coupons (2×5 cm^2). After 6 days of incubation at 37°C, *S.* Typhimurium biofilms had distinctive and greater relative fluorescence intensity compared with *E. coli* biofilms. Kim et al. (2012) studied the biofilm formation and detection of a non-pathogenic *E. coli* strain on stainless steel, titanium and titanium-alloy using HSI (400 to 700 nm wavelength range). They reported that no biofilm formation was observed on titanium and titanium-alloy; however, the *E. coli* biofilm formed on the stainless steel surface had different absorbance spectral signatures than non-inoculated stainless steel. Kim et al. (2012) also reported that the absorbance spectra of an *E. coli* colony was different from that of an *E. coli* biofilm, which could be attributed to the presence of extracellular polysaccharides in biofilms.

HSI was also used to differentiate between the spores of nonpathogenic strains of *Bacillus anthracis* and *Bacillus globigii* using the CytoViva® HSI system at 400 to 1000 nm wavelength range (CytoViva®, n.a.).

HSI has also shown promising applications in the filed of food quality. HSI has been researched for detecting bitter pits and bruises in apples; bruises on cucumbers; *Penicillium digitatum* infection in citrus fruits; fecal contamination on apples, cantaloupes and pork; skin tumors on chicken carcasses; predicting peach ripeness and beef tenderness; discriminating ripeness levels of tomatoes; estimating oil and moisture contents in corn kernels; and evaluating pork quality and marbling level (Polder et al., 2002; Cogdill et al., 2004; Kim et al., 2004; Lu and Peng, 2006; Nicolaï et al., 2006; Qiao et al., 2007; Naganathan et al., 2008).

Chapter 3 - Research Questions

3.1 Experiment I: Radio Frequency Dielectric Heating

- Can radio frequency dielectric heating of inoculated (with 5-strain cocktail of *Cronobacter sakazakii* or *Salmonella* spp.) nonfat dry milks (high-heat and low-heat varieties) shorten the times required to achieve target temperatures (75, 80, 85 and 90°C) compared to the conventional heating method, and still have equivalent biological lethality?

3.2 Experiment II: Hyperspectral Imaging

- Can hyperspectral imaging be potentially used for the rapid identification and differentiation of various foodborne pathogens?

- Can hyperspectral imaging profiles be affected by the age of the bacterial cells?

- Can hyperspectral imaging be used to differentiate healthy microbial cells compared to lauric arginate (a food-grade antimicrobial) treated cells?

Chapter 4 - Experiment I: Validation of Radio Frequency Dielectric Heating System for Destruction of *Cronobacter sakazakii* and *Salmonella* spp. in Nonfat Dry Milk

4.1 Abstract

Cronobacter sakazakii and *Salmonella* spp. have been associated with human illnesses from consumption of contaminated nonfat dry milk (NDM), a key ingredient in powdered infant formula and many other foods. *C. sakazakii* and *Salmonella* spp. can survive the spray-drying process if milk is contaminated after pasteurization, and the dried product can be contaminated from environmental sources. Radio frequency dielectric heating (RFDH) is a faster and more uniform process of heating low-moisture foods. The objective of this study was to design a RFDH process to achieve target destruction (log reductions) of *C. sakazakii* and *Salmonella* spp. The thermal destruction parameters (D-values) of *C. sakazakii* and *Salmonella* spp. in NDM (high-heat, HH; and low-heat, LH) were determined at 75, 80, 85, or 90°C using a thermal-death-time (TDT) disk method and the z-values were calculated. Time and temperature requirements to achieve specific destruction of the pathogens were calculated from the thermal destruction parameters, and the efficacy of the RFDH process was validated by heating NDM using RFDH to achieve the target temperatures and holding the product in a convection oven for the required period of time. Linear regression was used to determine the D-values and *z*-values. D-values of *C. sakazakii* in HH- and LH-NDM were 24.86 and 23.0 min at 75°C, 13.75 and 7.52 min at 80°C, 8.0 and 6.03 min at 85°C, and 5.57 and 5.37 min at 90°C, respectively. D-values of *Salmonella* spp. in HH- and LH-NDM were 23.02 and 24.94 min at 75°C, 10.45 and 12.54 min at 80°C, 8.63 and 8.68 min at 85°C, and 5.82 and 4.55 min at 90°C, respectively. The predicted

and observed destruction of *C. sakazakii* and *Salmonella* spp. were in agreement, indicating that the organisms' behavior was similar regardless of the heating system (conventional *vs.* RFDH). Radio frequency dielectric heating can be used as a faster and more uniform heating method to achieve the target temperatures for a post-process lethality treatment of NDM prior to the packaging.

4.2 Introduction

Nonfat dry milk (NDM) is a widely used dairy product that can be consumed directly or used as an ingredient in various food products, including powdered infant formula (PIF). Neither NDM nor PIF are sterile, and the possibility of post-pasteurization contamination of milk cannot be ruled out (Olsen et al., 2004; Brooks, 2010). *Cronobacter sakazakii* and *Salmonella* spp. have been associated with several sporadic outbreaks due to contaminated NDM and PIF (Bowen and Braden, 2006; Cahill et al., 2008; Astley, 2012) and have been isolated from NDM and PIF products (Louie et al., 1993; Iversen and Forsythe, 2004; Flynn, 2011a). Although *C. sakazakii* and *Salmonella* spp. cannot survive milk pasteurization (Read et al., 1968; Osaili et al., 2009), they can survive spray-drying temperatures if post-pasteurization contamination of the milk occurs (LiCari and Potter, 1970; Arku et al., 2008).

C. sakazakii and *Salmonella* spp. have been classified as class "A" pathogens (clear evidence of causality) in PIF by the Food and Agricultural Organization and World Health Organization of the United Nations (FAO/WHO, 2004a, b). *C. sakazakii* can result in life-threatening infections in infants such as bacteremia, meningitis, sepsis, cerebritis, and necrotizing enterocolitis (FAO/WHO, 2004a; Fiore et al., 2008; Lehner, 2010), and *Salmonella* spp. can cause severe diarrhea in infants that can result in death (FAO/WHO, 2004a).

Radio frequency dielectric heating (RFDH) is an electro-heating process in which electric energy is converted into electromagnetic waves that subsequently generate volumetric heat within a product (Rowley, 2001; Piyasena et al., 2003; Marra et al., 2009). Foods consist of dipoles (such as water) and ions (such as salts), which contribute to heat generation within a food product during RFDH (FDA, 2000). During RFDH treatment, a food product is placed between the capacitor plates of an RFDH unit and an alternating electric field is applied. Frictional heat is generated within the food product due to: (i) dipole rotation (the dipoles in the food align themselves along the electric field and oscillate continuously along the changing electric field), and (ii) ionic depolarization (the ions in the food move toward the opposite charged regions of the electric field and migrate continuously according to the changing electric field) (Piyasena et al., 2003; Ramaswamy and Tang, 2008; Marra et al., 2009). Because radio frequencies can interfere with radar and communication systems, the frequencies approved by Electromagnetic Compatibility Regulations and Federal Communications Commission for Industrial, Scientific, and Medical applications are: 13.56 ± 0.00678, 27.12 ± 0.16272, and 40.68 ± 0.02034 MHz (FDA, 2000; Rowley, 2001; Marra et al., 2009). The advantages of RFDH over conventional heating are better volumetric heating, more uniform heating, greater penetration power, lower surface overheating, fewer cold spots, shorter treatment time, improved energy efficiency, and better process control (FDA, 2000; Marra et al., 2009).

Although RFDH is used in baking, drying, and defrosting, commercial applications for food pasteurization and sterilization are scarce in North America (FDA, 2000; Rowley, 2001; Ramaswamy and Tang, 2008). Research on RFDH application for the thermal inactivation of foodborne pathogens has been explored in mashed potatoes for *Clostridium sporogenes* (Luechapattanaporn et al., 2004), scrambled eggs for *C. sporogenes* (Luechapattanaporn et al.,

2005), milk for *E. coli* and *Listeria innocua* (Awuah et al., 2005), ground beef for *E. coli* (Guo et al., 2006), apple cider for *E. coli* (Geveke and Brunkhorst, 2008), and pork luncheon meat for *Bacillus cereus* and *C. perfringens* (Byrne et al., 2010). Literature on *C. sakazakii* and *Salmonella* spp. in NDM or PIF suitable for commercial application is non-existent. Prior research on destruction of these pathogens in NDM and PIF was done either in liquid/rehydrated systems or at temperatures ≤70°C (Iversen at al., 2004; Kim and Park, 2007; Walsh et al., 2011). Therefore, the objective of this study was to determine the thermal processing parameters (decimal reduction times, D-values at 75, 80, 85, and 90°C; and thermal resistance constants, *z*-values) of *C. sakazakii* and *Salmonella* spp. in high-heat (HH) and low-heat (LH) NDM using a water bath and thermal-death-time (TDT) disks; and to validate an RFDH system for decontamination of NDM. Because HH- and LH-NDM are used for different applications in various food products, both were included in this study.

4.3 Materials and Methods

4.3.1 Experimental Design

A 5-strain cocktail of *C. sakazakii* or *Salmonella* spp. was used in this study because both of the organisms have been implicated in outbreaks from contaminated NDM. D- and *z*-values of *C. sakazakii* or *Salmonella* spp. in HH-NDM (ConAgra Foods, Menomonie, WI) and LH-NDM (Dairy America, Fresno, CA) were first determined using custom-designed stainless steel TDT disks (6.0 cm diameter and 0.5 cm height; University of Nebraska, Lincoln, NE) and a hot-water bath (Precision Scientific, Chicago, IL). On the basis of results obtained from the TDT disks, the RFDH experiment was designed to validate microbial destruction. The TDT disks and RFDH studies were considered independent experiments; HH- and LH-NDM were inoculated and heat-

treated randomly. Three replications were conducted for all treatments, and each microbial

enumeration was done in duplicate. Linear regression graphs were plotted using SAS version 9.1

(SAS Institute Inc., Cary, NC). All comparisons for *C. sakazakii* or *Salmonella* spp. in HH- and

LH-NDM at respective temperatures were conducted with the Student's t-test at $P \leq 0.05$ in SAS

9.1.

4.3.2 Bacterial Cultures

C. sakazakii and *Salmonella* spp. strains were selected based on risk and involvement in

outbreaks associated with NDM or PIF, isolated from food processing plants, and used in

published research. *C. sakazakii* 29544 and BAA-894 were obtained from the American Type

Culture Collection (ATCC; Manassas, VA), and one environmental isolate and two processing-

plant isolates of *C. sakazakii* were obtained from the Applied Food Safety Laboratory,

University of Nebraska (Lincoln, NE). *Salmonella* serotypes Agona BAA-707, Tennessee 10722,

and Typhimurium 13311 were obtained from the ATCC, and *Salmonella* Montevideo and

Senftenberg (processing plant isolates) were obtained from the Food Safety and Defense

Laboratory, Kansas State University (Manhattan, KS). All strains were confirmed using API 20E

biochemical identification strips (Biomérieux, Durham, NC).

4.3.3 Inoculum Preparation and Inoculation Procedure

All organisms were individually grown on tryptic soy agar (TSA; Difco, Becton

Dickinson Company, Sparks, MD), and isolated colonies were maintained at 4°C on TSA plates.

An individual colony of each strain was transferred from TSA to 10 mL of tryptic soy broth

(TSB; Difco, Becton Dickinson Company, Sparks, MD) and incubated at 37°C for 24 h. A loop

(25 µL) of each strain was individually transferred into 40 mL of TSB in centrifuge tubes and

statically incubated at 37°C for 24 h. Strains were then centrifuged at 5,520 × *g* at -4°C for 15

min, the supernatant was discarded, and pellets were reconstituted in 40 mL of 0.1% peptone

water (Difco, Becton Dickinson Company, Sparks, MD). Five strains of *C. sakazakii* or

Salmonella spp. were mixed and transferred into separate sanitized spray bottles (300 mL).

High-heat or LH-NDM in sanitized plastic tubs (9.4 L, Rubbermaid, Atlanta, GA) was

mist-inoculated with the 5-strain mixed cocktails of either *C. sakazakii* or *Salmonella* spp. by

spraying one squirt (~0.8 mL) per 10 g NDM. Inoculated NDM was then dried with open lids at

37°C for 24 h (to achieve the original moisture content of NDM; data not presented), then

ground using a food processor (Ninja, Euro-Pro Operating LLC, Boston, MA) for 1 min to

homogenize the inoculum.

4.3.4 Heat Treatment

4.3.4.1 Thermal-death-time Disks

Thermal-death-time disks (Figure 4.1) used in this study were similar to those used by Jin

et al. (2008); however, the disks in this study were manufactured from stainless steel instead of

aluminum. Eight grams of inoculated NDM were placed in sanitized TDT disks, sealed, and

transferred into a hot-water bath (pre-set at 75, 80, 85, or 90°C). Temperatures of samples

contained within TDT disks and the hot-water bath temperature were monitored using

thermocouples (Hypodermic Needle Probes, Omega Engineering Inc., Stamford, CT) and data

logger (Measurement Computing USB-TC and MCC DAQ Software, Norton, MA). Once the

target temperatures were reached, samples were held for 0 to 80 min, and were then transferred

from the hot-water bath into a cold-water bath (~4°C) at specific time intervals. The processed

samples were cooled to ~10°C, then immediately enumerated for viable *C. sakazakii* or

Salmonella spp.
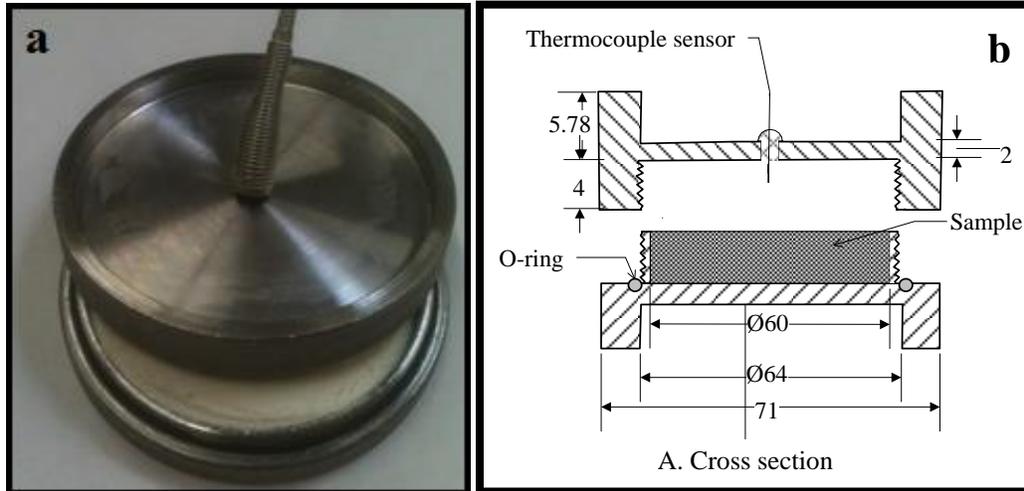
Figure 4.1 Thermal-death-time (TDT) disk: a) containing inoculated nonfat dry milk and connected to a thermocouple; b) schematics of the TDT system

4.3.4.2 Radio Frequency Dielectric Heating

Inoculated HH-NDM (5 g) or LH-NDM (7 g) was placed in a sanitized circular container (Aqua Lab, Decagon, Pullman, WA), covered with a lid, then wrapped in Parafilm M (Pechiney Plastic Packaging, Chicago, IL). The sealed, inoculated container was placed at the center of the circular high-density polyethylene tray (0.3 cm thickness, 22.4 cm diameter, and 2.1 cm height; Dynalab Corp., Rochester, NY) along with a non-inoculated sample container (Figure 4.2.a) to monitor the temperature using a fiber-optic probe (T1, Neoptix Inc., Québec, Canada) and a data logger (Reflex, Neoptix Inc., Québec, Canada). The circular tray was then filled with non-inoculated HH- or LH-NDM, and the product surface in the tray was leveled (with a 30-cm ruler) to a height of 2.1 cm (Figure 4.2.b).

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Figure 4.2 Inoculated and non-inoculated (connected with fiber-optic probe) nonfat dry milk in sealed containers in high-density polyethylene tray for radio frequency dielectric heating: a) before covering with non-inoculated nonfat dry milk; b) after covering with non-inoculated nonfat dry milk

Samples were heated in the RFDH unit (Figure 4.3; Strayfield Limited, Theale, Berkshire, England) set at 27.12 MHz frequency, 3 kW power, and 120 mm electrode distance until target temperatures were achieved. Samples were then quickly transferred to a convection oven (Thelco, GCA/Precision Scientific, Schaar Scientific Company, Chicago, IL) pre-set at the designated temperatures for specific time periods. Processed samples were then transferred to a cold-water (~4°C) bath. The samples were cooled to ~10°C, then immediately enumerated for viable *C. sakazakii* or *Salmonella* spp.

Figure 4.3 Radio frequency dielectric heating unit

4.3.5 Microbial Enumeration

The treated NDM products (5 or 7 g) were transferred to a stomacher bag, to which 45 or

43 mL of peptone water (0.1%) was added. Samples were stomached (Smasher, AES

Laboratoire, Bruz, France) for 1 min, and serial dilutions (1:10) were subsequently prepared

using peptone water diluent. Sample homogenates were spread-plated on TSA and incubated at

37°C for 24 h. Non-inoculated HH- and LH-NDM were also plated on TSA to quantify the

background microbial load and detect the presence of natural contamination by *C. sakazakii* and

Salmonella spp.

4.3.6 Determination of D- and z-values

Regression lines were generated from the log of viable microbes *vs*. time, and D-values were calculated as absolute values of the inverse of the slopes. For *z*-values, regression lines were generated from the log of D-values *vs.* temperatures, and *z*-values were calculated as absolute values of inverses of the slopes (Al-Holy et al., 2009).

4.3.7 Observed vs. Predicted Microbial Destruction

On the basis of thermal destruction parameters (obtained from the TDT disks study), times required to achieve a 3-log cycle reduction (predicted log reduction) of *C. sakazakii* and *Salmonella* spp. in HH- and LH-NDM were calculated. The RFDH study was then conducted to validate the log reduction (observed log reduction) of *C. sakazakii* and *Salmonella* spp. in HH- and LH-NDM at respective temperatures for calculated times. The observed reductions of *C. sakazakii* and *Salmonella* spp. were plotted against predicted reductions and compared.

4.4 Results and Discussion

Mean times required for NDM to achieve the target temperatures in the TDT disks and RFDH unit from an initial temperature of ~27°C are presented in Table 4.1. For all the temperatures, the time required to achieve the target temperatures was lower ($P \leq 0.05$) in the RFDH unit compared with TDT disks (Table 4.1). These results confirmed that RFDH can heat a low-moisture food product faster than conventional heating methods. In the TDT disks, heat was transferred mainly by conduction between the stainless steel TDT disks and NDM, whereas heat was generated within NDM due to dielectric heating in the RFDH unit.

Table 4.1 Time (min)[1] required to achieve target temperatures for nonfat dry milk in thermal-death-time (TDT) disks and by radio frequency dielectric heating (RFDH) unit

Temperature (°C)	75	80	85	90
TDT disks	7.94 ± 0.96*	9.51 ± 1.11*	8.86 ± 0.72*	10.72 ± 0.87*
RFDH	4.26 ± 0.23*	4.54 ± 0.32*	4.95 ± 0.30*	5.50 ± 0.38*

[1] Mean ± SE (n = 3)

* Values within a column are different ($P \leq 0.05$)

The non-treated initial total plate counts in HH- and LH-NDM were 2.05 ± 0.05 and 2.41 ± 0.06 log cfu/g (n = 3), respectively (which were within the standards; of <4 log cfu/g; USDEC, 2010), and the samples were negative for *C. sakazakii* and *Salmonella* spp. (detection limit of 1 log cfu/g). The thermal inactivation of *C. sakazakii* and *Salmonella* spp. in HH- and LH-NDM using TDT disks are presented in Figures 4.4 and 4.5. The TDT curves (log of D-value *vs.* temperature) were used to calculate *z*-values (Figure 4.6).
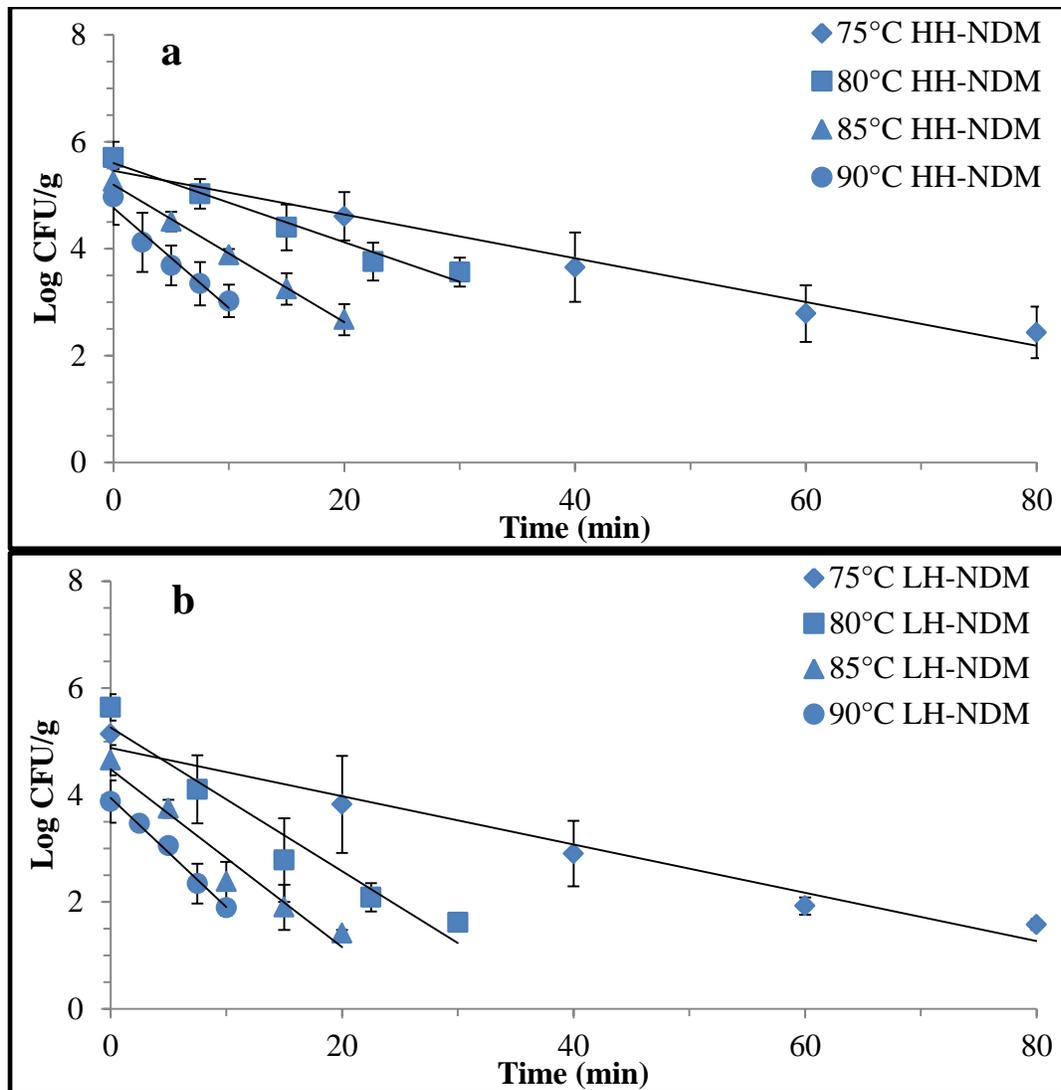
Figure 4.4 Thermal inactivation of *Cronobacter sakazakii* using thermal-death-time disks at 75, 80, 85 and 90°C in a) high-heat (HH) and b) low-heat (LH) nonfat dry milk (NDM)
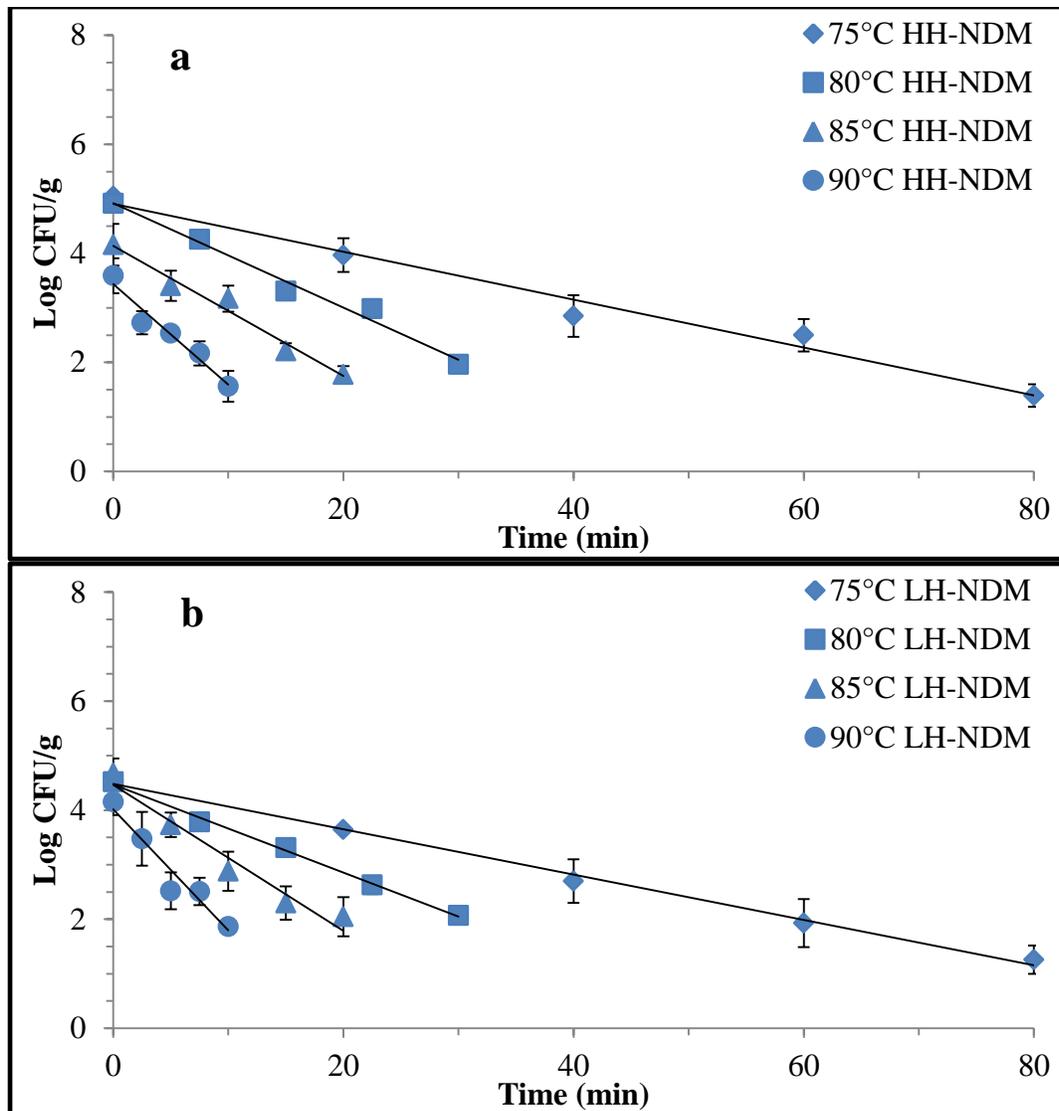
Figure 4.5 Thermal inactivation of *Salmonella* spp. using thermal-death-time disks at 75, 80, 85 and 90°C in a) high-heat (HH) and b) low-heat (LH) nonfat dry milk (NDM)
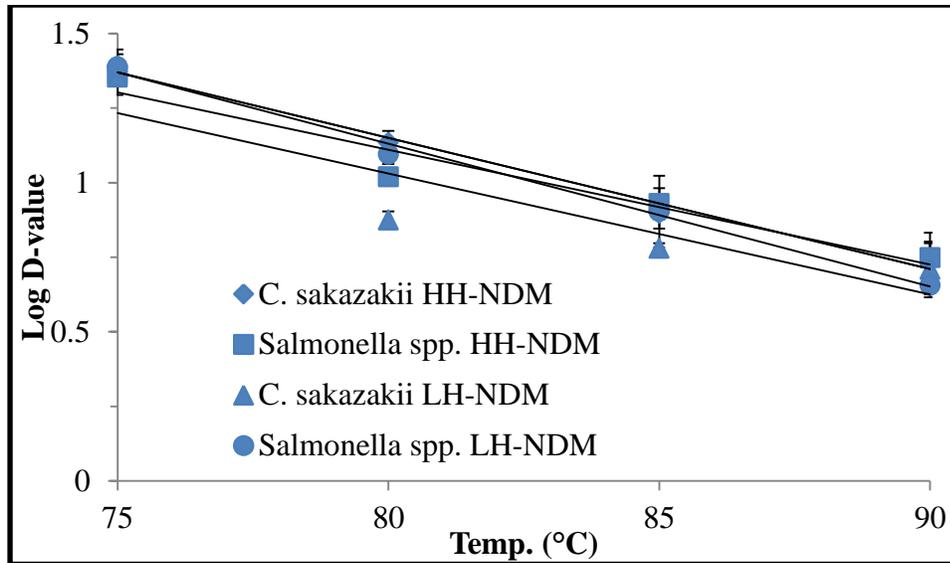
Figure 4.6 Log D-value *vs.* temperature for *Cronobacter sakazakii* and *Salmonella* spp. in high-heat (HH) and low-heat (LH) nonfat dry milk (NDM) using thermal-death-time disks

The initial pre-treatment counts of *C. sakazakii* or *Salmonella* spp. in inoculated NDM were ~6.0 log cfu/g. The D-values of *C. sakazakii* or *Salmonella* spp. for HH- and LH-NDM at the same temperatures were similar ($P > 0.05$), except for C. *sakazakii* in HH- and LH-NDM at 80°C (Table 4.2). The D-values of *C. sakazakii* and *Salmonella* spp. varied from 23.00 to 26.25 min at 75°C, 7.52 to 13.75 min at 80°C, 6.03 to 8.68 min at 85°C, and 3.05 to 5.82 min at 90°C, respectively (Table 2). The *z*-values of *C. sakazakii* in HH- and LH-NDM were 23.77 and 26.28°C, respectively, whereas *z*-values of *Salmonella* spp. in HH- and LH-NDM were 26.92 and 20.92°C, respectively (Table 4.2).

Table 4.2 D-values (min)[1] and *z*-values (°C)[1] of *Cronobacter sakazakii* and *Salmonella* spp. in high-heat (HH) and low-heat (LH) nonfat dry milk (NDM) heated in thermal-death-time disks

Temperature	*C. sakazakii*		*Salmonella* spp.	
(°C)	HH-NDM	LH-NDM	HH-NDM	LH-NDM
75	24.86 ± 2.33	23.00 ± 3.17	23.02 ± 1.53	24.94 ± 3.50
80	13.75 ± 1.22*	7.52 ± 0.48*	10.45 ± 0.15	12.54 ± 0.98
85	8.00 ± 0.93	6.03 ± 0.24	8.63 ± 1.04	8.68 ± 2.61
90	5.57 ± 0.79	5.37 ± 1.24	5.82 ± 1.18	4.55 ± 0.33
z-value	23.77 ± 3.70	26.28 ± 4.73	26.92 ± 3.65	20.92 ± 0.40

[1] Mean ± SE (n = 3)

* Values within a row and under respective bacterium are different ($P \leq 0.05$)

Observed log reductions were in agreement with predicted log reductions (Figure 4.7), except observed log reductions of *C. sakazakii* in HH- and LH-NDM at 90°C and *Salmonella* spp. in HH-NDM at 90°C were >1.5 log cfu/g compared with predicted reductions. Overall, the predicted and observed log reductions for *C. sakazakii* and *Salmonella* spp. were similar using TDT disks and RFDH methods, indicating that the destruction of organisms is mainly due to the thermal effect. Radio frequency dielectric heating reduced the time to achieve the target temperatures compared with conventional heating. Geveke et al. (2007), however, reported that RFDH treatment of pulp-free orange juice at 21.1 kHz and 60°C reduced the *E. coli* K12 population by 2.1 log cfu/mL, whereas *E. coli* population did not decrease when juice was heated at 60°C using the conventional method, suggesting that non-thermal effects could contribute to these differences.
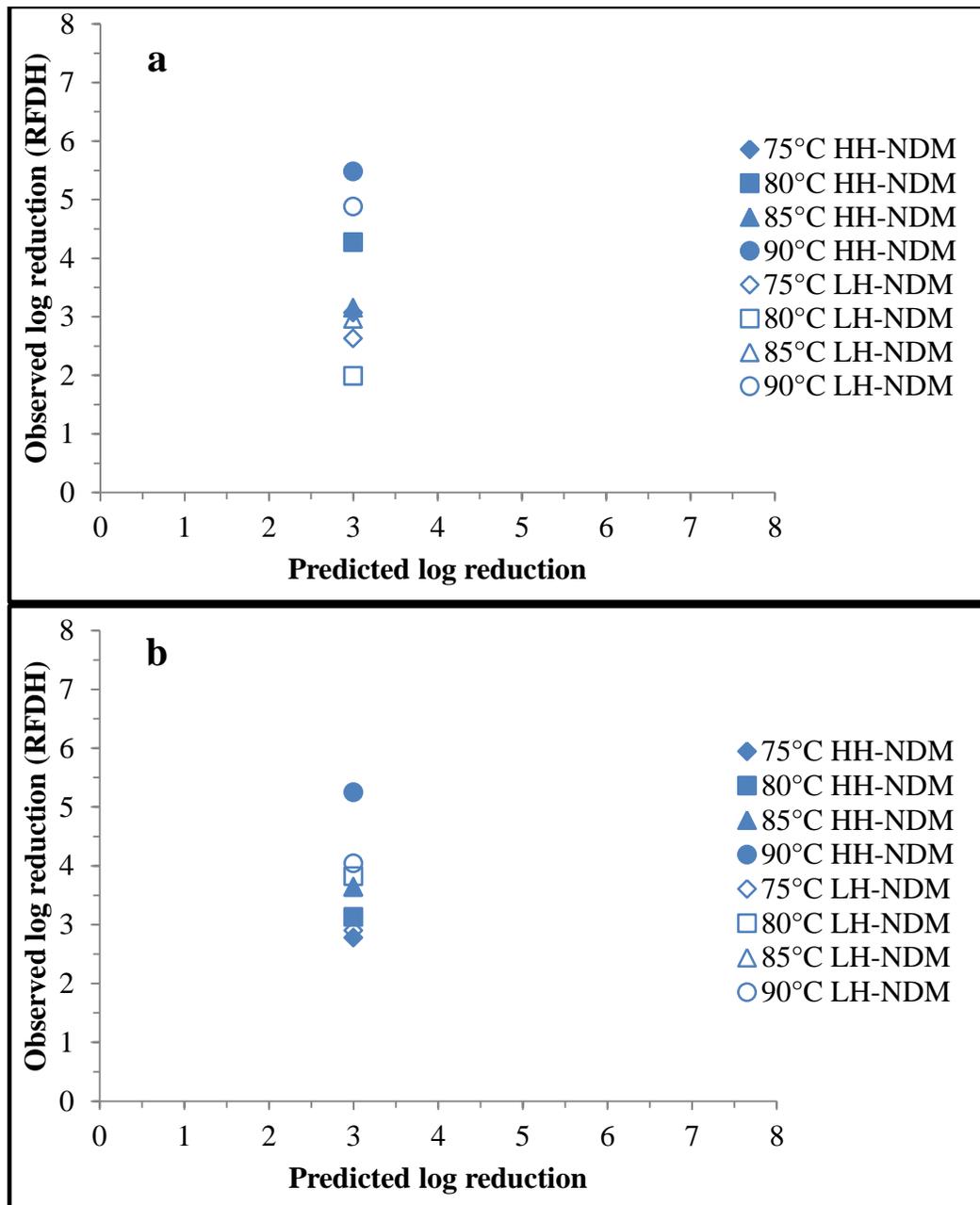
Figure 4.7 Predicted *vs.* observed log reductions of: a) *Cronobacter sakazakii* and b)

***Salmonella* spp. in high-heat (HH) and low-heat (LH) nonfat dry milk (NDM)**

Geveke and Brunkhorst (2008) reported that an *E. coli* K12 population in apple cider was

reduced by 4.8 log cfu/mL by RFDH at 60°C and 21 kHz compared with no reduction using

conventional heating at 60°C. Ukuku et al. (2012) suggested that non-thermal effects of RFDH on microbial cells could be due to a decrease in the microbial cell surface hydrophobicity and loss of negative ions.

Thermal inactivation data of *C. sakazakii* and *Salmonella* spp. in NDM or foods with low water activity (at the temperatures investigated in this study) are not available in the literature. The published thermal destruction parameters for *C. sakazakii* and *Salmonella* spp. in various dry products were conducted either at temperatures ≤ 70°C or as rehydrated products (such as infant formula). Lound et al. (2011) reported that D-values of non-acid-adapted *Salmonella* Typhimurium in powdered egg albumin heat-treated in a hot water bath were 99.56, 43.90, and 15.54 min at 72, 77, and 82°C, respectively. These D-values were greater than the D-values for *Salmonella* spp. in NDM at 75, 80, and 85°C in this study. Perhaps the differences in *Salmonella* spp. serovars/strains used and/or inoculation procedures [dried at 50°C after spray inoculation before heat treatment by Lound et al. (2011) *vs.* dried at 37°C after spray inoculation in this study], could have contributed to the differences in D-values. In addition, the compositional (proteins, carbohydrates, fat, etc.) differences between egg albumin and NDM could contribute to these differences in the D-values of *Salmonella* spp. VanCauwenberge et al. (1981) reported variability in the $D_{49°C}$ for *Salmonella* spp. (8 different serotypes) in corn flour, ranging from 18 to 594 min.

Luechapattanaporn et al. (2004) reported that D-values of *Clostridium sporogenes* (PA3679) in mashed potatoes ($a_w = 0.998$), using TDT tubes submerged in a heated oil bath, ranged from 2.16 to 0.61 min at 115.6 to 121.1°C. From this data, they calculated F_o values, which validated the microbial destruction using RFDH. Luechapattanaporn et al. (2005) used the same procedure to validate D-values in the RFDH, but with scrambled eggs as the medium;

moreover, both the Luechapattanaporn et al. (2004, 2005) studies maintained the samples in a RFDH unit, submerged in a water chamber to reduce the fringe (or edge) effect, during the entire process. In our study, samples were transferred to a convection oven for process time after attaining the target temperatures in RFDH. Mashed potatoes and scrambled eggs obviously had greater moisture contents than NDM (<5%), plus the microorganisms differed. Products with greater moisture contents have greater dielectric constants and loss factors, which result in greater conversion of radio frequencies into thermal energy compared with products with low moisture contents (Komarov et al., 2005).

Chen et al. (2013) studied the functional properties of HH- and LH-NDM by heating NDM in a RFDH unit to 75, 80, 85, and 90°C, and holding the samples in a convection oven up to 125.67, 57.75, 25.0, and 11.50 min, respectively. They reported that the whey protein nitrogen index (WPNI) of heat treated HH-NDM was similar to that of a non-heated control (1.48 mg/g) and ranged from 1.32 to 1.48 mg/g, except WPNI of HH-NDM heated at 80°C for 57.75 min, and 85°C for 10 and 25 min (1.32 to 1.34 mg/g) were lower than that of non-heated control. Whereas, WPNI of LH-NDM samples heated at temperatures ≥80°C (5.78 to 6.48 mg/g) were lower than that of a non-heated control (7.24 mg/g). Although WPNI of some NDM differed ($P \leq 0.05$) from the non-heated controls, all samples maintained their classification standards (>6 mg/g for HH-NDM, and <1.5 mg/g for LH-NDM), except for LH-NDM heated at 85°C for 25 min. Nitrogen solubility index (NSI) of all heated NDM samples at temperatures ≥80°C were lower (89.0 to 87.9% and 88.3 to 89.4% for HH- and LH-NDM, respectively; $P \leq 0.05$) than that of non-heated controls (90.6 and 93.3% for HH- and LH-NDM, respectively). Chen et al. (2013) also reported that color of HH-NDM was not affected by the heat treatment, except HH-NDM heated at 90°C for 0 min (b* = 13.27) was yellower than the non-heated control (b* = 11.46).

LH-NDM was also not affected by the heat treatment, except LH-NDM heated at 75 and 85°C for 25 and 10 min, respectively (a* = -2.51 and -2.44, respectively) were greener than non-heated controls (a* = -1.89), and LH-NDM heated at 90°C for 0 min (b* = 14.43) was yellower than the non-heated control (b* = 12.27).

4.5 Conclusions

The RFDH treatment can be used as a means of heating NDM to shorten its overall post-process lethality treatment prior to packaging to minimize food safety risks. The effects of RFDH on the functional properties of NDM, such as whey protein nitrogen index, solubility, color, and flowability, should also be evaluated. Thermal resistance of *C. sakazakii* and *Salmonella* spp. in NDM should be further validated under commercial conditions.

4.6 Acknowledgements

Chapter 5 - Experiment II: Hyperspectral Imaging of Various Foodborne Pathogens for Rapid Identification and Differentiation, and Effect of Age and Lauric Arginate Treatment of Pathogens on Hyperspectral Imaging

5.1 Abstract

Rapid identification and differentiation of foodborne pathogens is of vital importance to ensure food safety. Presence of contaminated food products on a retail shelf not only poses a threat of foodborne illnesses or outbreaks but could also result in enormous economic losses to food manufacturers and retail establishments. Although there are well-established traditional microbiological methods for the detection and identification of foodborne pathogens, they are cumbersome and slow (from 4 to 7 days to give confirmatory results). Scientists all over the globe are striving to develop novel techniques for the rapid detection and identification of pathogens. Hyperspectral imaging (HSI) is an immerging technology in food science that has a great potential in the field of food safety and quality. Hyperspectral imaging provides both spatial and spectral information of a sample by combining imaging with spectroscopy. The objective of this study was to generate hyperspectral graphs of common foodborne pathogens, and to develop and validate prediction models for the classification of these pathogens. Four strains of *Cronobacter sakazakii*, 5 strains of *Salmonella* spp., 8 strains of *Escherichia coli*, and 1 strain each of *Listeria monocytogenes* and *Staphylococcus aureus* were used in the study. Principal component analysis and *k*NN (*k*-nearest neighbor) classifier model were used for the classification of hyperspectra of various bacterial cells, which were then validated using the cross-validation technique. Classification accuracy of various strains within genera including *C. sakazakii*, *Salmonella* spp. and *E. coli*, respectively was 100%; except within *C. sakazakii,* strain

BAA-894, and within *E. coli,* strains O26, O45 and O121 had 66.67% accuracy. When all strains were studied together (irrespective of their genus) for the classification, only *C. sakazakii* P1, *E. coli* O104, O111 and O145, *S.* Montevideo and *L. monocytogenes* had 100% classification accuracy; whereas, *E. coli* O45 and *S.* Tennessee were not classified (classification accuracy of 0%). Strains of *E. coli* O157:H7 and *L. monocytogenes* used to study the affect of age on HSI had 100% classification accuracy at 12, 16, 20 and 24 h, suggesting that bacterial cells have distinguished hyperspectral signatures at different incubation times; however, no regular trend in hyperspectral graphs or intensities was observed with the increase of incubation time. Lauric arginate treatment of *C. sakazakii* BAA-894, *E. coli* O157, *S.* Senftenberg, *L. monocytogenes* and *S. aureus* significantly affected their hyperspectral signatures and treated cells could be differentiated from the healthy, non-treated cells. With future HIS validation studies including greater variety and number of strains, HSI potentially can be implemented as a rapid identification and classification technique for various foodborne pathogens at the presumptive level. This procedure likely can be incorporated into commonly utilized detection/identification protocols to enhance their accuracy and to reduce false negative sample screenings i.e. directly identify presumptive pathogen colonies on crowded agar plates which can be picked and further confirmed using immune- or molecular-based methods.

5.2 Introduction

Hyperspectral imaging (HSI), originally designed for remote sensing, has found its application in various fields such as astronomy, agriculture, pharmaceutical and medicine (Gowen et al., 2007; Smith, 2012). Hyperspectral imaging uses near-infrared (NIR) spectra (400 to 1000 nm) to acquire both spatial and spectral information of a sample by combining imaging with spectroscopy (Gowen et al., 2007; Dale et al., 2013). During HSI, hyperspectral images of a

sample are captured at various pre-defined wavelengths; and reflectance, transmittance, absorbance or fluorescence (depending on the molecular and chemical structure of a sample) is measured over this wavelength range to generate hyperspectral graphs. These hyperspectral graphs (used to develop a library for different organisms) can then be used for the rapid identification of a sample.

Hyperspectral imaging has a great potential in the area of rapid detection and identification of foodborne pathogens. Although at the present state of the technology, HSI cannot replace the traditional microbiological identification methods, but it can be used effectively at presumptive levels for the identification of pathogens. Rapid and early detection of pathogens in food is vital at every stage of processing; especially for 'zero tolerance' pathogens such as *Escherichia coli* O157:H7 and Big Six Shiga toxin producing *E. coli* (STEC; O26, O45, O103, O111, O121 and O145) in ground beef, and *Listeria monocytogenes* in ready-to-eat foods (Buchholz and Mascola, 2001; FSIS, 2012a, b). Another sensitive area where early and faster detection of pathogens is vital is the detection of *Salmonella* spp. and *Cronobacter sakazakii* in powdered infant formula manufactured for newborns and infants.

Previous research done utilizing HSI for the rapid identification and differentiation of various pathogens has been conducted on a macro scale using bacterial colonies grown on a media agar, but studies at micro scale (at the single bacterial cell level) are not present in the literature. Using HSI, Yoon et al. (2013a, b) were able to identify Big Six STEC grown on Rainbow Agar as pure and mix cultures at accuracy levels from 80 to 100%. Fiore et al. (2010) were able to discriminate maize kernels infected with *Aspergillus niger* and *A. flavus* from uninfected kernels using HSI. The U.S. Department of Agriculture's Agriculture Research Service (ARS) developed and successfully tried (under commercial conditions) a HSI system to

detect and separate fecal contaminated poultry carcasses from the good carcasses (Harrington, 2009). Factors like morphology of colonies grown on media agar and presence of food components on food surfaces, along with individual or clusters of bacterial cells, can interfere with HSI and affect the hyperspectra. Therefore, HSI of bacteria at the cellular level should be studied to exclude interfering factors in generating hyperspectra of targeted bacteria.

Lauric arginate (LAE) is a generally recognized as safe (GRAS) food-grade antimicrobial exhibiting activity against a wide range of pathogens (Saini et al., 2013). Lauric arginate has been studied for its use in food as an antimicrobial treatment and as a sanitizer for food contact surfaces (Saini 2012; Becerril et al., 2013). According to the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture, LAE can be used in various meat and poultry products up to 200 ppm by weight of the finished product (FSIS, 2013b). Lauric arginate disrupts the plasma membrane of bacterial cells, which alters the metabolic processes and inhibits normal cellular cycles (Bakal and Diaz, 2005). Therefore, it is important to study if inactive or sublethally injured bacterial cells (by the action of an antimicrobial such as LAE) can be differentiated from active and live bacterial cells using HSI.

Therefore, the main objective of this research was to study if hyperspectral graphs/signatures obtained from HSI of individual bacterial cells could be used for the rapid presumptive identification and differentiation of various strains of pathogenic bacteria. This included the development and validation of classification models for common foodborne pathogens. Affect of culture age and LAE treatment of various strains on hyperspectral signatures of these bacteria were also studied.

5.3 Materials and Methods

5.3.1 Experimental Design

To study if HSI can be used for the rapid identification and differentiation of various foodborne pathogens, 4 strains of *C. sakazakii*, 5 strains of *Salmonella* spp., 8 strains of *E. coli*, and 1 strain each of *L. monocytogenes* and *S. aureus* were used in the study. All strains were individually grown on tryptic soy agar (TSA; Difco, Becton Dickinson Company, Sparks, MD) for isolation, and hyperspectral images of these cells from the isolated colonies were captured. These images were then used to generate hyperspectral graphs of respective bacterial cells that were stored in a reference library. Principal component analysis (PCA) and *k*NN (*k* Near Neighbor) classifier model were used for the classification of hyperspectra of various pathogens, followed by the "cross-validation" technique. To study the classification of hyperspectral graphs on the basis of age during growth, one Gram positive (*L. monocytogenes*) and one Gram negative (*E. coli* O157:H7) bacteria were grown on tryptic soy broth (TSB; Difco, Becton Dickinson Company, Sparks, MD), and HSI was conducted every 4 h from 12 to 24 h. For LAE treatment, one strain from each genus was grown in TSB for 24 h and then treated with 2,000 ppm LAE followed by HSI. Three replications were conducted for each strain for generating hyperspectral graphs; and within each replication, HSI of various strains were conducted randomly.

5.3.2 Culture Propagation

The seventeen Gram negative (4 *C. sakazakii,* 5 *Salmonella* spp. and 8 Shiga toxin-producing *E. coli*) and two Gram positive (*L. monocytogenes* and *S. aureus*) foodborne pathogenic bacteria used in this study are presented in Table 5.1. All strains were selected on the basis of risk and involvement in foodborne disease outbreaks, or isolated from the environment

and food processing facilities. All cultures were stored on TSA at 4°C; and confirmed using

API® 20E (for *C. sakazakii, Salmonella* spp. and *E. coli* strains; Biomérieux, Durham, NC),

API® *Lister* (for *L. monocytogenes*) and API® Staph (for *S. aureus*).

Table 5.1 Gram-positive and Gram-negative bacteria used in the study

Bacteria	Strain/Serotype	Source
Cronobacter sakazakii	BAA-894	ATCC*
	Environmental isolate (E1)	University of Nebraska, Lincoln
	Processing plant isolate 1 (P1)	University of Nebraska, Lincoln
	Processing plant isolate 2 (P2)	University of Nebraska, Lincoln
Salmonella spp.	Agona BAA-707	ATCC
	Tennessee 10722	ATCC
	Typhimurium 13311	ATCC
	Montevideo	Kansas State University
	Senftenberg	Kansas State University
Escherichia coli	O26	University of Nebraska, Lincoln
	O45	University of Nebraska, Lincoln
	O103	University of Nebraska, Lincoln
	O104	University of Nebraska, Lincoln
	O111	University of Nebraska, Lincoln
	O121	University of Nebraska, Lincoln
	O145	University of Nebraska, Lincoln
	O157	Kansas State University
Listeria monocytogenes	SLR 2249	ATCC
Staphylococcus aureus	25923	ATCC

* American Type Culture Collection

5.3.3 Bacterial Cell Samples Preparation

Bacterial cell sample preparation for HSI is summarized in Figure 5.1. A loop (1 μL)

from stock cultures stored on TSA was individually transferred into 10 mL TSB, and incubated

for 24 h at 35°C for *L. monocytogenes* and *E. coli*, and 37°C for *C. sakazakii*, *Salmonella* spp.

and *S. aureus*. Incubated strains were then individually streaked on TSA for isolation, and incubated for 24 h at respective temperatures. For each strain, one loop from an isolated colony was mixed in 1 mL filtered sterilized (0.2 μL) HPLC grade water (Fisher Scientific, Pittsburg, PA) in a micro-centrifuge tube by vortexing for ~1 min. Ten μL of vortexed samples were individually transferred on clean and sanitized 1 mm glass slides (Fisherfinest®, Fisher Scientific, Pittsburgh, PA), air dried in a biosafety cabinet (SterilGARD® II, The Baker Company, Sanford, ME) for ~5 min, and used for HSI analysis.

5.3.4 Affect of Age on Hyperspectral Imaging

To study the affect of age on hyperspectral imaging, *L. monocytogenes* and *E. coli* O157:H7 were selected (representing a bacterium from Gram-positive and Gram-negative groups, respectively). A loop from the stock culture of both bacteria on TSA were individually transferred to 10 mL of TSB tubes and incubated at 35°C for 24 h. A loop from these incubated TSB tubes was then transferred into a fresh 10 mL TSB tubes (four tubes per bacteria), and incubated at 35°C for 24 h. During incubation HSI were conducted at times: 12, 16, 20 and 24 h. At each sampling time inoculated TSB tubes were centrifuged at 4960 □ g at - 4°C for 15 min, supernatant was discarded, and pellets were washed with 0.1% peptone (Difco, Becton Dickinson Company, Sparks, MD) solution, and centrifuged again. After centrifugation supernatant was discarded, and one loop from the pellet in centrifuge tube was used for HSI as described in Figure 5.1.

```
┌──────────────────────────────────────────────────────────────┐
│        Transfer one loop (1 µL) stock culture on TSA to 10 mL TSB        │
│                              ↓                                 │
│              Incubate for 24 h at 37°C                        │
│                              ↓                                 │
│              Streak for isolation on TSA                      │
│                              ↓                                 │
│              Incubate for 24 h at 37°C                        │
│                                                                │
│              One colony (1 µL loop)                           │
│                              ↓                                 │
│        Vortex in micro-centrifuge      ←    1 mL filtered     │
│             tube for ~ 1 min                sterilized (0.2 µm)│
│                              ↓              HPLC grade water   │
│        Transfer 10 µL on a clean sanitized glass slide (1 mm) │
│                              ↓                                 │
│            Air dry in Biosafety Cabinet (~ 5 min)            │
│                              ↓                                 │
│              Hyperspectral Imaging                           │
│                              ↓                                 │
│        Save Hyperspectral Image and Graph in Library         │
└──────────────────────────────────────────────────────────────┘
```

Figure 5.1 Bacterial cell sample preparation for hyperspectral imaging

5.3.5 Lauric Arginate Treatment

For LAE (as Ethyl Lauroyl Arginate HCL; Vedeqsa Inc., Spain) treatment, one strain

from each genus was selected: *C. sakazakii* BAA-894, *E. coli* O157:H7, *L. monocytogenes*, *S.*

aureus and *Salmonella* Senftenberg. Based on the previous research conducted on LAE

treatment of veal carcasses in our laboratory, in which ~2,000 ppm LAE solution was used to

achieved a final 200 ppm LAE by weight of beef cut being treated, 2,000 ppm LAE

concentration was selected for this study. A loop from the stock culture of all bacteria on TSA were individually transferred into 10 mL of TSB and incubated for 24 h at respective temperatures. For all strains, a loop from the inoculated TSB was then transferred to 10 mL TSB tubes and again incubated for 24 h at respective temperatures. For each strain, after inoculation, 3 mL of inoculated TSB was mixed with 6 mL of non-inoculated TSB and 1 mL of LAE (to achieve the final concentration of 2,000 ppm) and stored for 5 min at 4°C. After LAE treatment, 5 mL of D/E Neutralizing broth was immediately added to the tubes (to neutralize the solution and arrest the antimicrobial affect of LAE). Tubes were then centrifuged at 4960 □ g at **-** 4°C for 15 min, supernatant was discarded and pellets were washed with 0.1% peptone solution, and centrifuged again. After centrifugation supernatant was discarded, and one loop from the pellet in centrifuge tube was used for HSI analysis of each strain (as described in Figure 5.1).

5.3.6 Hyperspectral Graphs Generation

A CytoViva® microscope system (CytoViva, Inc., Auburn, AL) used for capturing hyperspectral images in the study is presented in Figure 5.2. Environment for Visualizing Images (ENVI, Exelis Visual Information Solutions, Boulder, CO) software version 4.4 was used for analyzing acquired hyperspectral images and generating hyperspectral graphs. Hyperspectral images of individual bacterial cells on air-dried glass slides were acquired by focusing the microscope at 4,000 × magnification, and ENVI settings at: 0.5 s exposure time, low grain, and low spatial and spectral resolutions (as recommended by CytoViva® personal communication). Hyperspectral images were acquired using 'push broom' (also known as 'line scan') technique in which the glass slide supporting the dried culture on the motorized stage of microscope was moved across the field of view of the microscope during image acquisition. Using ENVI, three bacterial cells were selected from the acquired images as regions of interest (ROI). Average

scattering values at respective wavelengths of these three ROI were used to generate hyperspectral graphs at wavelengths ranging from 400 to 1,000 nm (at wavelength interval of ~1.29 nm resulting in 465 wavelength bands). These graphs were then stored in a reference library for future use and comparison/classification.

Figure 5.2 CytoViva® Hyperspectral Imaging Microscope System. 1: Halogen light source; 2: Optical microscope; 3: Optical Camera; 4: VNIR spectrophotometer; 5: Motorized stage; 6: Controller for stage; 7: Computer

5.3.6 Principal Component Analysis, Classification and Validation of Hyperspectral Graphs

Pre-processing is the first important step in statistical analyses and classification of a spectral data set; however, no general guidelines or rules exist for selecting a particular pre-processing technique for a specific type of a data set (Scott et al., 2007). The type of pre-

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processing technique used for a particular data set should aim to provide the best possible classification accuracy. For this study, spectral data/graphs were first pre-processed by reducing the number of wavelength bands from 465 (ranging from 400 to 1,000 nm) to 255 (ranging from 425.57 to 753.84 nm). Hyperspectral graphs were then pre-processed by normalizing the y-axis (scattering value) from values 0 to 1 (Scott et al., 2007), with '1' being the brightest point on the ROI and '0' being the darkest point. The following equation was used to calculate normalized scattering values (Scott et al., 2007):

$$\underline{Xij} = [Xij - min(Xj)]/[max(Xj) - min(Xj)]$$

where, $\underline{Xij}$ is normalized scattering value at a particular wavelength

Xij is actual scattering value at a particular wavelength

$min(Xj)$ is minimum scattering value

$max(Xj)$ is maximum scattering value

Unsupervised PCA of normalized hyperspectral graphs was conducted to reduce the dimensionality and redundancy in the data set (scattering values at the respective wavelengths) for the classification (Jun et al., 2009). The new coordinates of the spectral data with reduced dimensions are called principal components (PC), which explain the variability within the data (Del Fiore et al., 2010). The PCs explaining 99% of the variability in the data set were used for the further analysis and classification of hyperspectral graphs. Principal component analysis plots for various sets of hyperspectral graphs were plotted using the first two PCs for approximate visualization of differences and similarities among the strains. The mathematical model was constructed using the following equation (Romía and Bernàrdez, 2009):

$$X = TP^T + E$$

where, X (K × N) data set matrix is reduced to a much smaller number of A variables called principal components (PC), T (N × A) is a matrix containing A scores for PCs, P (K × A) that containing the A loadings for the PCs and E (K × A) the residual matrix of the model.

The *k*NN classifier modeling technique was used for the classification of different sets of strains, where *k* value was kept at 3. The *k*NN is a supervised classification technique used most commonly for data sets with little prior knowledge of data distribution. For the *k*NN classification, the training model requires sorting the training data, and the distances of samples from the training set with respect to the unclassified sample is calculated and the unclassified sample is assigned to the group of the nearest *k* neighbors (Scott et al., 2007). In the kNN classification model, "*k*" is referred to as the number of close neighbors or samples to be considered for the grouping of the unclassified sample. The value of "*k*" can be any positive numeric value; however, values from 1 to 3 are commonly used (Scott et al., 2007; Yoon et al., 2013a, b). These classification models were then validated using the cross-validation technique.

5.4 Results and Discussion

An example of an image of bacterial cells as visible under the field of view of the microscope and acquired by the CytoViva® microscope system is presented in Figure 5.3a. Using ENVI software, this view was further zoomed at two levels as presented in Figures 5.3b and 5.3c. The appearance of bacterial cells at different zoom levels after the ROI were selected for acquiring hyperspectral graphs are presented in Figures 5.3d, 5.3e and 5.3f. The average time required to acquire images was ~5 min. The captured images were clarified using various image-

clarifying tools available in ENVI for the better visualization of bacterial cells; however, clarification of images did not affect the hyperspectral signatures of bacterial cells or other pixels in the images. Based on the preliminary work and personal communication with CytoViva® personnel, wavelength range from 425.57 to 753.84 nm was selected for PCA and kNN classifier modeling because wavelengths below 425.57 nm and above 753.84 nm were overlapping for all bacterial strains and no useful information could be utilized for the differentiation and classification purposes.

During the preliminary study, it was observed that following protocols for making proper dilutions of bacterial cells using sterilized HPLC grade water and air drying of slides were important activities in order to capture images with less concentrated or overlapping bacterial cells. An example of a hyperspectral image with overcrowded bacterial cells is presented in Figure 5.4a; whereas, a hyperspectral image without air-drying bacterial cells is presented in Figure 5.4b. These blurred images with overcrowded bacterial cells made selecting the ROI difficult and might not represent the real hyperspectra of the selected ROI.
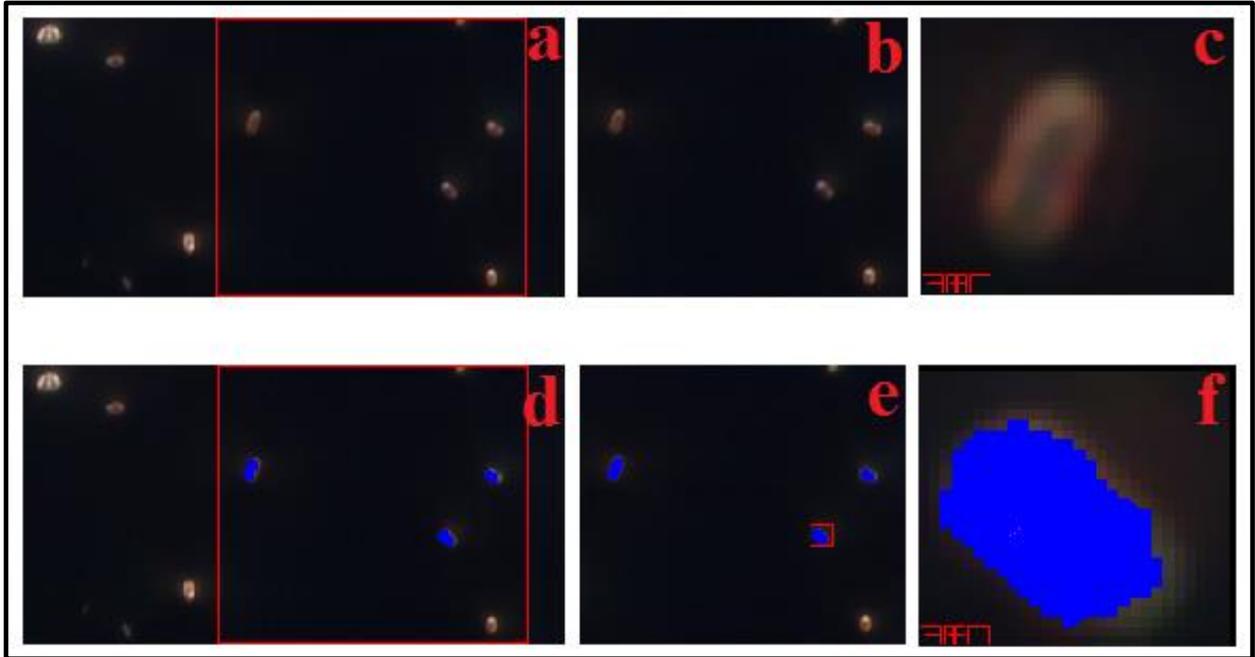
Figure 5.3 Hyperspectral Images of *Listeria monocytogenes* **at 4,000 × magnification. a and d: images of non-selected and selected regions of interest (ROI) as appears under the field of view of microscope, respectively; b and e: images of non-selected and selected ROI at first zoom, respectively; c and f: images of non-selected and selected ROI at second zoom, respectively**
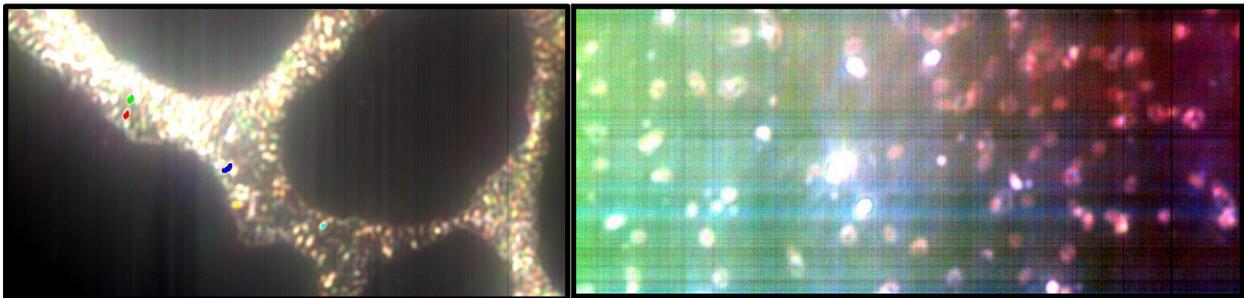
Figure 5.4 Hyperspectral Images *Escherichia coli* **O157:H7 at 4,000 × magnification. a: image with overcrowded bacterial cells; b: image captured with air drying slide**

To study the variation of hyperspectral graphs/signatures within a genus, HSI of different strains of *C. sakazakii*, *E. coli* and *Salmonella* spp. was conducted. Classification models of these strains developed using PCA and *k*NN classifier were cross-validated. Hyperspectral graphs of various strains of *C. sakazakii* are presented in Figure 5.5a. The graph indicates that *C. sakazakii* P1 had a different scattering pattern (shape of the curve) at wavelengths <615 nm compared to the other strains; however, at wavelengths >615 nm, the scattering patron of *C. sakazakii* P1 was similar to that of *C. sakazakii* E1. Although *C. sakazakii* BAA-894 and P2 had different intensities, the scattering pattern of both strains were similar throughout the wavelength range (Figure 5.5a). The PCA plot of *C. sakazakii* strains shows that *C. sakazakii* P1 was grouped on the negative (left) side of the PC1, while *C. sakazakii* E1 was grouped around the center, and *C. sakazakii* 894 and P2 were grouped on the positive (right) side of PC2 (Figure 5.6). Classification accuracy of *C. sakazakii* E1, P1 and P2 was 100%; however, *C. sakazakii* BAA-894 had classification accuracy of 66.67% (where *C. sakazakii* BAA-894 was misclassified as *C. sakazakii* P2 at 33.33%; Table 5.2). These results suggested that hyperspectral signatures of *C. sakazakii* strains were different from each other, and HSI could be used for the rapid presumptive identification and differentiation of various *C. sakazakii* strains studied in this research, except that *C. sakazakii* BAA-894 had 33.33% chance of being misclassified as *C. sakazakii* P2.

Within *Salmonella* spp., at wavelengths <600 nm, *S.* Montevideo had a different scattering pattern compared to the other strains; however, at wavelengths >600 nm, all strains had similar scattering patterns but different scattering intensities (Figure 5.5b). Cross-validation of PCA (Figure 5.7) and *k*NN classification shows 100% classification accuracy for all *Salmonella* strains (Table 5.2), indicating that each strain had distinguishable hyperspectral signatures and can be differentiated using the developed protocol with 100% accuracy.
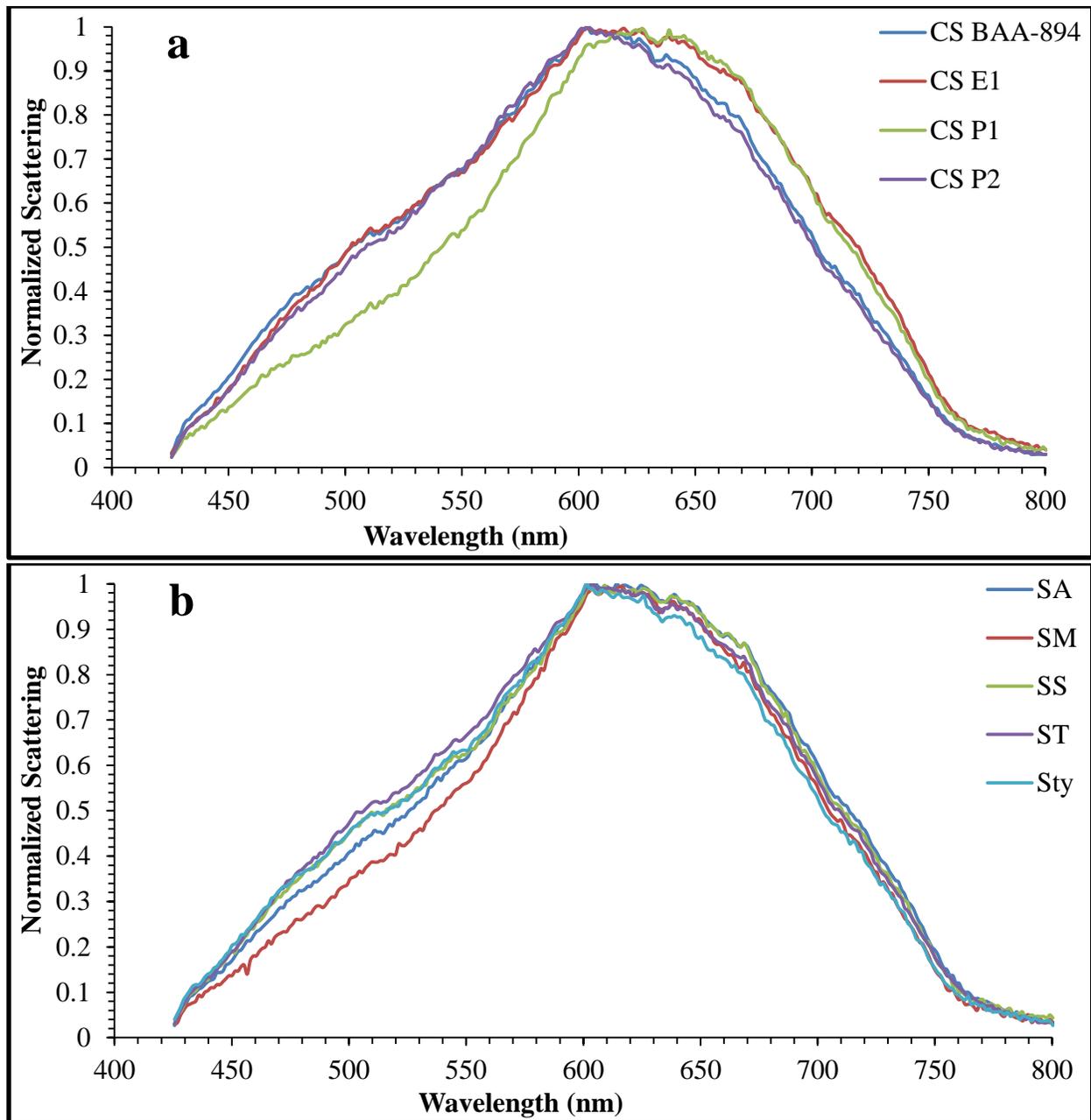
Figure 5.5 Hyperspectral graphs of: a) *Cronobacter sakazakii* **(CS) BAA-894, environment isolate (E1), and two food processing plant isolates (P1 and P2); b)** *Salmonella* **Agona BAA-707 (SA), Montevideo (SM), Senftenberg (SS), Tennessee 10722 (ST) and Typhimurium 13311 (STy)**
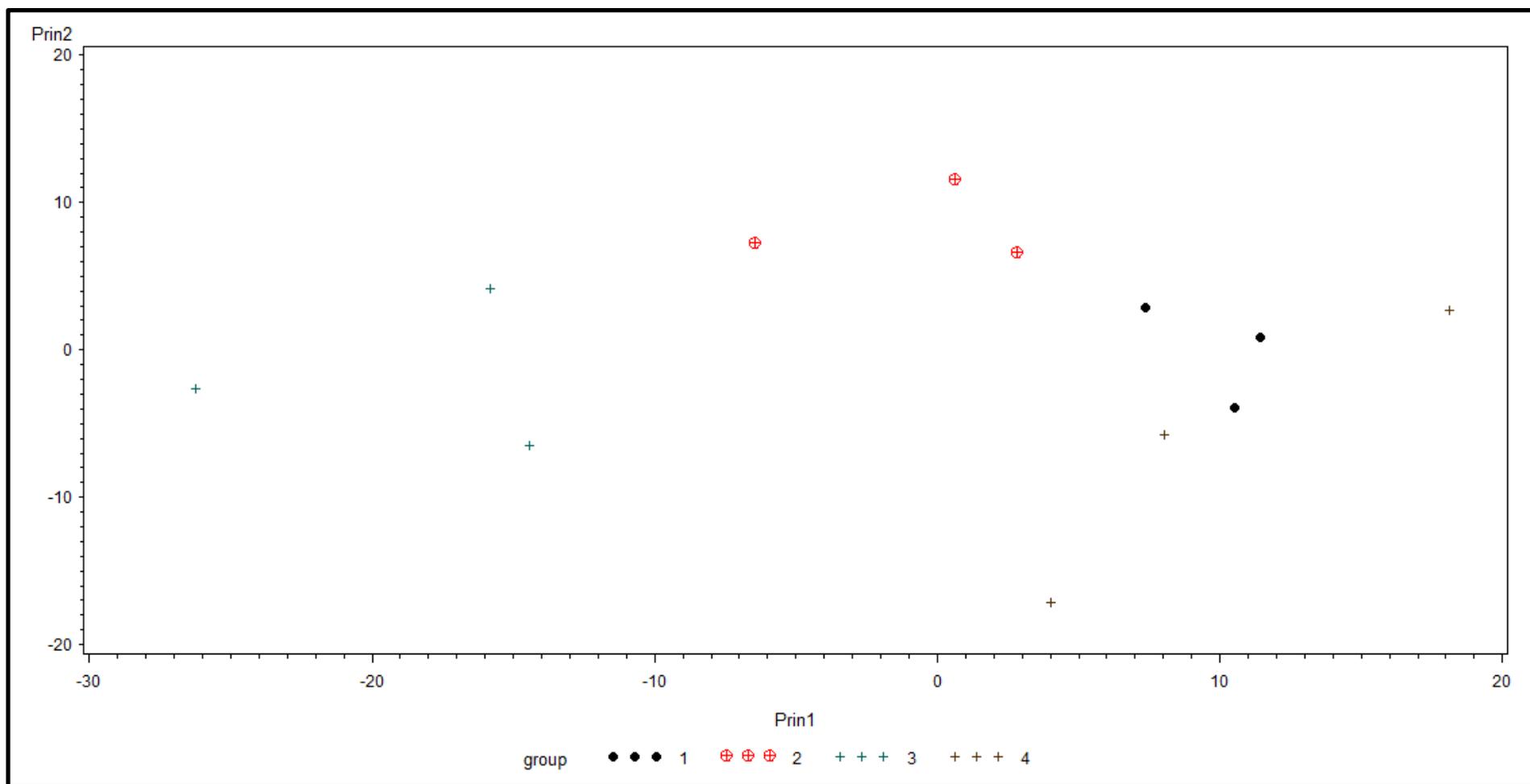
Figure 5.6 Principal component analysis (PCA) plot showing first two principal components (Prin1 and Prin2) of hyperspectral graphs of

Cronobacter sakazakii **BAA-894 (1), environment isolate (2), and two food processing plant isolates (3 and 4)**
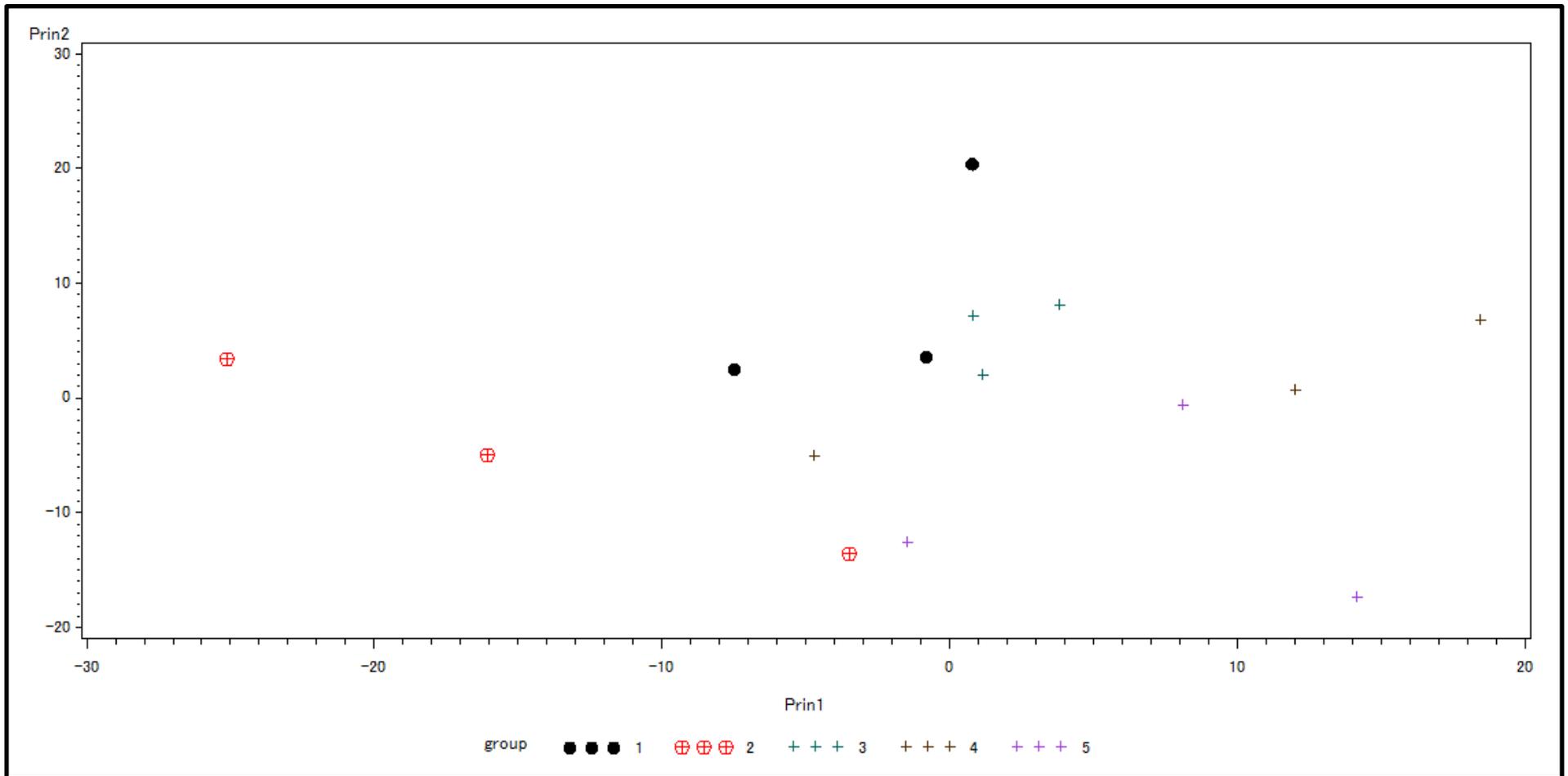
Figure 5.7 Principal component analysis (PCA) plot showing first two principal components (Prin1 and Prin2) of hyperspectral graphs of

Salmonella **Agona BAA-707 (1), Montevideo (2), Senftenberg (3), Tennessee 10722 (4) and Typhimurium 13311 (5)**

Table 5.2 Classification accuracy of various strains of *Cronobacter sakazakii*, *Salmonella* spp. and *Escherichia coli* within the respective genus obtained from cross-validation of principal component analysis and *k*NN (k-nearest neighbor, k = 3) classification*

Bacteria	Strain/Serotype	% Classification Accuracy	Misclassified As (%)
Cronobacter sakazakii	BAA-894	66.67	CS-P2 (33.33)
	E1	100	-
	P1	100	-
	P2	100	-
Salmonella spp.	SA	100	-
	SM	100	-
	SS	100	-
	ST	100	-
	STy	100	-
Escherichia coli	O26	66.67	EC-O103 (33.33)
	O45	66.67	EC-O121 (33.33)
	O103	100	-
	O104	100	-
	O111	100	-
	O121	66.67	EC-O45 (33.33)
	O145	100	-
	O157	100	-

* **CS**: *Cronobacter sakazakii*; **EC**: *Escherichia coli*; **E1**: environmental isolate of *C. sakazakii*; **P1** and **P2**: processing plant isolates of *C. sakazakii*; **SA**: *S.* Agona BAA-707; **SM**: *S.* Montevideo; **SS**: *S.* Senftenberg; **ST**: *S.* Tennessee 10722; **STy**: *S.* Typhimurium 13311

Among *E. coli* serotypes, although all serotypes had a different intensity of scattering, they followed a similar pattern throughout the wavelength range (Figure 5.8a). Approximate grouping of various serotypes of *E. coli* can be visualized from the PCA plot presented in Figure 5.8. Except *E. coli* O26, O45 and O121, which had classification accuracy of 66.67%, classification accuracy of all other *E. coli* serotypes was 100% (Table 5.2). *Escherichia coli* O26, O45 and O121 were misclassified as *E. coli* O103, O121 and O45, respectively at 33.33%.
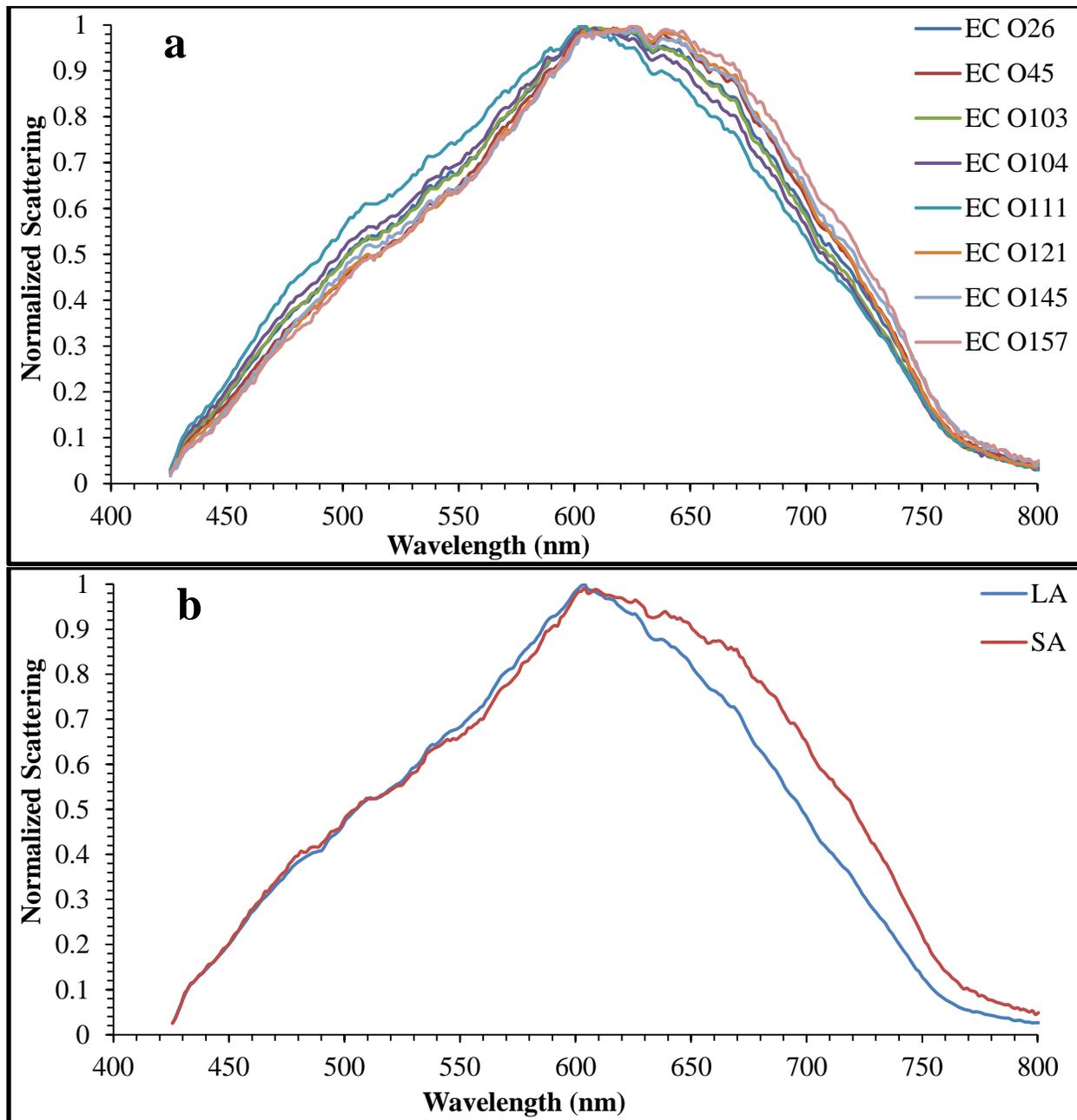
Figure 5.8 Hyperspectral graphs of: a) *Escherichia coli* **(EC) O26, O45, O103, O104, O111, O121, O145 and O157; b)** *Listeria monocytogenes* **SLR 2249 (LA) and** *Staphylococcus aureus* **25923 (SA)**
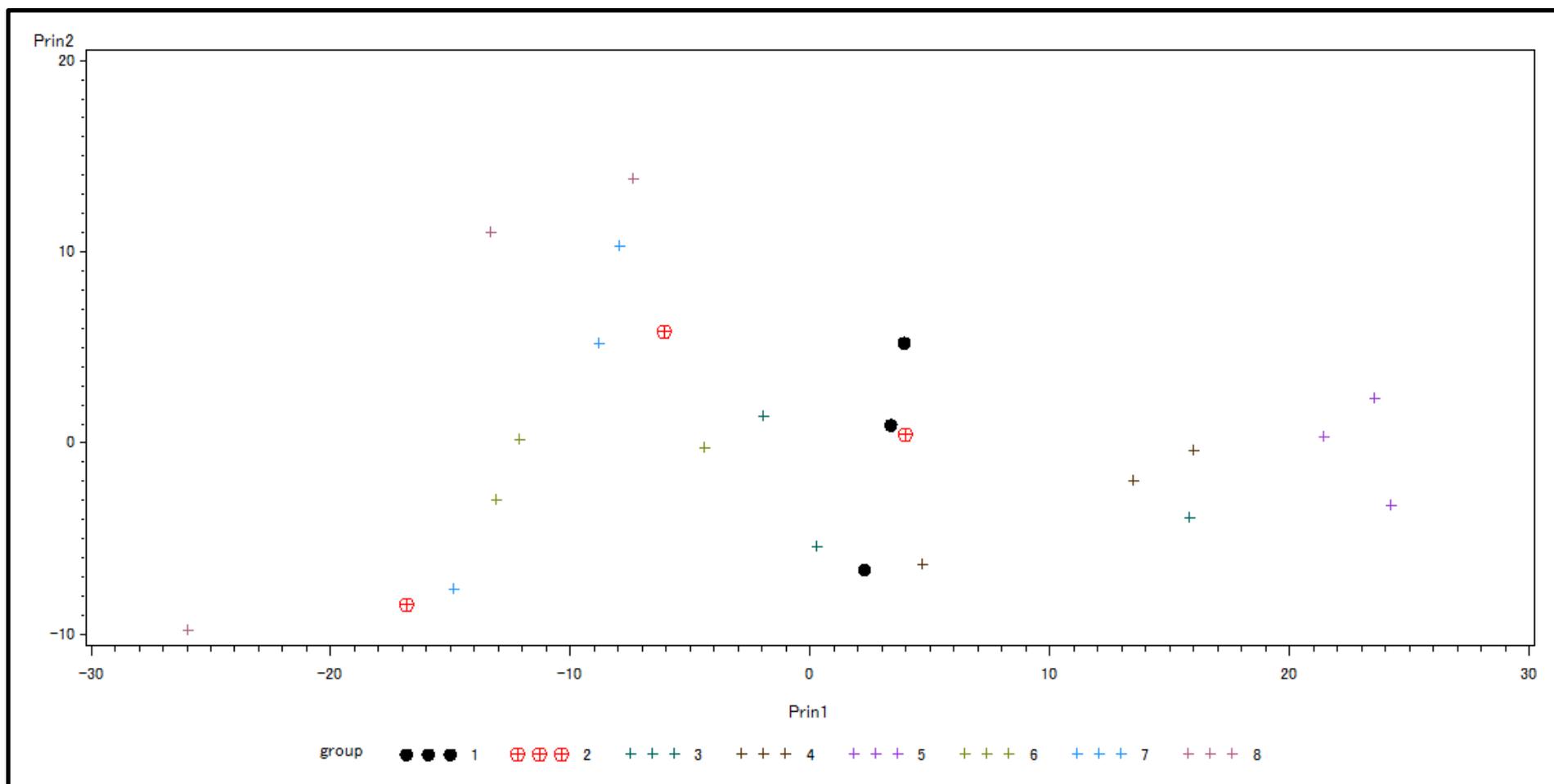
Figure 5.9 Principal component analysis (PCA) plot showing first two principal components (Prin1 and Prin2) of hyperspectral graphs of

Escherichia coli **O26 (1), O45 (2), O103 (3), O104 (4), O111 (5), O121 (6), O145 (7) and O157 (8)**
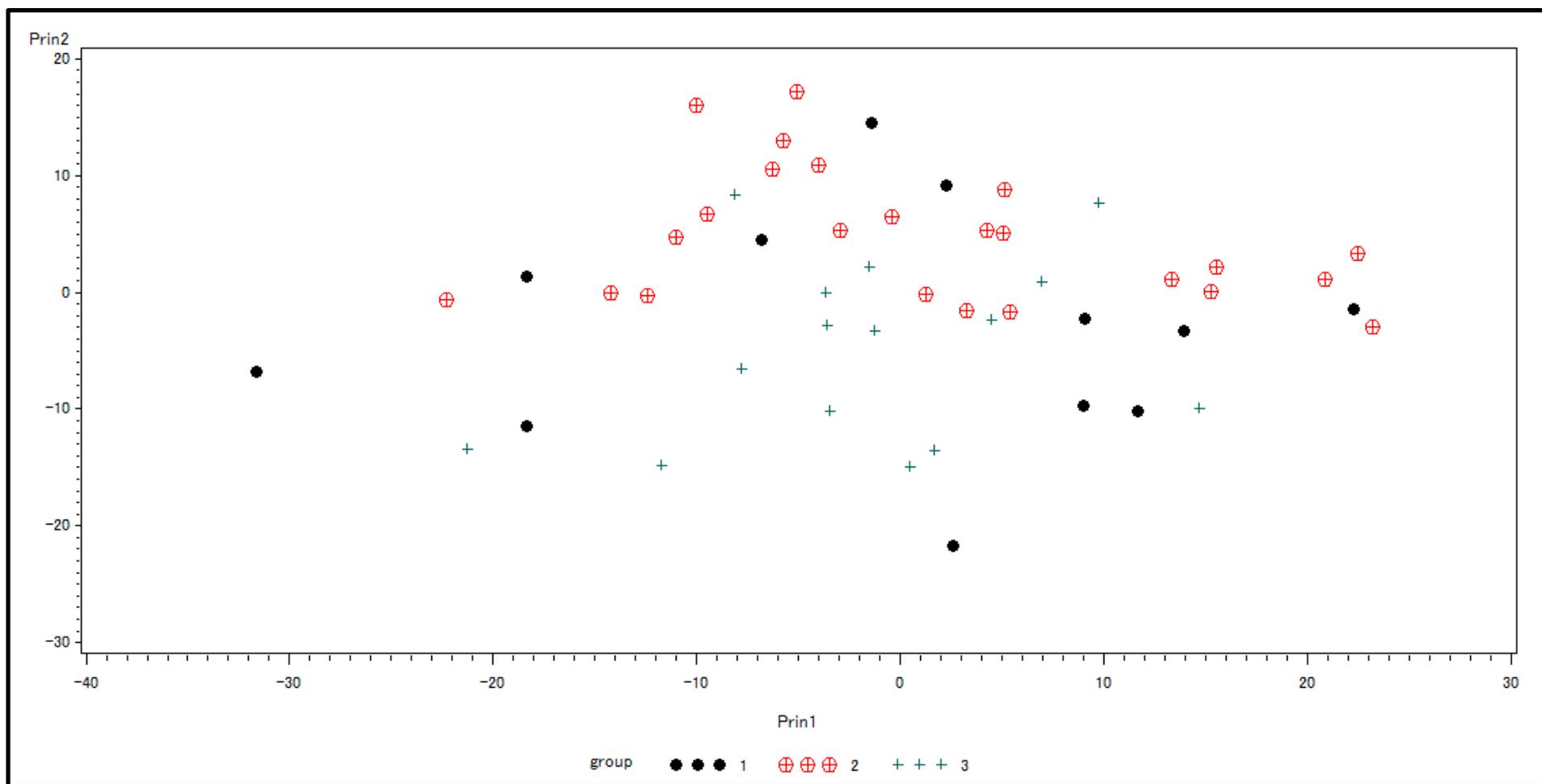
Figure 5.10 Principal component analysis (PCA) plot showing first two principal components (Prin1 and Prin2) of hyperspectral graphs of four strains of *Cronobacter sakazakii* (1); eight strains of *Escherichia coli* (2); five strains of *Salmonella* spp. (3)
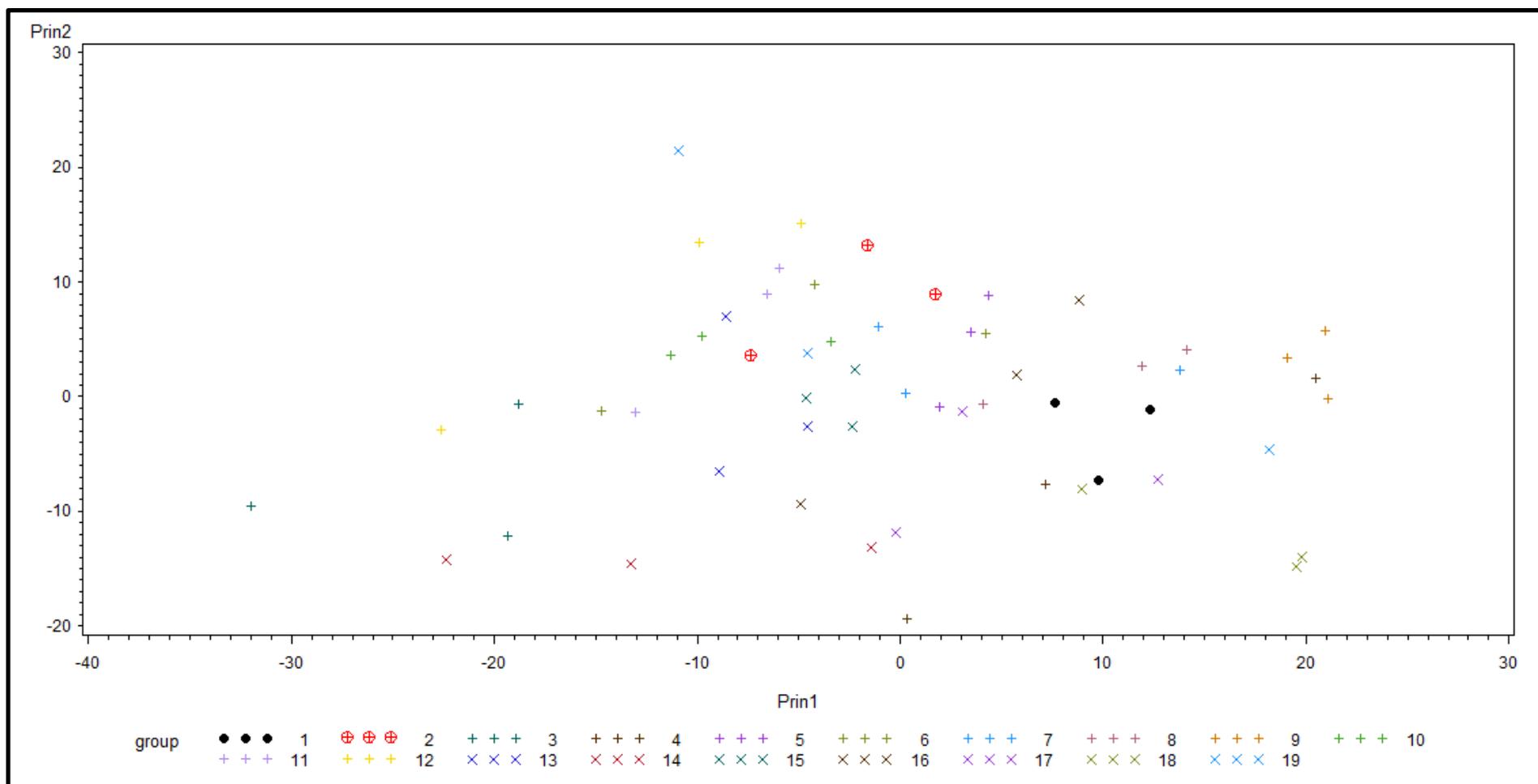
Figure 5.11 Principal component analysis (PCA) plot showing first two principal components (Prin1 and Prin2) of hyperspectral graphs of *Cronobacter sakazakii* **BAA- 894 (1), E1 (2), P1 (3), P2 (4);** *Escherichia coli* **O26 (5), O45 (6), O103 (7), O104 (8), O111 (9), O121 (10), O145 (11), O157 (12);** *Salmonella* **Agona BAA-707 (13), Montevideo (14), Senftenberg (15), Tennessee (16), Typhimurium (17);** *Listeria monocytogenes* **(18);** *Staphylococcus aureus* **(19)**

To study if HSI could be used to differentiate various bacteria at the genus level, PCA (Figure 5.10) and *k*NN classification followed by cross-validation was conducted using hyperspectra of various strains of *C. sakazakii*, *E. coli* and *Salmonella* spp. Overall, low values of classification accuracy were obtained for *C. sakazakii* (58.33%) and *Salmonella* spp. (20%); however, *E. coli* had 79.17% classification accuracy. This poor classification accuracy could be explained from the results presented in Table 5.2. As each strain within a genus had distinguished hyperspectral signatures and was different from other strains in the same genus, it is difficult to group together various strains within a genus on the basis of *k*-NN modeling technique used in this study.

To study the efficacy of HSI to differentiate bacteria when different strains from different genera are analyzed together, PCA (Figure 5.11) and *k*NN classification of hyperspectral graphs of various strains of *C. sakazakii*, *E. coli* and *Salmonella* spp., and *L. monocytogenes* and *S. aureus* was conducted. Cross-validation accuracy of the classification of different strains is presented in Table 5.3. Overall, classification accuracy of these strains was not good. Only *C. sakazakii* P1, *S.* Montevideo, *E. coli* O104 and O111, and *L. monocytogenes* had 100% classification accuracy; whereas, all other strains were classified at accuracy of ≤66.67%. *Salmonella* Tennessee and *E. coli* O45 were the most poorly classified strains with an accuracy of 0%.

Effect of age of *E. coli* O157 and *L. monocytogenes* on hyperspectra is presented in Figure 5.12. Although overall shapes of hyperspectra at various incubation times (culture age during 24 h incubation) for respective bacteria were similar (with different scattering intensities at different temperatures), no trend of increase or decrease in scattering intensities with increase of incubation time was observed. However, classification accuracy for both bacteria evaluated at

various incubation times was 100%. This suggested that hyperspectra of evaluated bacteria at different incubation times were distinguishable and HSI could be used to predict the age of bacterial cells.

Table 5.3 Classification accuracy of various strains of *Cronobacter sakazakii*, *Salmonella* spp. and *Escherichia coli*, and *Listeria monocytogenes* and *Staphylococcus aureus* when analyzed together obtained from cross-validation of principal component analysis and *k*NN (k-nearest neighbor, k = 3) classification*

Bacteria	Strain/Serotype	% Classification Accuracy	Misclassified As (%)
Cronobacter sakazakii	BAA-894	33.33	CS-P2 (33.33), EC-O104 (33.33)
	E1	33.33	Others (66.67)
	P1	100	-
	P2	33.33	CS-BAA-894 (33.33), Others (33.33)
Salmonella spp.	SA	33.33	EC-O45 (33.33), EC-O103 (33.33)
	SM	100	-
	SS	66.67	Others (33.33)
	ST	0	EC-O26 (33.33), EC-O121 (33.33), Others (33.33)
	STy	33.33	CS-BAA-894 (66.67)
Escherichia coli	O26	66.667	ST (33.33)
	O45	0	EC-O121 (33.33), Others (66.67)
	O103	66.67	ST (33.33)
	O104	100	-
	O111	100	-
	O121	66.67	EC-O45 (33.33)
	O145	100	-
	O157	66.67	EC-O121 (33.33)
Listeria monocytogenes	SLR 2249	100	ATCC
Staphylococcus aureus	25923	66.67	LM (33.33)

* **CS**: *Cronobacter sakazakii*; **EC**: *Escherichia coli*; **E1**: environmental isolate of *C. sakazakii*; **P1** and **P2**: processing plant isolates; **LM**: *Listeria* monocytogenes; **SA**: *S.* Agona BAA-707; **SM**: *S.* Montevideo; **SS**: *S.* Senftenberg; **ST**: *S.* Tennessee 10722; **STy**: *S.* Typhimurium 13311
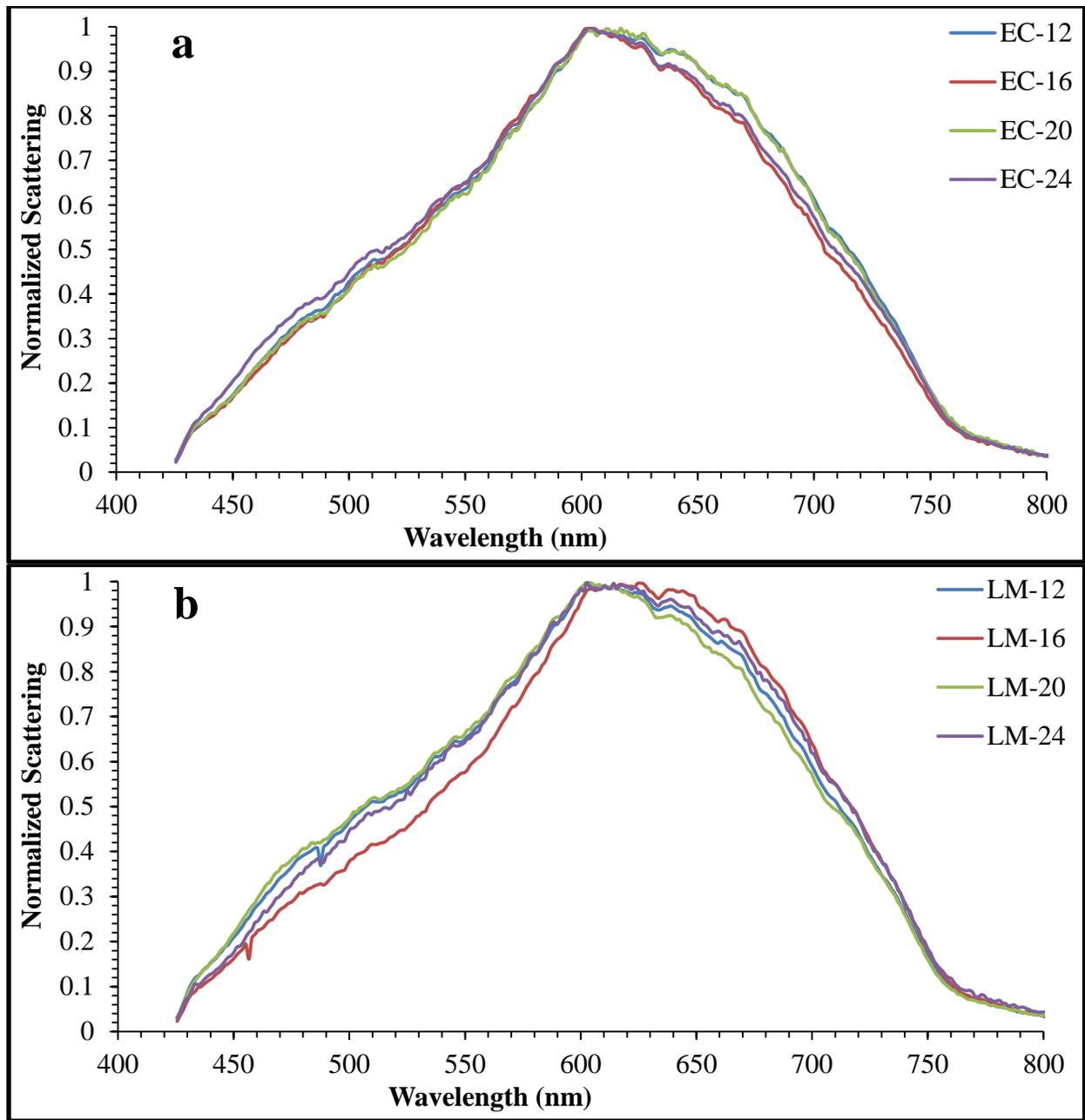
Figure 5.12 Hyperspectral graphs of growth in tryptic soy agar at 35°C at 12, 16, 20 and 24 h for: a) *Escherichia coli* **O157 (EC); b)** *Listeria monocytogenes* **SLR 2249 (LM)**

Becerril et al. (2013) reported that bacterial cell membranes were the main targets of LAE treatment, which resulted in the inactivation of cells by the disruption of cell membranes. Using a scanning electron microscope, they also demonstrated that cell membrane disruption resulted in structural changes in bacterial cells. As expected in this study, no differences were observed between the hyperspectral images of LAE treated and non-treated bacterial cells of various strains at $4,000 \times$ magnification. However, LAE treatment of various strains significantly affected their hyperspectral signatures. As seen in Figure 5.13, LAE treated strains had different intensities over the entire range of wavelengths compared to their corresponding non-treated strains. Classification accuracy of LAE treated and non-treated within each bacterium was 100%; therefore, suggesting that LAE treated bacterial cells can be differentiated from healthy and non-treated cells. No specific trend in the change of hyperspectra of various strains due to LAE treatment along the wavelengths was observed; however, overall differences in scattering intensities between LAE treated and non-treated strains were greater at >600nm.
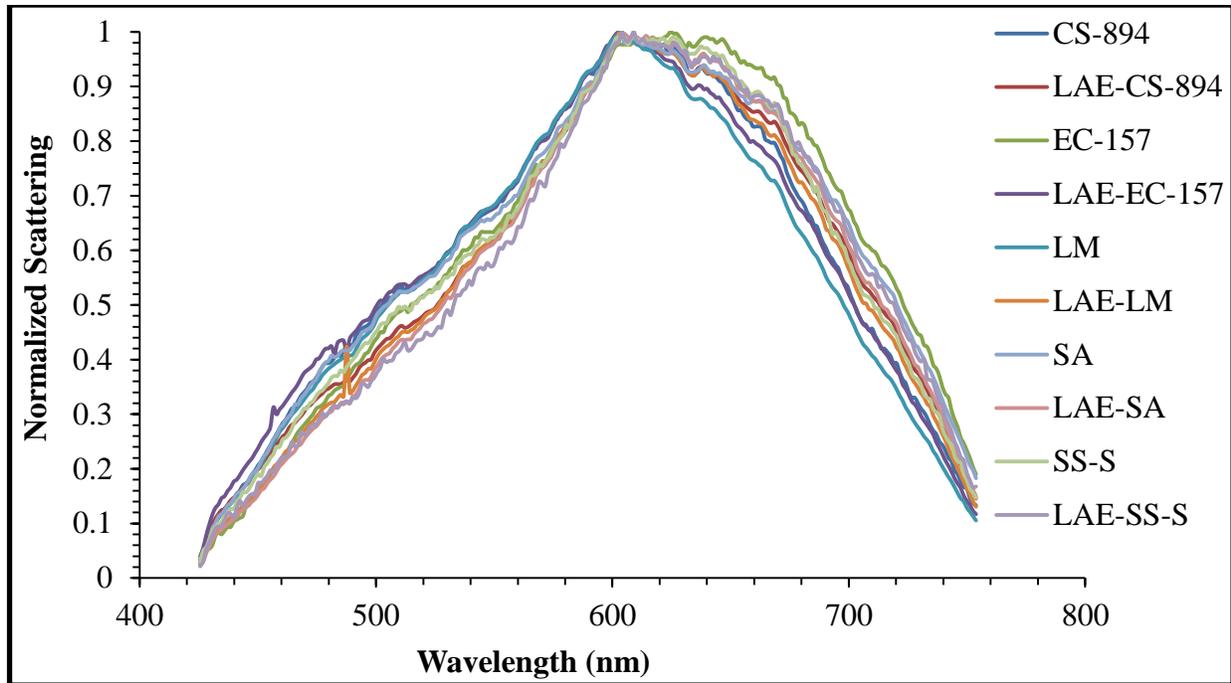
Figure 5.13 Hyperspectral graphs of lauric arginate (LAE) treated and non-treated

Cronobacter sakazakii **BAA-984 (CS-894),** *Escherichia coli* **O157 (EC-157),** *Listeria*

monocytogenes **SLR 2249 (LM),** *Staphylococcus aureus* **25923 (SA) and** *Salmonella*

Senftenberg (SS-S)

Yoon et al. (2013b) used HSI over the wavelength of 400 to 1000 nm (with 1.29 nm

wavelength separation) for the identification and differentiation of pure culture STEC colonies

grown on Rainbow agar at 37°C for 24 h. They used PCA-*MD* (Mahalanobis distance) and PCA-

*k*NN (k = 3) models for the classification prediction of STEC colonies; and applied hold one out

and *k*-fold CV (cross-validation) techniques for the validation of classification models. Yoon et

al. (2013b) reported that *E. coli* O111 and O121 showed the highest classification accuracy of

>99%, irrespective of classification modeling technique used; whereas, *E. coli* O26, O45 O103

and O145 showed the accuracy between 84 to 100% depending on the preprocessing technique

used.

In another study, Yoon et al. (2013a) used HSI (at 400 to 1000 nm wavelength range) and various prediction models for classification of STEC colonies plated on Rainbow agar as mixed cultures. They concluded that the best prediction model was kNN classification when standard normal variate and detrending, first derivative and spectral smoothing were used as preprocessing techniques. Using this kNN model, Yoon et al. (2013a) reported that classification accuracy of *E. coli* O26, O45, O103, O111, O121 and O145 was 95.80, 100, 88.54, 100, 100 and 91.93%, respectively. Yoon et al. (2013a) also concluded that hyperspectral graphs from wavelengths 750 to 1,000 nm did not provide important information for the classification and differentiation purposes of bacterial strains. These conclusions are in agreement with the current study's findings because hyperspectral graphs generated in this study at wavelengths ranging from ~750 to 1,000 nm for different bacterial strains were overlapping and indistinguishable, and could not be utilized for the classification purposes. Yoon et al. (2013a) also expressed a concern regarding morphology of bacterial colonies that could interfere with HSI and hence affecting hyperspectral signatures.

CytoViva® conducted a study to identify and differentiate spores of *Bacillus globigii* and *B. anthracis* on the basis of their hyperspectral signatures. They reported that normalized hyperspectra of spores of two *Bacillus* strains had significantly different shapes and peaks (CytoViva®, n. a.). However, in the current study, we observed very similar shaped hyperspectra and peaks of strains (but with different scattering intensities) within a same genus. These differences between CytoViva® (n. a.) and our results could be attributed to the differences in the biochemical structure of spores and vegetative bacterial cells. Bacterial cells consist of basic cellular components common to most of the prokaryotes (such as cell membrane, cytoplasm and DNA); whereas, bacterial spores have entirely different structure consisting of different proteins

(compared to cells) and seven different layers over the core of spores (Montville and Matthews, 2005f).

5.5 Conclusions

Overall classification accuracy of strains used in this study was acceptable when *k*NN classification model and cross-validation was applied within a specific genus. However, poor classification of various strains when compared together strongly suggests a need of further investigation, and use of different classification models and validation techniques, to eliminate the factors responsible for poor classification. After the validation of these HSI protocols, bacterial cells from various food matrices should also be studied for the feasibility of practical application of the developed HSI protocols. Hyperspectral signatures of pathogens such as STEC should also be studied when grown on selective medium agar (like Posse Agar) to develop a protocol to select colonies on the agar plates based presumptive HSI results for further confirmatory analysis such as PCR.

5.6 Acknowledgements

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Appendix A - Statistical Analysis System (SAS) Codes

SAS Code Used for Experiment I

SAS Code Used for Generating Regression Lines for Calculating D- and z-Values

```
dm "output;clear;log;clear;";
options linesize=95 pagesize=50 nodate pageno=1;

filename path 'E:\KSUconsulting\Minto\RFDH - SAS Data - Minto.xls';

%macro reg(tit,sheet);
title &tit;
proc import
datafile=path out=one
replace dbms=excel;
range=&sheet;
run;
proc transpose data=one out=two name=rep;
var R1-R3;
by time;
```

```
run;
proc print data=two;
run;
proc reg data=two;
model col1=time;
run;
proc plot data=two;
plot col1*time;
run;
%mend;

%reg('90c2min','Sheet1$A4:D9')
%reg('85c10min','Sheet1$A13:D18')
%reg('80c10min','Sheet1$A22:D27')
%reg('75c15min','Sheet1$A31:D36')


run;
quit;
```

Student's t-test at $P \leq 0.05$ Used for Comparing D- and z-Values

```
data one;
input wb rf;
cards;
29.41 28.49
23.42 20.33
21.74 24.15
;

proc ttest data=one;
paired wb*rf;
run;
```

SAS Code Used for Experiment II

```
options ls=100 ps=65 pageno=1;
dm 'log;clear;output;clear;';

filename jay 'F:\Minto';

title1 'PCA';
```

```sas
data sensor;
  infile jay (HSI-pure.txt)
      lrecl=5512 delimiter ='09'x firstobs=2;

  input  classtype $ feature1-feature255 group;

 if group = 4 or group = 12 or group = 16 then delete;
         run;



ods listing close;
ods output Eigenvalues=evals;
proc princomp data = sensor out=sensor_pca;
  var feature:;
  run;
ods listing;



data _null_;
  set evals;
  if cumulative >.99 then counter+1;
   if counter=1 then do;
     call symput('prinmx',compress(number));
     end;
  run;



%put &prinmx;



data _null_;
  set evals;
  if  Proportion = 0 then counter+1;
  if counter=1 then do;
    call symput('samplesize',compress(number));
    end;
  run;



%put &samplesize;



proc print data = sensor_pca;
  var classtype prin1-prin&prinmx;
```

```
   run;


data pca_group;
   merge sensor_pca sensor;
   drop _name_ feature:;
 run;


proc gplot data= pca_group;
  plot prin2*prin1 = group;
  symbol1 v=dot c=black;
  symbol2 v= '+' c=red;
  title2 'A plot showing the first two principal components'
       '(PC-1 and PC-2) obtained for meat samples';
  run;
 quit;
proc discrim data=pca_group method=npar k=3 posterr pool=no crossvalidate;

  var prin1-prin&prinmx;
  run;
```