Utilization of Intense Pulsed Light for the Microbial Decontamination of Low-Moisture Foods

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Dedication

This dissertation is dedicated to the loving memory of my grandpa Robert Hugh Jacobchick, whose infectious laughter and loving nature are greatly missed. Thank you for being such a positive influence in my life and instilling in me at an early age that anything is possible if you put in the hard work and are dedicated to your craft.

Abstract

This work aimed to develop a continuous intense pulsed light (IPL) system for the nonthermal pasteurization of low-moisture foods (LMFs). In the last few years, LMFs have been implicated in multiple foodborne outbreaks and caused severe illness in thousands of people. This system should aid in the reduction of food recalls and to assist the food industry to meet the rising consumer demands for safe, minimally processed foods.

The approach included evaluating various inoculation methodologies to understand how each one impacts the desiccation tolerance and homogeneity of bacteria following sample equilibration, identifying various treatment parameters and how they affect the efficacy of IPL to eliminate pathogenic bacteria in LMFs, and finally to test the treatment parameters from powdered foods and apply them to a larger, more irregularly shaped food matrix.

The results show that IPL can be used to rapidly decontaminate different types of LMFs. Treatment times of less than 30 seconds resulted in ~3-log reduction of Cronobacter sakazakii in nonfat dry milk, and treatment times of 120 seconds resulted in at least a 1.5-log reduction on most microorganisms in hard red wheat (HRW). Results indicate that, especially in HRW, treatment times can be extended without negatively impacting functional properties.

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List of Abbreviations

AACC	American Association of Cereal Chemists
ANOVA	Analysis of Variance
AOCS	American Oil Chemists' Society
ATCC	American Type Culture Collection
BBB	Blood Brain Barrier
BGPI	Broth-Grown Pelletized Inoculation
BGSI	Broth-Grown Spray Inoculation
BSPL	Broad Spectrum UV Light
CDC	(US) Centers for Disease Control and Prevention
CFU	Colony Forming Unit
CPD	Cyclobutane-Pyrimidine Dimers
DBD	Dielectric Barrier Discharge
DI	Deionized
ECDC	European Centre for Disease Prevention and Control
EFSA	European Food Safety Agency
EHEC	Enterohemorrhagic *Escherichia coli*
EPA	Environmental Protection Agency
ERH	Equilibrated Relative Humidity
FAO	Food and Agriculture Organization (United Nations)
FDA	(US) Food and Drug Administration
FoodNet	Foodborne Diseases Active Surveillance Network
GMP	Good Manufacturing Practices
HIPL	High-Intensity Pulsed UV Light
HRW	Hard Red Wheat
IPL	Intense Pulsed Light
LGLI	Lawn-Grown Liquid Inoculation
LMF	Low Moisture Foods
NDT	Nondestructive Testing
NFDM	Nonfat Dry Milk
NRRL	Agriculture Research Service Culture Collection
OK	Oh and Kang
OTA	Ochratoxin A
PEF	Pulsed Electric Field
PIF	Powdered Infant Formula
PL	Pulsed Light
PPCO	Polypropylene Copolymer
PUV	Pulsed UV Light
PWL	Pulsed White Light
RH	Relative Humidity
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SASP	Small Acid Soluble Proteins
SD	Standard Deviation
SMA	Skim Milk Agar

STEC	Shiga Toxin-producing *Escherichia coli*
TLC	Thin-Layer Chromatography
TSA	Tryptic Soy Agar
TSAYE	Tryptic Soy Agar with Yeast Extract
TSB	Tryptic Soy Broth
TSBYE	Trypic Soy Broth with Yeast Extract
UCAR	University Corporation for Atmospheric Research
UK	United Kingdom
US	United States
UV	Ultraviolet
WHO	World Health Organization
YOPI	Young, Old, Pregnant, and Immunocompromised

Chapter One
LITERATURE REVIEW

General Introduction

Every year, almost 1 in 10 people (~600 million) around the world fall ill after the consumption of contaminated food, and some 420,000 individuals die. Children under the age of 5 account for an alarming 40% of the foodborne disease burden, resulting in 125,000 deaths each year (WHO, 2015). What this means is that almost 30% of the reported deaths each year are young children. Access to safe and nutritious foods is an essential factor in increasing the quality of life and promoting good health. With the world's food supply chain now spanning and crossing national borders, it is easier than ever for countries to supplement their food supply with safer and nutritious food. However, there still needs to be a foundation of collaboration between governments, food producers, and consumers. One of the significant hurdles in countries experiencing food safety issues is the cost of imported foods. Most families cannot afford the higher prices and continue to consume unsafe food. Continued consumption of unsafe food results in a vicious cycle of disease and malnutrition. This cycle affects not only the entire population but particularly the most susceptible demographics of the population, YOPI's, the Young, Old, Pregnant, and Immunocompromised (WHO, 2019). Countries are seeking to import products from elsewhere to meet the rising demand for safe food and to supplement their food supply during food shortages (FAO, 2018). Amongst these imports are low-moisture foods (LMFs).

LMFs, especially powdered foods, are becoming popular amongst consumers due to their convenience and versatility either by direct consumption or in food preparation.

Two of the defining features of a LMF are, long shelf life and that they do not directly support the growth of microorganisms, especially foodborne pathogens. This has to do with LMFs inherently having low water activity (a_w). However, their low a_w does not mean that they are safe for consumption. Within the last few years, LMFs have been the cause of multiple foodborne outbreaks and subsequent recalls. One of the first multistate recalls and outbreaks, from foodborne pathogen contamination with wheat flour, happened in 2016 when General Mills detected Shiga toxin-producing *Escherichia coli* (STEC) O121 and O26 in their flour produced at a Kansas City, Missouri facility. After the investigation was over, the CDC reported that there were a total of 63 illnesses across 24 states (CDC, 2016b). Since then, there have been multiple flour recalls, as well as recalls of powdered milk, whey powder, spices, and seasonings. *Salmonella* spp. is the most common foodborne pathogenic organism responsible for recalls and foodborne outbreaks in LMFs. Two other foodborne pathogens of significant concern in LMFs are *Cronobacter sakazakii* and spores of *Bacillus cereus*. *C. sakazakii* infections have been on the rise and have led to multiple recalls of powdered milk around the world. *B. cereus* is a hardy, spore-forming bacterium known for its toxin production and is a microorganism frequently found on grains, especially rice.

To meet the rising consumer demand for high-quality, minimally processed foods, multiple non-thermal processing techniques are currently being investigated for use in LMFs. Amongst these processing techniques is pulsed light. Pulsed light has proven itself useful in solid and some powdered foods already; however, almost all the research occurred on small laboratory scale systems. The work presented here is the first to employ a continuous pulsed light apparatus for the non-thermal decontamination of

vegetative cells, and spores-forming bacteria in LMFs and the detrimental effect IPL causes on microorganisms.

1.1 Foodborne Pathogens

Thirty-one major pathogens cause most of the foodborne illnesses in the United States. These include bacteria, parasites, and viruses. In the years between 2000-2008, these 31 pathogens caused 9.4 million foodborne illnesses, 55,961 hospitalizations, and 1,351 deaths. Of those 1,351 deaths, nontyphoidal *Salmonella* spp. accounted for 28% of total deaths, followed by *Toxoplasmosis gondii* (24%), *Listeria monocytogenes* (19%), and norovirus (11%) (Scallan et al., 2011).

The Foodborne Diseases Active Surveillance Network, a.k.a. FoodNet has been utilized by the CDC to track foodborne illness trends since 1996. In 2018, they found that Shiga toxin-producing *E. coli* infections saw a -12% change in the incidence of O157-associated, and a +25% increase in non-O157-associated illnesses, respectively. Of the top 3 *Salmonella* serotypes, a -10% decrease was observed for Enteritidis, a +10% increase for Newport, and a +4% increase for Typhimurium compared with the 2015-2017 average (CDC, 2019a; Tack et al., 2019). In the last three years, there has been an increased incidence of foodborne pathogen illnesses. However, every year, we are getting better at surveillance, leading to quicker identifications of patterns of illness and linking those to food products. As a result of faster awareness and response to foodborne illness, the incidences of reported foodborne illnesses have increased (Nyachuba, 2010). Since 2009, *Salmonella* infections account for 40% of the total confirmed bacterial foodborne infections with *Campylobacter* causing the second most foodborne illnesses, with 33%

(Figure 1.1). This accounts for a total of 76,333 confirmed cases of *Salmonella* and 63,340 cases of *Campylobacter* from food since 2009 (Table 1.1) (Tack et al., 2019).

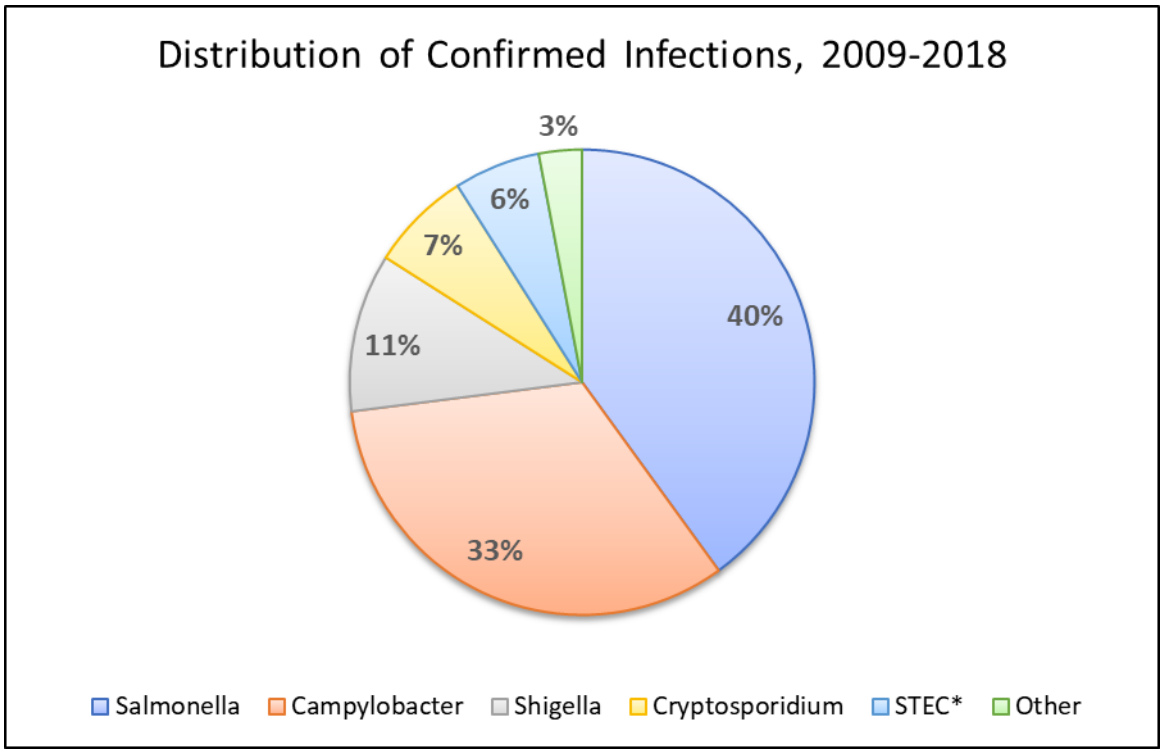


Figure 1.1: Distribution of confirmed bacterial foodborne illnesses by percentage in the United States from the years 2009-2018.
*Shiga toxin-producing *Escherichia coli*
Source of data (CDC, 2019b)

Table 1.1: Distribution of confirmed cases of bacterial foodborne infections in the United States from the year 2009-2018

Pathogen	**Cases**
Salmonella	76,333
Campylobacter	63,340
Shigella	20,384
Cryptosporidium	13,010
STEC*	11,853
Other	5,998

*Shiga toxin-producing *Escherichia coli*
Source of data (CDC, 2019b)

As described in the environmental stressors section (1.3), pathogens can survive common manufacturing stressors such as pH, desiccation, temperature, chemical disinfectants, and pasteurization (Barron and Forsythe, 2007; Breeuwer et al., 2003; Burgess et al., 2010; Gandhi and Chikindas, 2007; Hanson et al., 2005; Sagripanti and Bonifacino, 1999). Their inherent ability to survive is concerning because if the surviving organisms are in a high enough concentration, their consumption by the consumer leads to foodborne illness and subsequent product recalls. Some pathogens have low-infectious doses to cause human disease, Infectious Dose (ID_{50}), 1000 colony forming units (CFU) for *Cronobacter sakazakii* (Parra-Flores et al., 2015), as low as one CFU for *Salmonella* spp. (Hammack, 2017), and 10-100 cells for Enterohemorrhagic *Escherichia coli* O157: H7 (Feng et al., 2013), depending on age, the health of host and strain variation. The low infectious dose of pathogens is concerning for food safety since even if the food industry can significantly reduce pathogens in their product, just a few surviving pathogenic cells can cause illness in consumers.

Not all these pathogens are of concern in LMFs. Foodborne infections caused by *Vibrio* spp. called vibriosis, occurs upon the consumption of raw or undercooked seafood. These bacteria typically are found in oysters and other shellfish in warm coastal waters, with the three most common species being *V. parahaemolyticus*, *V. vulnificus*, and *V. alginolyticus* (CDC, 2019e). *Cryptosporidium* causes a disease called cryptosporidiosis in which those infected with the parasite experience watery diarrhea, nausea, dehydration, fever, and stomach cramps. In individuals with healthy immune systems, symptoms usually last between 1-2 weeks. Recreational waters in the United States are a common reservoir and point of infection in the United States, and the CDC reports that between

2001-2010, that *Cryptosporidium* was the leading cause of waterborne disease outbreaks (CDC, 2017a).

Campylobacter causes campylobacteriosis and is one of the most causes of diarrhea in the United States. FoodNet estimates that 14 out of every 100,000 people are diagnosed with campylobacteriosis every year, with most cases going undiagnosed or unreported. Every year, more than 1.3 million people fall ill from *Campylobacter* infections. *C. jejuni* is the most common cause of human *Campylobacter* illnesses; however, other *Campylobacter* species (such as *C. coli* and *C. fetus*) can also cause human illness. 33% of raw chicken bought from retailers tested positive for *Campylobacter* in 2014, and considering that fewer than 500 cells of *Campylobacter* can make someone sick, CDC reports that a single drop of raw chicken juice has the potential to cause illness (CDC, 2017b).

1.2 Low-Moisture Foods

Within the last decade, the number of food recalls linked to low-moisture foods (LMFs) contaminated with pathogens has been on the rise (Beuchat et al., 2013; Dey et al., 2013; Finn et al., 2013; Podolak et al., 2010; Van Doren et al., 2013). Beuchat et al. defined LMFs as a food product with a water activity (a_w) < 0.85 (Beuchat et al., 2013), while Blessington et al. defined them as having a water activity of <0.70 (Blessington et al., 2013). Either way, they are naturally low in moisture and are made from foods higher in moisture that has undergone a deliberate drying processing such as spray-drying and dehydration. As of late, they are becoming increasingly common as ingredients in further

processed foods or even just through direct consumption by humans and animals without a cooking step (Beuchat et al., 2013; Finn et al., 2013).

LMFs can be divided into nine main categories: 1.) Cereals and grains; 2.) Confections and snacks; 3.) Dried fruits and vegetables; 4.) Dried protein products; 5.) Honey and preserves; 6.) Nuts and nut products; 7.) Seeds for consumption; 8.) Spices and dried herbs; 9.) Specialized nutritional products (Table 1.2). Not only are LMFs convenient for consumption since many do not require refrigeration, but they also have a longer shelf life. Compared with other food products with higher a_w values, LMFs do not support the growth of pathogenic organisms and are less susceptible to microbial spoilage (Cordier, 2014; Maltini et al., 2003). Even though LMFs do not support the growth of pathogenic microorganisms, some foodborne pathogens have demonstrated the ability to survive in low a_w environments and remain viable for months to years in LMFs (Beuchat et al., 2013; Finn et al., 2013; Podolak et al., 2010).

Manufacturing processes, such as thermal treatments, can reduce native organisms/pathogens. However, high sugar/fat content, when combined with low a_w, is thought to significantly increase microbial survival in LMFs (Beuchat et al., 2013; Finn et al., 2013). Factors such as inappropriate handling, post-processing contamination, lack of Good Manufacturing Practices (GMPs), and poor ingredient control/handling can lead to product contamination before packaging (Podolak et al., 2010). In the last five years (2014-2018), there have been numerous outbreaks (Table 1.3) and recalls (Table 1.4) related to low moisture foods such as spices, herbs, infant formula, milk powder, flour, and seasonings (FDA, 2019). Pathogens and foodborne outbreaks are not the only

concern in LMFs; spoilage organisms and spore-forming bacteria can be detrimental to the food industry.

Table 1.2: Low-moisture food categories

Category	Foods
1.) Cereals and grains	Whole and milled grains Rice and rice products Cereals and cereal products
2.) Confections and snacks	Cocoa, chocolate products, and other confections/confectionary products Snacks
3.) Dried fruits and vegetables	Dried fruits Dried vegetables Dried/dehydrated mushrooms Dried seaweed
4.) Dried protein products	Dried dairy products Dried egg products Dried meat other than sausages/salamis/jerky
5.) Honey and preserves	Honey, jams, syrups
6.) Nuts and nut products	Tree nuts Peanuts and peanut products Mixed and unspecified nuts
7.) Seeds for consumption	Sesame seeds Tahini Halva/helva Other and unspecified seeds
8.) Spices and dried herbs	Fruit/seed based Root-based Herb/leaf-based Bark/flower based Mixed/unspecified Tea
9.) Specialized nutritional products	Lipid-based nutrient supplements Dried /powdered nutrient supplements

Adapted from (FAO and WHO, 2014)

Table 1.3: Selected foodborne disease outbreaks in low-moisture foods, 2014-2018

Year	Product	Location	Pathogen	Cases	Source
2014	Chia Powder	United States, Canada	*Salmonella* Newport, Oranienburg, others	94 infected, 20 hospitalized	(Harvey et al., 2017)
2014	Cashews (cashew cheese)	United States	*Salmonella* Stanley	17 infected, Three hospitalized	(CDC, 2014b)
2014	Nut butter (almond and peanut)	United States	*Salmonella* Braenderup	Six infected, Five hospitalized	(CDC, 2014c)
2014	Dietary supplements (Solgar ABC Dophilus powder)	United States	*Rhizopus oryzae*	One infected, Infant who died	(FDA, 2014)
2014-2015	Dried vegetable spice mixes	Sweden	*Salmonella* Enteritidis phase-type 13a	174 infected	(Jernberg et al., 2015)
2015	Raw sprouted nut butter	United States	*Salmonella* Paratyphi B variant	13 infected, Ten hospitalized	(CDC, 2016a)
2016	Pistachios	United States	*Salmonella* Montevideo and Senftenberg	11 infected, Two hospitalized	(FDA, 2016)
2016	Flour	United States	*E. coli* O121 and O26	63 infected, 17 hospitalized, 1 HUS	(CDC, 2016b)
2016	Organic shake and meal replacement (powder)	United States	*Salmonella* Virchow	33 infected, Six hospitalized	(CDC, 2016c)
2016-2017	Sesame seeds/tahini	Greece, Germany, Czech Republic, Luxembourg	*Salmonella* unknown serotype and Enteritidis	40 infected, 38 affected with one asymptomatic	(ECDC, 2017)
2017	Soy nut butter	United States	*E. coli* O157: H7	32 infected, 12 hospitalized, 9 HUS	(Hassan et al., 2019)
2017	Flour	Canada	*E. coli* O121	29 infected, Eight hospitalized	(Morton, 2017)
2018	Dried coconut	United States	*Salmonella* Typhimurium	14 infected, Three hospitalized	(CDC, 2018d)
2018	Kellogg's Honey Smacks Cereal	United States	*Salmonella* Mbandaka	135 infected, 34 hospitalized	(CDC, 2018c)
2018	Kratom	United States	*Salmonella* I 4,[5],12:b:-, Javiana, Okatie, or Thompson	132 infected, 38 hospitalized	(CDC, 2013b)

Abbreviation: HUS= Hemolytic Uremic Syndrome

[a] Exact number of hospitalizations not verified

Table 1.4**:** List of LMF recalls in the United States due to bacterial contamination in the past five years (2014-2018)

Year	Food Product	Company	Bacteria
2014	Brown rice flour	Lundberg Family Farms	*Salmonella*
	Carob powder	Dancing Star LLC, Earth Circle Organics, Hummingbird Wholesale, Sunburst Superfoods, Sunfood, Glaser Organic Farms, and Z Natural Foods	*Salmonella*
	Cashew Pieces	Chetak New York	*Salmonella*
	Chia and flax seed powder	Health Matters America, Inc.	*Salmonella*
	Chili powder	Fernandez Chile Company, Inc., Anhing Corporation, and US Trading company	*Salmonella*
	Chopped walnuts and pecan cookie pieces	John B. Sanfilippo & Son, Inc.	*Salmonella*
	Dietary supplements	New England Greens LLC	*Salmonella*
	Dried Roach (Vobla) Fish	S&S Food Import Corp.	Potential for *Clostridium botulinum* growth
	Ground annatto	Miravalle Foods, Inc.	*Salmonella*
	Ground black pepper	Gel Spice Company	*Salmonella*
	Ground Malabar Pepper	Olde Thompson, Inc.	*Salmonella*
	Ground oregano	McCormick & Company	*Salmonella*
	Herbal supplement (Full Spectrum Cilantro)	Swanson Health Products	*Salmonella*
	Macadamia nuts	Marathon Ventures, Inc.	*Salmonella*
	Nut butter	nSpired Natural Foods, Inc.	*Salmonella*
	Organic black peppercorns	Sprouts Farmers Market, Inc., Vitamin Cottage Natural Food Markets Inc., and Frontier Natural Products Co-op	*Salmonella*
	Oven Smoked Atlantic Salmon Stix, chili mango flavor	St. Mary's River Smokehouses	*Listeria monocytogenes*
	Peanut butter and cranberry crunch nutrition bars	Perfect Bar	*Salmonella*
	Peanut butter, cheese, salsa, and spreads	Parker's Farm Acquisition, LLC	*Listeria monocytogenes*
	Premium sliced smoked Atlantic salmon	Vita Food Products, Inc	*Listeria monocytogenes*
	Shark cartilage complex dietary supplement	AMS Health Sciences, LLC	*Salmonella*
	Shelled walnuts	Belleville Farmer's Market and Sun Tree LLC	*Listeria monocytogenes*
	Sprouted chia seed powder products	Navitas Naturals	*Salmonella*
	Superfood protein medley (sprouted chia seed powder)	Oriya Organics	*Salmonella*
	Sweet basil (dried)	Lisy Corporation	*Salmonella*

	Sweet paprika powder	TJX Companies, Inc.	*Salmonella*
	Walnuts	Sherman Produce	*Listeria monocytogenes*
2015	Beef jerky treats	Grill-Phoria LLC	*Salmonella*
	Bleached all-purpose flour	Navajo Pride, LLC	*Salmonella*
	Caribbean Nut and Fruit Mix	Vitamin Cottage Natural Food Markets Inc.	*Salmonella*
	Cashew kernels	The Hemisphere Group, Inc.	*Salmonella*
	Cashew split	Maya Overseas Foods, Inc.	*Salmonella*
	Chopped walnuts	John B. Sanfilippo & Son, Inc.	*Salmonella*
	Coriander powder and Organic coriander powder	Chetak New York LLC and Nijay International, Inc.	*Salmonella*
	Ground flaxseed meal	Zenobia Company LLC	*Salmonella*
	Island fruit and nut trail mix/bulk macadamia nuts	Rocky Mountain Foods, Inc./Various Brands	*Salmonella*
	Italian pine nuts	World Variety Produce, Inc.	*Salmonella*
	Mixed nuts and mixed nuts + cranberry mix	Aurora Products, Inc and Hannaford Supermarkets	*Salmonella*
	Nut butter spreads	JEM Raw Chocolate, LLC	*Salmonella*
	O'Coconut (including hemp and chia)	Nutiva	*Salmonella*
	Organic garlic powder	Vitamin Cottage Natural Foods Markets, Inc.	*Salmonella*
	Organic steel cut oats and chia with flax and rye flakes	Homestat Farm	*Salmonella*
	Organic sunflower seed	Freeland Foods, Inc.	*Salmonella*
	Pecanettes	Stone Mountain Pecan Company	*Salmonella*
	Pine nuts	Superior Nut & Candy Co. and Waymouth Farms, Inc.	*Salmonella*
	Raw cashews	Grand BK Corp.	*Salmonella*
	Raw hazelnuts	Fairway "Like No Other Market"	*Salmonella*
	Raw macadamia nuts and products	Whole Foods Market, Texas Star Nut and Food Co. Inc., Kanan Enterprises, Texas Pecan Company Inc., Aurora Products, Inc., Vitamin Cottage Natural Food Markets, Inc., Mahina Mele Farms, Sid Wainer and Son, and Sincerely Nuts Inc.	*Salmonella*
	Seasonings	The Kroger Co.	*Salmonella*
	Seed mix	Freeland Foods, Inc.	*Salmonella*
	Various products manufactured with garlic powder	Frontier Co-op	*Salmonella*
	Walnut halves and pieces	First Source, LLC.	*Salmonella*
	Walnut pieces	Eillien's Candies Inc.	*Salmonella*
	Walnuts	Hines Nut	*Salmonella*

	Walnuts and trail mixes	Aurora Products, Inc.	*Salmonella*
2016	Asafoetida powder	Shakti Group USA LLC	*Salmonella*
	Black pepper powder	Summit Import Corp.	*Salmonella*
	Boost tea (organic spinach powder)	Awareness Corp.	*Salmonella*
	Chocolate products	Asher's Chocolates/Lewistown, Inc.	*Salmonella*
	Cookie dough and products containing cookie dough (e.g., ice cream)	Nutrisystem Everyday, LLC, Blue Bell Ice Cream, Publix Super Markets, Wells Enterprises, Inc., Chocolate Shoppe Ice Cream Company, Inc., AC Creamery Inc., McConnell's Fine Ice Creams, ISB Food Group, LLC, Cedar Crest Specialties, Inc., Agave Dream, House of Flavors, Inc., Aspen Hills, LLC, and Kraft Heinz Company	*Listeria monocytogenes*
	Flaxseed powder	Health Matters America	*Salmonella*
	Flour and products made with flour	General Mills, Molly & Drew, Continental Mills, Kerry Inc., International Commissary Corporation, Hampton Creek, and Rabbit Creek Products	*Escherichia coli* O121 Updated to include several sub-types
	Fried rice	Hy-Vee, Inc.	*Listeria monocytogenes*
	Granola	Wild Blue Yonder Foods	*Salmonella*
	Macadamia nuts and nut butter	Mahina Mele Farms, LLC and Living Tree Community Foods, Marathon Ventures, Ashland Food Co-op, Marin Foods Specialties, Inc., and Snyder's-Lance, Inc.	*Salmonella*
	Milk powder and products containing milk powder (e.g., baking mixes, chips, mac and cheese, cheddar cheese seasoning)	Publix Super Markets, Stonewall Kitchen, LLC, Valley Milk Products LLC, Shearer's Foods LLC, Deep River Snacks, Tree House Foods, Inc., New Hope Mills, Fourth Street Barbecue, Inc./Packing Division, Brand Castle LLC, Old Dutch Foods Inc, Poppies International Inc., Houdini Inc, Williams-Sonoma, Snyder of Berlin, Herrs Foods Inc, Dawn Food Products, House-Autry Mills, Inc., Route 11, Bickel's Snack Foods Inc., Southeastern Mills Inc., TreeHouse Foods, and Miskesell's Potato Chip Company	*Salmonella*
	Organic Hojicha Tea	Frontier Co-op	*Salmonella*
	Organic Turmeric Ginger Green Tea	The Republic of Tea	*Salmonella*
	Pistachios (shelled and in-shell) and pistachio products	Braga Organic Farms, International Foodsource LLC, Lipari Foods, LLC, Country Life Natural Foods, Kanan Enterprises, Inc., Wonderful Pistachios, Texas Star Nut and Food Co., Inc., American Gourmet, Crescent Specialty Foods Inc., H-E-B, Rand on's Home Style Foods, Inc.	*Salmonella*

	Raw cashew pieces	Heritage International (USA) Inc.	*Salmonella*
	Sambar powder	SHRI SHIVA Foods Inc.	*Salmonella*
	Spiced herbal tea	CVS Pharmacy	*Salmonella*
	Sunflower kernels, snacks containing sunflower kernels, and products made with sunflowers (e.g., salads, spreads, butter, Cajun mix)	TreeHouse Foods, Inc., Giant Eagle, Hickory Harvest Foods, SunOpta, The Kroger Co., Brown & Haley, Publix Supermarkets Inc., Creative Snacks Co., Schulze and Burch Biscuit Co., Dakota Style Foods, Inc., Rucker's Makin' Batch Candies, Inc., Rucker's Wholesale and Service Co., Vending Nut Co., The Quaker Oats Company, Papa John's Salad and Produce, Inc., Hy-Vee, Inc., Eillien's Candies Inc., E.S. Foods, C. J. Dannemiller Co., First Source, Kashi Company, Troyer Cheese Inc., General Mills, Nu Life Market, ACH Food Companies, Inc. Windy Acres Candy & Nut Company, Clif Bar & Company, Post Consumer Brands, Atkins Nutritionals, Inc., The Hershey Company, MaraNatha, NoGii, Creative Snacks Co., Bounce USA, Honey Bunchie, LLC, Ernest Eats, Atlantic Spice Company Inc, and Figi's Companies, Inc.	*Listeria monocytogenes*
	Walnuts and walnut-containing products	Publix Super Markets and United Natural Trading LLC d/b/a Woodstock Farms Manufacturing, and HMSHost	*Listeria monocytogenes*
2017	Adobo seasoning	Goya de Puerto Rico	*Salmonella*
	Almonds, almond butter, and products containing almonds	GoMacro, Hampton Farms, and NOW Health Group Inc.	*Listeria monocytogenes*
	Biscuit dough	T. Marzetti Company	*Listeria monocytogenes*
	Cappuccino snack mix	Dutch Valley Food Distributors, Inc.	*Salmonella*
	Cashews, cashew butter, and products containing cashews	NOW Health Group Inc., Ava's, Wildway LLC, and Bulletproof 360	*Listeria monocytogenes*
	Chickpeas and products made with chickpeas	Chic-a-Peas LLC and House of Thaller,	*Listeria monocytogenes*
	Chili seasoning (jalapeño,	Conagra Brands, Inc., Frito-Lay, and Phoenix Food, LLC	*Salmonella*
	Dates	United Natural Trading LLC	*Listeria monocytogenes*
	Dried apricots and products containing dried apricots	Publix Super Markets	*Listeria monocytogenes*
	Flour	Smucker Foods of Canada Corp	*Escherichia coli* O121
	Ginger powder	Lords Organic	*Salmonella*
	Macadamia nuts and products containing macadamia nuts	Kroger	*Listeria monocytogenes*
	Milk powder and products containing milk	Continued from 2016s Valley Milk products recall:	*Salmonella*

	powder	Palmer Candy Company, Hostess Brands, LLC, Tupperware U.S., Inc., and Hy-Vee, Inc.	
	Oats and products made with oats	Garden of Light Inc.	*Listeria monocytogenes*
	Soy nut butter and granola products	SoyNut Butter Co. and Pro Sports Club	*Escherichia coli* O157: H7
	Sunflower seeds and products made with sunflower seeds	Hudson Valley Foods, Inc. and Bhu Foods,	*Listeria monocytogenes*
	Sweet basil seeds	L. A. Lucky Import & Export	*Salmonella*
	Tarragon	Organic Spices, Inc. d/b/a Spicely Organics	*Salmonella*
2018	Almond butter	Inspired Organics, LLC	*Listeria monocytogenes*
	Biscuits (continued form 2017)	T. Marzetti Company and Hom/Ade Food, Inc.	*Listeria monocytogenes*
	Cake mixes	Conagra Brands	*Salmonella*
	Cardamom pods green	Starwest Botanicals	*Salmonella*
	Coconut flour	King Arthur Flour Company	*Salmonella*
	Dietary supplements	Arthri-D, LLC and Break Ventures/California Basics	*Salmonella*
	Fruit and nut mixes	Hickory Harvest Foods	*Listeria monocytogenes*
	Kratom powder	PDX Aromatics DBA Kraken Kratom, Phyloextractum, Soul Speciosa, Tamarack Inc., NutriZone, LLC, Triangle Pharmanaturals LLC, Club 13, NGB Corp, Maya Distribution, LLC, Badger Botanicals, LLC, Pious Lion, Gaia Ethnobotanical, LLC, Blissful Remedies, Zakah Life, LLC, and World Organix, LLC	*Salmonella*
	Macadamia nuts	Mauna Loa Macadamia Nut Corporation	*Escherichia coli*
	Nutritional yeast	Oregon Food Bank	*Listeria* species
	Organic amaranth flour	Bob's Red Mill Natural Foods	*Salmonella*
	Peanut butter crunch cereal	The Quaker Oats Company	*Salmonella*
	Pistachios	Bazzini LLC and Barcelona Nut Company	*Salmonella*
	Pumpkin seeds	Oregon Food Bank	*Listeria* species
	Shredded and grated coconut	Evershing International Trading Company and Global Commodities Corporation	*Salmonella*
	Sunflower butter	Inspired Organics, LLC	*Listeria monocytogenes*
	Tahini	Ziyad Brothers Importing and Achdut LTD	*Salmonella*
	Whey powder and products containing whey powder (e.g., crackers, cereal)	Kellogg Company, Mondelēz Global LLC, Pepperidge Farm, Flowers Foods, Inc., and Associated Milk Producers, Inc.	*Salmonella*

Source of recalls: (FDA, 2019)

1.3 Environmental Stress

Bacteria face a variety of environmental stressors throughout their life cycle, and microorganisms either adapt or perish from stressors. Evolving to respond to these ever-changing environmental conditions provides bacteria with a selective advantage as well as increasing their chance for survival. Stressors such as desiccation, nutrient limitation, reactive oxygen species, temperature extremes, pH, starvation, and osmolarity are all environmental stressors that bacteria may encounter (Figure 1.2). Bacteria require adequate growth temperatures and access to nutrients to grow yet can remain viable for extended periods when exposed to unfavorable environments. Understanding how bacteria adapt, survive, and overcome different and often unique processing environments throughout the food industry is essential in understanding how to keep them in check and reduce their impact on the food industry.

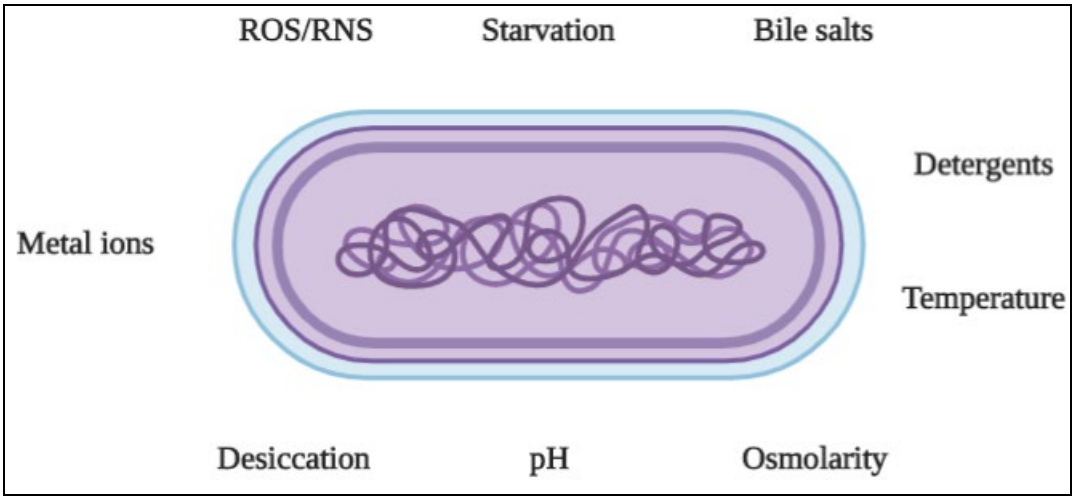


Figure 1.2: Common stressors that bacteria may encounter during the processing of food
ROS/RNS= Reactive Oxygen Species and Reactive Nitrogen Species
Adapted from (Runkel et al., 2013)

1.3.1 Effects of Water Activity (a_w) on Bacteria and Desiccation tolerance

Water activity (a_w) measures the state of water in foods (Scott, 1957) and is the ratio of the water vapor pressure of a sample divided by that of pure water at the same temperature. Another way to define a_w is by the percent equilibrium relative humidity (ERH) divided by 100. At this equilibrium, food does not gain or lose water to the surrounding air.

When determining a_w, a solution of pure water has an a_w of 1.00, and something that is considered completely dry has an a_w of 0.00. Upon the addition of solutes (such as sugar and salt), the water activity decreases (Forsythe, 2007). Bacteria have minimum water activity levels required to sustain growth, and below this minimum value, they are unable to grow (Table 1.5). Typically speaking, Gram-positive bacteria are less sensitive to lower a_w than Gram-negative bacteria (Montville and Matthews, 2008). However, even if growth does not occur, Gram-negative bacteria such as *Cronobacter sakazakii* and *Salmonella* can remain viable in low-a_w foods for extended periods such as years (Lin and Beuchat, 2007; Santillana Farakos et al., 2013).

Table 1.5: List of the minimum a_w required for growth of pathogenic bacteria and spoilage organisms

Microorganism	**Minimum a_w**
Clostridium botulinum E	0.97
Escherichia coli (STEC)	0.95
Clostridium perfringens	0.95
Salmonella spp.	0.95
Clostridium botulinum A, B	0.94
Vibrio parahaemolyticus	0.94
Bacillus cereus	0.93
Listeria monocytogenes	0.92
Staphylococcus aureus (anaerobic)	0.90
Staphylococcus aureus (aerobic)	0.86

Mycotoxigenic pennicilia and some yeasts	0.80-0.85
Halophilic bacteria and mycotoxigenic *Aspergilli*	0.75-0.80
Xerophilic molds and *Saccharomyces bisporus*	0.65-0.75
Osmophilic yeasts and a few molds	0.60-0.65

Adapted from (Meter Group, 2017)

One of the most common and effective processing methods employed to inactivate microorganisms is thermal energy. However, some bacteria exhibit increased thermal resistance in low-moisture environments when compared to high-moisture environments (Laroche et al. , 2005). Once contaminated low-moisture food begins to encounter high temperatures, the a_w of the food will differ from that of the bacterial cell. This osmotic imbalance leads to an exchange of water between the food and the bacterial cell. In foods, microbes oriented at the surface-air interface start to metabolize due to their ability to access water from hygroscopic surfaces or the surrounding air (Brown, 1976; Stone et al., 2016). High relative humidity has the potential to extend microbial metabolism if high enough. Bacteria possess the ability to adjust their internal a_w to that of the surrounding food matrix (Syamaladevi et al., 2016). Syamaladev et al. showed that bacterial cells, especially cocci, were able to equilibrate to their surrounding environment quickly (fractions of a second to seconds). *Bacillus* spp., a rod-shaped bacteria, take about 65% longer to equilibrate when compared to coccoid shaped bacteria. The calculations used by Syamaladev et al. were all based on a constant diffusion coefficient of vapor pressure inside the cell, which could change from cell to cell, thus impacting bacterial responses to desiccation (Syamaladevi et al., 2016). Shuxiang et al. found that *Salmonella* Enteritidis and its surrogate *Enterococcus faecium* had $D_{80°C}$ (min) values of

159.31 and 281.78, respectively, at 0.11 a_w vs. $D_{80°C}$ (min) of 10.37 and 12.71 at 0.50 a_w. The authors of the study hypothesize that when bacteria equilibrate to their surroundings (i.e., loss of water from the cell), the formation of increased stability protein structures that have the potential to thwart thermal denaturation may occur (Liu et al., 2018).

Certain bacteria, such as those in the family *Enterobacteriaceae*, are exceptionally well adapted to survive in desiccated conditions. These desiccation-tolerant organisms, dubbed anhydrobiotes, are not just limited to microorganisms; plants and invertebrates can be anhydrobiotes as well. Normally, cells that undergo desiccation experience protein misfolding, membrane fracturing, cytoplasm overcrowding, and metabolic arrest leading to their eventual death (Tapia et al., 2015). In response to desiccation, bacteria accumulate osmoprotectants such as trehalose (Edelson-Mammel et al., 2005; Leslie et al., 1995; Lin and Beuchat, 2007; Potts, 2001; Reina-Bueno et al., 2012; Tapia and Koshland, 2014; Tapia et al., 2015; Welsh and Herbert, 1999; Zhang and Yan, 2012), and some researchers have proposed the "water replacement hypothesis" to describe how desiccation-tolerant cells can survive under desiccated environments (Crowe et al., 1988; Leslie et al., 1995). In this proposed microbial mechanism, osmoprotectants replace the shell of water that surrounds macromolecules, protecting their membranes and proteins from damage induced by dehydration (Crowe et al., 1998; Potts, 1994; Potts, 2001). *Cronobacter sakazakii*, a member of the *Enterobacteriaceae* family that is well known for its desiccation tolerance, can utilize potassium and glutamate as temporary osmoprotectants (Du et al., 2018).

Through a process called vitrification, bacteria utilize trehalose and sucrose to form glasses that stabilize the drying cytosol. Vitrification minimizes damage to cellular

structures caused by stress, protecting their biological capabilities during subsequent rehydration upon consumption or reconstitution (Leslie et al., 1995; Sun and Leopold, 1997). In order to survive in a desiccated state for an extended period, bacteria must maintain this vitreous state or suffer from cytoplasmic crystallization, phase separation, and free radical oxidation (Sun and Leopold, 1997). The onset of these damaging conditions can result in loss of viability even after rehydration and receiving proper nutrients.

1.3.2 Oxidative Stress

The mere presence of reactive oxygen species (ROS), whether generated by disinfectants, non-thermal processing, or other means, has deleterious effects on bacterial cells. Processing plants frequently use chlorine as a disinfectant, which results in the formation of hydroxyl radicals that generates oxidative stress. When ROS forms, they damage bacterial proteins, lipids, and nucleic acid compounds (Cabiscol et al., 2000). Cysteine and methionine, both sulfur-containing amino-acids abundant in the cytoplasm and bacterial envelope, are the most susceptible to ROS and reactive chlorine species. In response to these deleterious compounds, bacteria produce catalases, peroxiredoxins, and superoxide dismutases to quickly try to modify/neutralize ROS before irreparable damage occurs (Ezraty et al., 2017).

SoxR is a transcriptional regulator that is activated by oxidation, and the transcription of *soxS* activates the Sox regulon. The sox regulon is comprised of the genes *sodA* (O_2^- detoxification), *nfsA* (prevents O_2^- formation), and *nfo* (DNA repair) (Daugherty et al., 2012). OxyR is also a transcription regulator that can afford a

protection mechanism for *Salmonella* against H_2O_2 stress (Christman et al., 1989; Daugherty et al., 2012). Once OxyR is activated by redox-active cysteine residues, more than 20 genes become transcribed. These transcribed genes are involved in DNA protection, disulfide bond formation, breakdown of H_2O_2, iron-sulfur cluster repair, and the removal of oxidized lipids (Calhoun and Kwon, 2011; Hébrard et al., 2009; McLean et al., 2010; Paget and Buttner, 2003; Spector and Kenyon, 2012). Together, SoxR and OxyR both activate Fur, which is an iron uptake repressor protein (Zheng et al., 1999).

Fur regulates iron uptake in bacteria, which retards the formation of hydroxyl radicals via the Fenton reaction. Via the Fenton reaction, iron, in its reduced form, converts H_2O_2 to •OH and Fe^{4+} (Touati, 2000). Through the regulation of iron uptake, the bacterial cell can control the formation of this extremely damaging hydroxyl radical (Touati, 2000; Zheng et al., 1999). In *S.* Typhimurium, researchers found that exposure to nitric oxide elicits an adaptive response by arresting reparation in cells and allowing for the survival under extreme oxidative stress conditions (Husain et al., 2008).

1.3.3 Biofilms, Persistent Foodborne Pathogens, and Virulence

Algae, fungi, archaea, and bacteria do not spend their entire lives as independent (planktonic) cells; they aggregate and coexist in communities called biofilms (Bär et al., 2002). Biofilms are composed of extracellular polymeric substances (EPSs), where cells embed themselves in the biofilm matrix, conferring a protective effect against desiccation (Bogino et al., 2013; Fei et al., 2017; Flemming et al., 2016; Yang et al., 2016). Bacterial fimbriae, primarily type 1 fimbriae produced by *E. coli* and *Salmonella enterica*, are vital during the initial cell-to-surface attachment (Beloin et al., 2004; Pratt and Kolter, 1998;

Van Houdt and Michiels, 2010). They play an essential role in the attachment of *Salmonella* Enteriditis to Teflon and stainless steel (Austin et al., 1998). Amongst *Enterobacteriaceae*, type 1 fimbriae are the most common adhesins; however, others such as curli fimbriae can enhance the capacity for biofilm formation (Ryu et al., 2004).

Biofilm formation occurs in a step-by-step process that starts by inorganic or organic molecules adsorbing onto a surface, creating a conditioning layer for bacterial attachment (Kumar and Anand, 1998). Microbial growth follows attachment, and this results in the subsequent production and secretion of EPS. Over time, this will result in the formation of a mature biofilm. In an abundance of nutrients, such as processing conditions just before spray-drying, biofilm formation can occur within just one hour in food processing facilities. As determined in biofilm formation in a food processing plant after an eight-hour shift, greater than 91% of bacteria are irreversibly attached (Flint et al., 1997).

Surface hydrophobicity plays a role in bacterial attachment. In general, an increase in surface hydrophobicity is thought to enhance microbial attachment, especially in *Bacillus* spores. Stainless steel is a favorite material used in processing plants because it is durable, resistant to corrosion and heat, and easy to clean/maintain. Unfortunately, it is also a hydrophobic material, and this means that spores (whether viable or not) can attach due to their hydrophobic outer coat proteins (Parkar et al., 2001). If damaged bacterial cells find their way to a biofilm and embed themselves within, they can use the conditioning layer to repair, grow and reproduce, further increasing the size of the biofilm. When these aggregates are no longer attached and slough off, they are known as

flocs and display many of the same characteristics/resistances as biofilms (Hall-Stoodley et al., 2004).

Traditionally, biocides such as sodium hypochlorite, have been used to disinfect processing plants; however, bacteria in biofilms are inherently more resistant to biocides than their planktonic counterparts. This resistance is a direct result of a few different mechanisms in biofilms, slow penetration of the biocide, altered physiology in biofilm cells, and the presence of persister cells (Kubota et al., 2009; Van Houdt and Michiels, 2010).

1.3.4 Bacterial Stationary Phase and Stress Resistance

Once nutrients required for growth are depleted, microorganisms in the air, water, soil, and food exist predominantly in stationary phase (Gefen et al., 2014). During stationary phase, the rate of bacterial growth equals the rate of cell death. In natural environments microbes inhabit, there is constant competition for a limited number of nutrients leading to a decrease in rapid growth and proliferation (Jaishankar and Srivastava, 2017). However, nutrient availability is not the only rate-limiting step for microbial proliferation; environmental stressors such as pH, temperature, osmolarity, and water activity also play significant roles. In response to environmental stressors, bacteria respond by entering stationary phase. Physiological changes to the cell anatomy increase peptidoglycan thickness in the cell wall and results in an accumulation of trehalose, nucleoid condensation, membrane fluidity reduction, and the cell takes on a more spherical appearance (Jaishankar and Srivastava, 2017; Nyström, 2004). During the transition to stationary phase, genes needed for metabolic growth processes are turned off,

and genes essential for survival are expressed. *E. coli* cells during stationary phase were found to express 20% of their overall genes at a higher level during stationary phase than during lag/log phase (Schellhorn et al., 1998). These stationary phase expressed genes are linked to normal stress responses such as osmotolerance, pH tolerance, thermotolerance, and DNA repair (Bohannon et al., 1991; Ishihama, 1997; Rava et al., 1999).

Persister cells are resistant bacterial cells that form in response to stress and are considered to be more resistant to many stress factors (Fisher et al., 2017; Van Houdt and Michiels, 2010). Studies have linked biofilm formation with persister presence (Brooun et al., 2000; Conlon et al., 2013; Keren et al., 2004; LaFleur et al., 2010; Spoering and Lewis, 2001). The formation of persister cells is thought to be due to the regulation and expression of specific, persister proteins (Lewis, 2001; Lewis, 2005; Lewis, 2007). Even if the wild type population is completely inactivated, persister cells can remain for extended periods.

Over time, enteric bacteria have evolved with tolerance mechanisms to survive acidic conditions. Once bacteria reach stationary phase, an alternative sigma factor, σ^s encoded by *rpoS,* enables cells to transport glutamate from the surrounding acidic environment (if available) into the cell. Upon internalization, glutamate decarboxylase converts glutamate to γ-amino butyric acid (Cheville et al., 1996; Small et al., 1994; Waterman and Small, 1996a). This amine provides a buffering effect and aids the cell in maintaining its internal pH homeostasis (Waterman and Small, 1996b). In *Salmonella* Typhimurium, σ^E plays a role in stationary phase survival, and when mutants lack the *rpoH gene* (the gene coding for σ^E), these mutant strains showed increased susceptibility to oxidative stress (Testerman et al., 2002).

1.4 Current Non-thermal Processing Technologies

Currently, thermal treatment is the most used food processing method due to its ease of use; however, the introduction of high heat to foods can adversely affect the organoleptic properties of LMFs. Non-thermal processing technologies have garnered much attention lately due to their ability to control microorganisms and cause minimal deleterious effects on LMFs (Barba et al., 2015). Multiple alternative decontamination methods have been investigated, such as pulsed electric field, ionizing radiation, "cold" plasma, pulsed light, and ozone. Each technology has its benefits and limitations, while others have poor consumer perception, have the potential to form mutagens carcinogens, or leave behind chemical residues. In this section, each non-thermal methodology will be briefly described by first introducing the methodology, what LMFs they have been used to decontaminate and their overall efficacy, and finally, how they impact microorganisms.

1.4.1 Pulsed Electric Field

Pulsed Electric Field (PEF) is a non-thermal pasteurization technology where the energy from a DC power supply is stored in a capacitor or bank of capacitors, and discharged, producing high-voltage, short-duration pulses of electricity. These pulses range from 10-80 kV/cm in voltage. Foods treated this way can be either static or flowing through a treatment chamber; however, most publications on microbial reduction relate to pumpable foods such as juice, milk, liquid eggs, and beer (Amiali et al., 2004; Amiali et al., 2007; Bermúdez-Aguirre et al., 2012; Cserhalmi et al., 2002; Dutreux et al., 2000; Elez-Martínez et al., 2004, 2005; Grahl and Märkl, 1996; Gurtler et al., 2011; Milani et al., 2015; Salvia-Trujillo et al., 2011; Zhao et al., 2013). While not commonly used in dry

or solid foods, some studies looked at PEF treatment of dark rye flour, although they only attained a 0.6-log reduction at field strengths higher than 20 kV/cm. Keith et al. reported that unless higher field strengths are applied via pressurization >260 kPa, the commercial applications of PEF for flour remains unlikely (Keith et al., 1998).

It is hypothesized that the discrepancy between microbial log reductions in liquid foods and flour could be due to the low moisture content, which influences the dielectric strength (Keith et al., 1998). The dielectric properties of foods influence the absorption of energy from high-frequency electric fields. However, even in whole-wheat flour with a moisture content of 12%, the dielectric constant and loss factor increased significantly with an increase in temperature, and the most pronounced effect was at 75°C and 85°C (Nelson and Bartley, 2002).

PEF treatment has an electroporation-like effect on bacterial cells, which leaves cells in three different physiological states: dead, sublethally injured, or intact. One of the limitations of PEF is that most bacterial spores and mold ascospores are resistant even at high-intensity exposure due to a lack of a cytoplasmic membrane. Gram-positive cells have a thicker peptidoglycan layer than Gram-negative cells, making them more resistant to PEF. The thin layer of peptidoglycan in Gram-negative bacteria results in a change in their membrane's electrical charge, making cells easier to inactivate through PEF treatment (Hülsheger et al., 1983; Schottroff et al., 2018). However, studies have shown that Gram-negative organisms such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella* serotypes Typhimurium and Senftenberg, and *Yersinia enterocolitica* are more resistant in acidic foods or media with a pH value of ~4.0 ((Álvarez et al., 2000; García et al., 2005, 2007; Somolinos et al., 2008). Arroyo et al. confirmed this by

demonstrating that *Enterobacter sakazakii* (now named *Cronobacter sakazakii*) was more resistant to PEF at a pH of 4.0 than at a pH of 7.0 (Arroyo et al., 2010). The increase in survivability is thought to be related to the presence of organic acids in the fruit juices tested (Arroyo et al., 2010). Exposure time (t_{PEF}) significantly impacts PEF efficiency, with some organisms demonstrating the ability to reseal pores in the membrane (seconds to hours) even when the membrane was damaged entirely (Pavlin et al., 2008; Teissie et al., 2005).

1.4.2 Ionizing Radiation

Ionizing radiation includes gamma rays, X-rays, and electron beam (or e-beam) and exists in the form of waves of shorter wavelengths less than 10^{-10} m (Figure 1.3) (Pillai, 2016). These shorter wavelengths are high energy, and their mode of bactericidal action on cells is by the direct destruction of chemical bonds. E-beam has the lowest penetration depth out of the three ionizing radiation types and distinguishes itself by bombarding the target sample with high-energy electrons (Sánchez-Maldonado et al., 2018). A linear accelerator generates these high-energy electrons by taking normal electrical currents and accelerating them to near the speed of light to generate a beam of electrons. In order to achieve uniform product exposure, the beam scans back and forth to generate a curtain of electrons (McKeen, 2012). Depending on the food processing application, e-beam has three energy levels, categorized by beam energy (megaelectronvolt, MeV), low (<1 MeV), medium (1-8 MeV), and high (8-10 MeV). Low-energy e-beam effectively sterilizes packing materials and seeds, medium-energy for packaged fruits and vegetables, and high-energy for spices, seafood, ingredients, and

packaged meats. Sterilization with levels higher than 10 MeV is possible; however, it would exceed the maximum allowable energy set by The U.S. Nuclear Regulatory Commission (NRC) (Pillai, 2016).

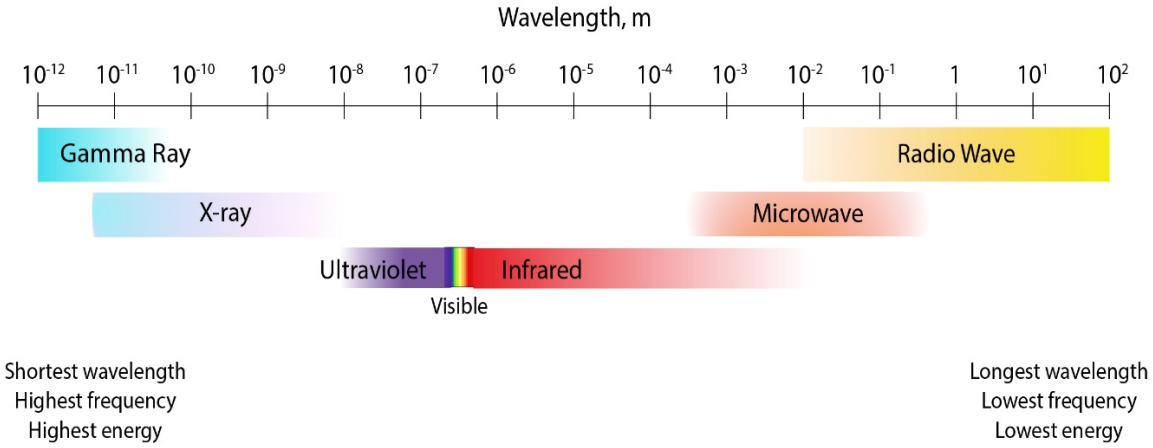


Figure 1.3: Electromagnetic spectrum spanning from gamma-ray to radio waves. Adapted from (NASA, 2013)

Gamma rays are emitted from the elements Cobalt-60 (most common) or Cesium-137 in their radioactive form. As Cobalt-60 decays, it produces energies of 1.17 and 1.31 MeV, which is higher than the 0.662 MeV generated by Cesium-137 (Parsons, 2012). When food products are exposed to gamma radiation, Cobalt-60 particles can penetrate uniformly into thicker materials and even packaged foods in shipping boxes. In this process, pallets of food are transported into a room that is evacuated of individuals and locked. Once operators are ready to treat a particular product, a lever system raises the source of radiation (Cobalt-60) from a pool of water where it is safely stored until it is needed. A conveyor system moves the product around the racks of Cobalt-60 until it has been exposed to gamma radiation for a preset period (Lambert and Martin, 2013). One downside to Cobalt-60 use is that it decays and becomes less effective over time, and in

food processing facilities, around 10% are replenished annually (Roberts, 2003). Unlike E-beam, gamma radiation has been used for decades in the food industry to sterilize fruits, meats, and vegetables.

Generating X-ray radiation occurs using linear or rhodotron-style accelerators and is produced when high energy electrons interact with the nuclei of high atomic numbers like tungsten (atomic number 74) or tantalum (atomic number 73). Upon interaction of the electrons with the atom, the electrons decelerate, and this results in the release of X-rays with electron energies of 5-6 MeV. Out of the three, x-ray radiation has the highest penetration depth and can pass through multiple rows of products (NDT Resource Center, 2009). Even though X-ray irradiation is a relatively new process (compared to gamma), it has been used in the food industry to treat foods such as oysters, milk, spinach, lettuce, tomatoes, shrimp, and fish (Moosekian et al., 2012).

Both X-ray and gamma rays share the same mechanistic bactericidal effect to damage DNA, and through the hydrolysis of water, disrupt metabolic functions in microorganisms (Clavero et al., 1994; Moseley, 1990; Pillai and Shayanfar, 2017). Food irradiation not only kills microorganisms, but also destroys insect eggs, prevents sprouting of vegetables, and delays the ripening of fruit (Abbas et al., 2011; Chéour, 2005; FDA, 2018). One benefit that irradiation has over other non-thermal technologies is that sterilization can be achieved in a food product. Foods sterilized by irradiation are typically useful for hospitals, which are an epicenter for immunocompromised individuals (B. Niemira, 2014; FDA, 2018). However, irradiation does impact the organoleptic properties of foods, and if irradiation treatments are decreased to minimize

these effects, they tend to have little to no effect on the deactivation of viruses and spores (Sánchez-Maldonado et al., 2018).

Spices are considered a high commodity food product traded around the world. Spices are not sterile products and come with microbiological concerns such as *Salmonella* spp., *B. cereus*, *Clostridium perfringens*, molds, and mycotoxins (Dennis et al., 2013). Even though ethylene oxide is currently being used to decontaminate spices, irradiation comes with the added benefit of increasing the shelf life of treated spices (Suhaj et al., 2006). Gamma irradiation has been used in LMFs, ranging from cardamom to peanut butter (Table 1.6). When treating perishables such as fruits and vegetables, an intermediate dose (1-10 kGy) of irradiation is enough to achieve pasteurization, and for sterilizing LMFs, doses range anywhere from 10 kGy up to 50 kGy depending on the product (Chizoba Ekezie et al., 2018).

Even though the FDA has deemed irradiated foods safe for consumption, consumer perception is an obstacle preventing more widespread usage in the food industry. Currently, as of 2016, there are more than 60 countries that utilize irradiation in some capacity for food safety (Maherani et al., 2016). The term irradiation confuses or alarms consumers since they associate it with radioactivity. Consumers and even the media are generally unaware of the benefits that irradiation brings to food safety (Eustice and Bruhn, 2012). Foods that are irradiated must be labeled with the international symbol for irradiation, the Radura symbol.

Table 1.6: Selection of ionizing radiation uses to decontaminate LMFs

Food Product	Source	Irradiation (kGy)	Microorganism	Starting (CFU/g)	Log reduction (CFU/g)	Reference
Alfalfa seeds	Gamma	Up to 2.8 kGy	*Salmonella* *Escherichia coli*	-	~2.8-log reduction ~4-log reduction	(Duncan et al., 2017)
Almonds	X-ray	0.431 kGy	*Salmonella* Enteritidis PT30 *Salmonella* Tennessee	8.40×10^8 7.73×10^7	5-log reduction 5-log reduction	(Jeong et al., 2012)
Almonds	Gamma	Up to 5 kGy	*Salmonella* Enteritidis PT30	-	4 log reduction	(Prakash et al., 2010)
Cardamom	Gamma	Up to 10 kGy	Total viable bacteria Total fungal count	1.3×10^4 1.3×10^3	Complete decontamination	(Sharma et al., 1984)
Cinnamon	Gamma	Up to 10 kGy	Total viable bacteria Total fungal count	2.1×10^3 3.1×10^2	Complete decontamination	(Sharma et al., 1984)
Clove	Gamma	Up to 10 kGy	Total viable bacteria Total fungal count	8.7×10^2 9.3×10^2	Complete decontamination	(Sharma et al., 1984)
Nutmeg mace	Gamma	Up to 10 kGy	Total viable bacteria Total fungal count	3.6×10^4 8.1×10^3	Complete decontamination	(Sharma et al., 1984)
Peanut butter	Gamma	1, 2, and 3 kGy	*Salmonella* Typhimurium	6.6×10^6	1.3- 4.0 log reduction	(Ban and Kang, 2014)
Peanut butter	E-beam	Up to 3.0 kGy	*Salmonella* Typhimurium *Salmonella* Tennessee	8.2×10^8 8.32×10^8	4.19 log reduction 5 log reduction	(Hvizdzak et al., 2010)
Pecans	E-beam	Up to 1.4 kGy	*Salmonella* Enteritidis PT30 *Salmonella* Senftenberg	$\sim 10^6$ $\sim 10^5$	4 log reduction 4 log reduction	(Cuervo et al., 2016)
Pepper	Gamma	5, 7.5 and 10 kGy	Total viable bacteria Total fungal count	1.4×10^7 8.7×10^2	Complete decontamination	(Sharma et al., 1984)
Pistachios	Gamma	Up to 5 kGy	*Salmonella* Typhimurium *Escherichia coli* O157: H7 *Listeria monocytogenes*	$\sim 10^6$	Below detection limit (1.0 log CFU/g)	(Song et al., 2019)
Tahini	Gamma	Up to 2 kGy	*Salmonella*	10^7 - 10^8	~4.6 log reduction	(Osaili et al., 2016)
Walnuts	X-ray	0.930 kGy	*Salmonella* Enteriditis PT30 *Salmonella* Tennessee	8.65×10^8 7.87×10^7	5-log reduction 5-log reduction	(Jeong et al., 2012)

Complete decontamination means that the total surviving population was reported as 0 CFU/g.
- Denotes that starting log concentration was not given.

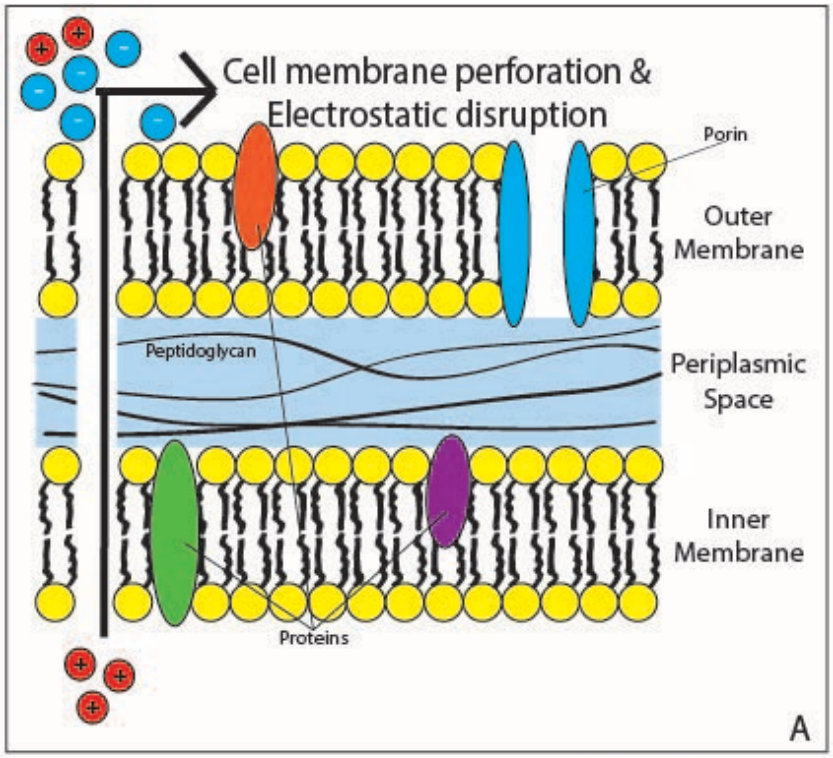
1.4.3 Non-thermal "Cold" Plasma

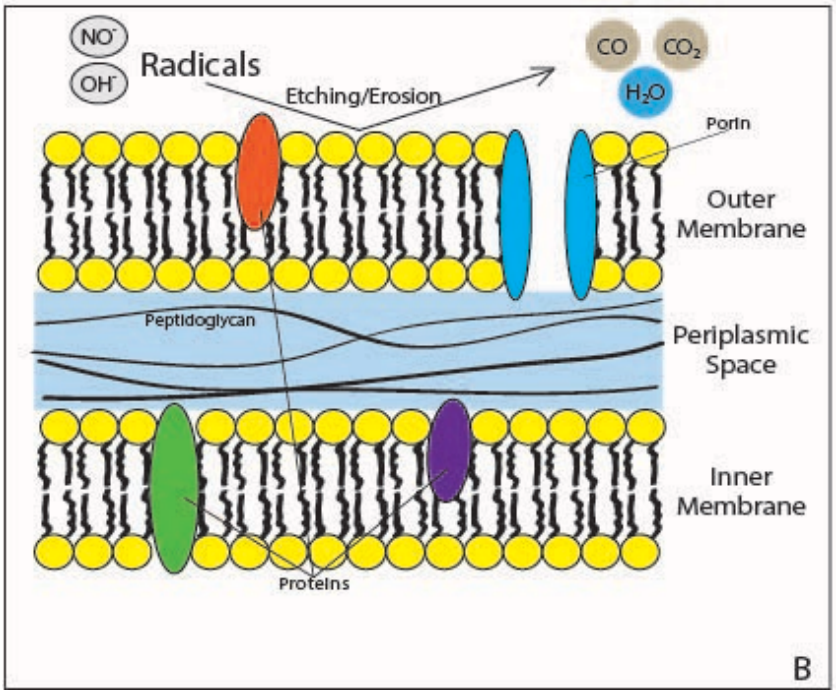
Plasma consists of a gas of total neutral charge, such as air, N_2, He, Ar, or Ne, that is entirely or partially ionized. The plasma generated consists of atoms, free electrons, ions, and photons in their fundamental or excited states (Misra et al., 2011). "Non-thermal plasma" in food processing refers to the mode of action that inactivates microorganisms without the use of a thermal component (Niemira, 2012). There are three types of plasma systems used in the food industry: direct, semi-direct, and indirect-treatment (Schlüter et al., 2013). Direct treatment, such as plasma jet and dielectric barrier discharges (DBD), results in the plasma generated coming in direct contact with the food. Semi-direct treatment, such as plasma-activated hydrogen peroxide, refers to when the resulting distance between the food and generated plasma is much larger than the mean free particle path. Indirect treatment, such as from an ozone generator, comes from plasma enclosed in a UV/VUV transparent reactor, and the plasma particles do not come in direct contact with the food (Schlüter et al., 2013).

Even though the mechanism of action of nonthermal plasma on the deactivation of microorganisms is poorly understood, it is believed to be caused by photodesorption and etching/erosion. Etching results from the adsorption of reactive species such as OH^- and NO^- that attack the components of the cell membrane. The plasma generated emits oxygen atoms or radicals, that upon combustion, cause an atom level erosion of the microorganism. UV radiation produced alongside plasma breaks chemical bonds in DNA strands by photon-induced desportion, dubbed photodesorption. Following desorption, atoms begin to form volatile particles (ex. CO, COOH, and CHx) that damage and cellular membranes and components (Delben et al., 2016; Laroussi, 2002; Moisan et al.,

2002; Sánchez-Maldonado et al., 2018; Schottroff et al., 2018). To visualize the effects of plasma on bacteria, including the impacts of ions, radicals, UV-light, and ROS/RNS on Gram-negative bacteria, refer to Figure 1.4.

Nonthermal plasma has been used for the decontamination of different LMFs with varying effectiveness (Table 1.7). The uses of this technology in the food industry are promising; however, there is a lack of standardization when using cold plasma, and a lack of standard treatment parameters. As such, it should be tailored towards the specific food product either by changing the working gas, utilizing a different plasma source, or by changing process parameters (Schlüter et al., 2013). One barrier for the use of nonthermal plasma on LMFs is that the different shapes and sizes of each food treated, which may result in a shadowing effect, such as peppercorns, which contain cracks, grooves, and pits. This shadow effect can reduce the efficiency of nonthermal plasma by obstructing microorganisms from the emitted UV photons, reactive species, and charged particles (Hertwig et al., 2015).


Cell membrane perforation &
Electrostatic disruption
Porin
Outer
Membrane
Peptidoglycan
Periplasmic
Space
Inner
Membrane
Proteins
A


NO
OH
Radicals
Etching/Erosion
CO
CO2
H2O
Porin
Outer
Membrane
Peptidoglycan
Periplasmic
Space
Inner
Membrane
Proteins
B

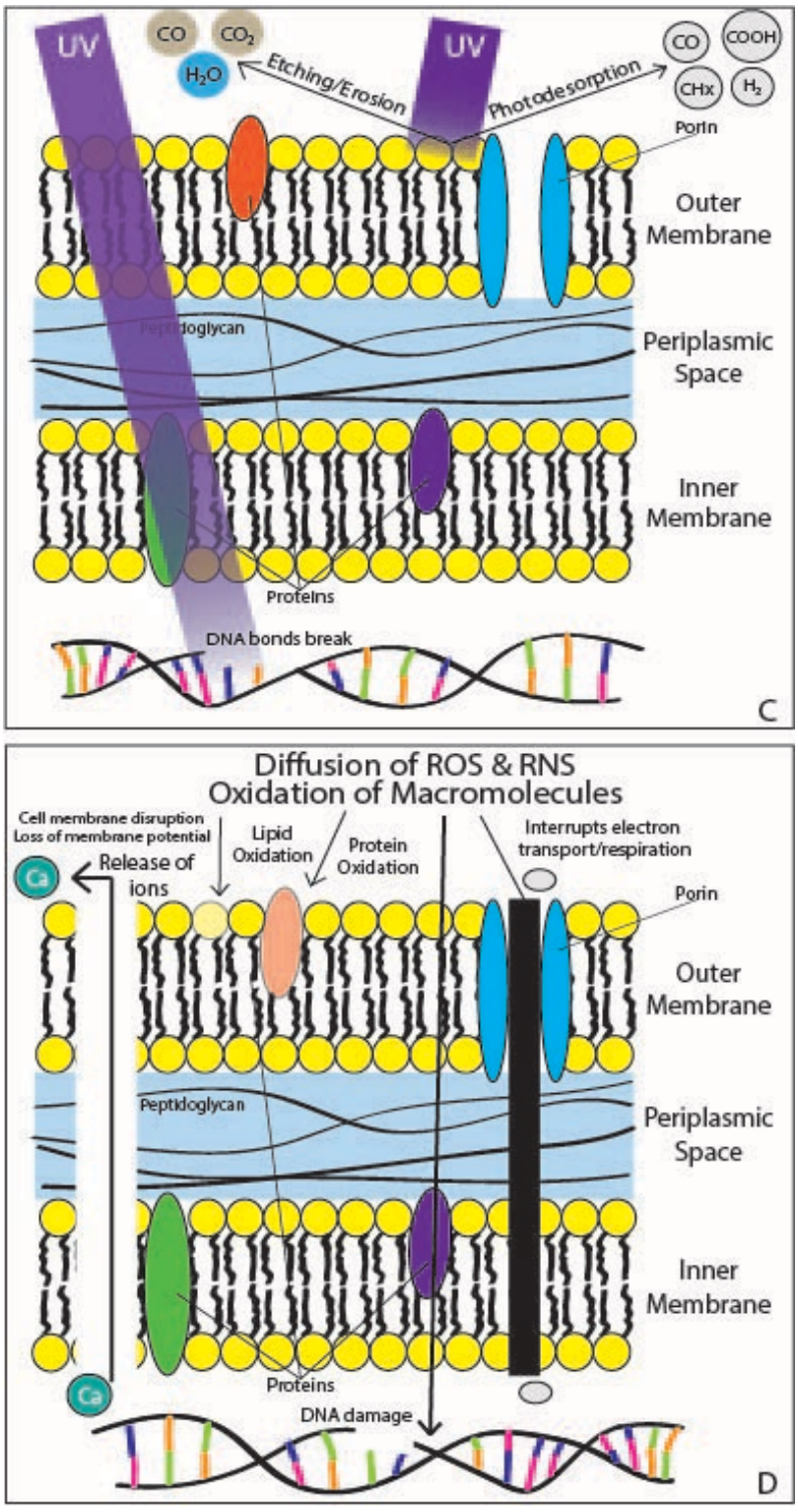


Figure 1.4: Effects of plasma on bacteria, including the impacts of ions, radicals, UV-light, and ROS/RNS on gram-negative bacteria. A) Ions, B) Radicals, C) UV-light, and D) ROS/RNS.
Adapted from (Schottroff et al., 2018)

Table 1.7: Non-thermal "Cold" plasma treated LMFs

Food product	Microorganism or toxin	Type of treatment/gas	Time	Findings	Reference
Nonfat dry milk	*Cronobacter sakazakii*	Cold atmospheric plasma/N_2	120 sec	3-log reduction	(Chen et al., 2019b)
Almonds	*Escherichia coli*	Dielectric-barrier discharge/ air	30 sec	5-log reduction	(Deng et al., 2007)
Hazelnuts, peanuts and pistachios	*Aspergillus parasiticus*	Low-pressure cold plasma/ SF_6	20 min	5-log reduction	(Basaran et al., 2008)
	Total aflatoxins (AFB1 and 2, AFG1 and 2)	Low-pressure cold plasma/ air		50% reduction	
Pepper seeds, paprika powder	Total mesophilic aerobic count	Remote plasma microwave-powdered/ air	60 min	>3-log reduction	(Hertwig et al., 2015)
Oregano powder				1.6-log reduction	
Brown rice cereal bars	*Aspergillus flavus*	Plasma jet/ Ar	20 min	4.25-log reduction	(Suhem et al., 2013)
Red pepper powder	*Aspergillus flavus*	Microwave-powered cold plasma/ N_2	20 min	2.5-log reduction	(Kim et al., 2014)
	Total aerobic bacteria			1-log reduction	
	Bacillus cereus spores	Microwave-powdered cold plasma/ $He\text{-}O_2$	30 min*	3.4-log reduction	

SF_6 = sulfur hexafluoride
* Denotes that log reduction was achieved after 90°C.

1.4.4 Pulsed Light

Pulsed light (PL) is a novel non-thermal technology developed to inactivate microorganisms present in a wide range of foods from juices, sugar syrups, milk, seafood, meat, vegetables, and fruits to powdered foods and ingredients such as nonfat dry milk, egg whites, whey and flour (Agüero et al., 2016; Aguiló-Aguayo et al., 2013; Artíguez and Martínez de Marañón, 2015a; Chaine et al., 2012; Cheigh et al., 2013; Chen et al., 2019a; Chen et al., 2018; Choi et al., 2010; Ganan et al., 2013; Hierro et al., 2009, 2012;

Huang et al., 2017; Ignat et al., 2014; Innocente et al., 2014; Izquier and Gómez-López, 2011; Krishnamurthy et al., 2007; Lasagabaster and De Marañón, 2012; Luksiene et al., 2012; Manzocco et al., 2014; Miller et al., 2012; Palgan et al., 2011; Pataro et al., 2011; Proulx et al., 2017; Ramos-Villarroel et al., 2012, 2014; Xu and Wu, 2016; Yi et al., 2017; Zenklusen et al., 2018). PL is also referred to by other names such as high-intensity broad-spectrum UV light (BSPL), high-intensity pulsed UV light (HIPL), pulsed UV-light (PUV), intense pulsed light (IPL), and pulsed white light (PWL) (Heinrich et al., 2016). An extensive list of published articles on pulsed light for use on liquids, vegetables, and fruits in the food industry is documented, but there is very little research on the use of PL on LMFs (Table 1.8). Pulsed light is most efficient when the treated product's appearance is as close to transparent as possible, where it can have maximum penetration effect with minimal scattering (Elmnasser et al., 2007).

Table 1.8: Types of LMFs treated with pulsed light

Food matrix	**Microorganisms**	**Treatment time**	**Log reduction (CFU/g)**	**Reference**
Alfalfa seeds	*Escherichia coli* O157: H7	135 pulses	0.94-1.82 log reduction	(Sharma and Demirci, 2003)
Black pepper	*Saccharomyces cerevisiae*	64 pulses	2.93 log reduction	(Fine and Gervais, 2004)
Corn meal	*Aspergillus niger* (S)	100 sec	1.35-4.95 log reduction	(Jun et al.,2003)
Egg white powder	*Cronobacter sakazakii* *Enterococcus faecium*	84 sec	5.30 log reduction 2.74 log reduction	(Chen et al., 2019a)
Ground black pepper	*Bacillus subtilis*	Ten flashes	0.80 log reduction	(Nicorescu et al., 2013)
Ground caraway	*Bacillus subtilis*	Ten flashes	0.80 log reduction	(Nicorescu et al., 2013)

Ground red pepper	*Bacillus subtilis*	Ten flashes	1.00 log reduction	(Nicorescu et al., 2013)
Honey	*Clostridium sporogenes* (S)	135 pulses	0.89-5.46* log reduction	(Hillegas and Demirci, 2013)
Infant food	*Listeria monocytogenes*		1-3 log reduction	(Choi et al., 2010)
Nonfat dry milk	*Cronobacter sakazakii* *Enterococcus faecium*	112 sec	5.27 log reduction 3.67 log reduction	(Chen et al., 2019a; Chen et al., 2018)
Powdered infant formula	*Cronobacter sakazakii*	4 ms	4 log reduction	(Choi et al., 2009)
Sugar syrup	*Bacillus subtilis* (S) *Saccharomyces cerevisiae* *Geobacillus stearothermophilus* (S) *Alicyclobacillus acidoterrestris* (S) *Aspergillus niger*	3 to 4 pulses	4.2 log reduction 5.4 log reduction >4 log reduction 3 log reduction 1.3 log reduction	(Chaine et al., 2012)
Wheat flour	*Saccharomyces cerevisiae*	64 pulses	0.7 log reduction	(Fine and Gervais, 2004)
Wheat flour	*Cronobacter sakazakii* *Enterococcus faecium*	112 sec	4.92 log reduction 2.79 log reduction	(Chen et al., 2019a)

(S) denotes spores were used instead of vegetative bacteria.
* They claim that even though they saw a log reduction, it was unable to inactivate the spores completely.

In order to generate pulsed light, electromagnetic energy accumulates in a capacitor for fractions of a second (nanoseconds to milliseconds) and is released in a broad spectrum of wavelengths (100-1,100 nm) (Figure 1.5); moreover, the energy density ranges from 0.01 to 50 $J \cdot cm^{-2}$ (Min and Zhang, 2005). The use of a shorter pulse generates a higher energy intensity, which translates into an increased germicidal effect.

Pulsed light is generated from inert gasses housed in a flash lamp, with xenon being the most common gas used in lamps (Dunn et al., 1989; Gómez-López et al., 2007; Sánchez-Maldonado et al., 2018). To describe the effect of pulsed light, these essential measurement parameter should always be measured and reported: fluence rate (F) [$W \cdot m^{-2}$], fluence (F) [$J \cdot m^{-2}$], number of pulses (n), pulse width (t) [s], exposure time ($t_{tot} = n*t$) [s], frequency ($f = 1/t$) [Hz] and peak power [W] (Heinrich et al., 2016).

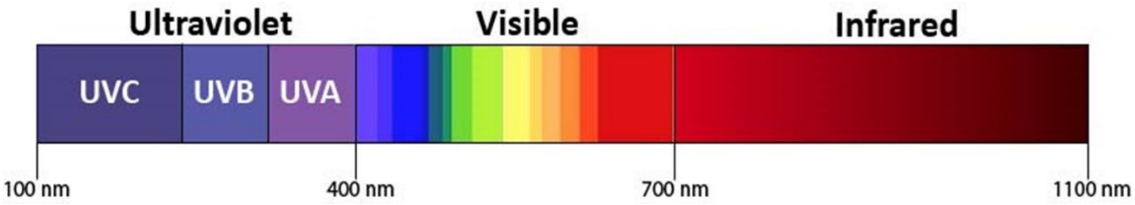


Figure 1.5: Range of light wavelengths generated by IPL
Adapted from (Montena, 2019)

Some limitations for pulsed light use in the food industry include a short penetration depth (1-2 mm), the variation of food particle uniformity, and cracks/creases/crevices in foods that can prevent IPL penetration, nutrient composition (high protein and lipids), and light scattering and reflection by particles (Gómez-López et al., 2007). Gómez-López et al. and Rajkovic et al. found that food matrices that are rich in fat and protein will impair the microbial decontamination efficacy of pulsed light (Gómez-López et al., 2005; Rajkovic et al., 2010). Proteins are thought to be able to absorb UV light at 280 nm (Hollósy, 2002). Aromatic residues (tryptophan, tyrosine, and phenylalanine) can capture UV light from ~250-298 nm, and this leads to a detrimental photochemical pathway that impacts the overall protein structure. Proteins contain disulfide bridges that can quench UV light since excitation triggers the ejection of

electrons from the side chains of aromatic residues, which are then captured by disulfide bridges and eventually result in a free thiol group in the protein (Teresa et al., 2012). This process could be beneficial in specific applications as pulsed light could be utilized to inhibit enzymatic activities (J. Dunn, 1996; Wang et al., 2017).

Pulsed light has a three-fold effect on bacteria: 1.) photochemical; 2.) photothermal; and 3.) photophysical. Photochemical, which damages DNA via UV light, is considered the primary target of pulsed light in bacterial cells. UV light is referred to either as UVA, UVB, or UVC. UVA, sometimes described as long-wave UV, is the closest radiation to visible light, and its wavelength spans from 320 to 400 nm. UVB spans from 280 to 320 nm. UVC, sometimes referred to as short wave UV, spans from 100 to 280 nm (UCAR, 2017). When referring to the germicidal capabilities of each wavelength region, UVA and UVB have longer wavelengths than UVC; therefore, they do not contain sufficient energy to penetrate bacterial cells. If bacterial cells become exposed to UVC for extended periods, UVC can penetrate the leading to cellular damage. Peak germicidal effectiveness of the pulsed light spectrum is at 265 nm, which lies in the UVC region. UVB, especially 280-315 nm, induces cytotoxic and mutagenic DNA lesions in cells. These lesions include cyclobutane-pyrimidine dimers (CPDs), 6-4 photoproducts, Dewar valence isomers, and strand breaks (Kim et al., 2016; Rastogi et al., 2010). The formation of these dimers distorts strands in the DNA helix leading to a conformational change, which interferes with DNA replication. However, if bacteria were not able to evolve to respond to UV exposure, they would not be able to survive on the earth, and as such, they have developed several highly conserved DNA repair mechanisms. These mechanisms are base excision repair, photoreactivation, UV-damage

endonuclease, nucleotide excision repair, and the SOS response (Goosen and Moolenaar, 2008; Krishna et al., 2007; Rastogi et al., 2010). Even though they have an inherent ability to withstand UV damage, Green et al. found that exposure to UV wavelengths between 200-320 nm inhibits their ability to perform photoreactivation (Green, et al., 2003).

Photothermal provides a lethal effect on bacteria when energy exceeds 0.5 $J \cdot cm^{-2}$, causing a temporary overheating of the bacterial cell leading to disruption (Wekhof, 2000). Bacteria absorb UV light differently than their surrounding medium (food matrix), and this difference leads to the vaporization of their internal water content leading to cell membrane disruption. Wekhof et al. demonstrated that following the exposure of an *Aspergillus niger* spore to pulsed light, a ruptured top to the spore occurred, and the inside of the spore was empty due to the leakage of internal contents (Wekhof et al., 2001). When UV wavelengths are absent, photothermal is considered the primary mode of action due to the rapid transfer of large amounts of thermal energy on the surface of the food product (Elmnasser et al., 2007).

Photophysical damage affects the bacterial membrane and overall cell composition. Takeshita et al. utilized both pulsed light and continuous UV on *Saccharomyces cerevisiae* and found that there was a higher concentration of eluted proteins from yeast cells in response to pulsed light exposure, demonstrating the potential for cell membrane damage post-pulsed light. Transmission electron microscopy of PL-treated yeast cells showed cell membrane distortion, change in yeast shape to more circular, and expanded vacuoles. When compared to continuous UV, yeast's cell structure

remained almost the same, further adding to their hypothesis of photophysical induced cell membrane damage (Takeshita et al., 2003).

1.4.5 Ozone

Ozone, also known as O_3 or triatomic oxygen, is a potent oxidant and virucide that has been approved for use as a sanitizer/disinfectant and has uses in food processing since 1997 (United States). In order to generate ozone, oxygen molecules are first dissociated by an energy source, typically by running a high voltage alternating current across a dielectric discharge gap, which contains the oxygen-bearing gas. This method, aptly named corona discharge method (Figure 1.6), is the most used in the food industry, with the other being UV radiation (188 nm). Free-radical oxygen species are generated, which then react with diatomic oxygen to form the ozone (EPA, 1999; Guzel-Seydim et al., 2004; Torlak et al., 2013). Ozone decomposes back to diatomic oxygen relatively quickly, with decomposition rates of 20-50 minutes in the atmosphere and 1-10 minutes in water. One of the benefits of ozone is that it is considered safe for the use on food products due to its residue-free oxidant properties (Allen et al., 2003).

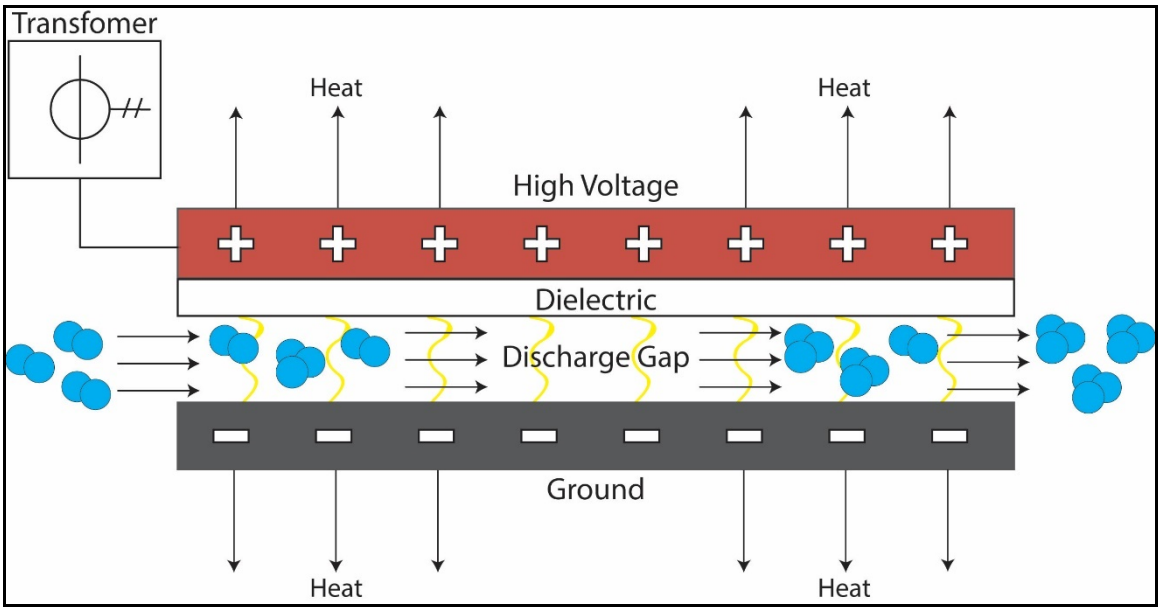


Figure 1.6: Corona discharge method with a current transformer
Adapted from (Oxidation Technologies, LLC, 2017)

Ozone is effective in the reduction of aflatoxins present in food such as peanuts, dried figs, red pepper, and corn (de Alencar et al., 2012; Inan et al., 2007; Luo et al., 2014; Zorlugenç et al., 2008). Fungal strains *Aspergillus flavus* and *Aspergillus parasiticus* are the primary producers of aflatoxins. Of the different aflatoxins produced, B1, B2, G1, and G2 are a health concern with B1 being the most toxic to humans (Maeba et al., 1988; McKenzie et al., 1997; Proctor et al., 2004). Multiple studies have demonstrated its effectiveness on the reduction of yeasts, molds, fungi, and any produced toxins while leaving no trace of ozone on the treated food (Akbas and Ozdemir, 2006; Freitas-Silva and Venâncio, 2010; Prudente and King, 2002; Proctor et al., 2004). Other studies have also demonstrated that ozone is useful in reducing microbial pathogens from the surface of LMFs and ground products such as ground black pepper and flaked red peppers (Table 1.9).

Table 1.9: Types of LMFs treated with ozone

Food Matrix	Microorganisms	Treatment conditions	Log reduction	Reference
Barley	Fungal spores and mycelia	Gaseous ozone 0.10 mg/min 5 min 20°C	96% reduction	(Allen et al., 2003)
Corn	Aflatoxin B_1	Gaseous ozone 12-16 g/m^3 96 h	92% reduction	(Prudente and King, 2002)
Dried figs	*Escherichia coli* *Bacillus cereus* *Bacillus cereus* (S)	Gaseous ozone 1 ppm 9 ppm for spores 360 min	3.5 log reduction 3.5 log reduction ~2 log reduction	(Akbas and Ozdemir, 2008b)
Flaked red pepper	*Escherichia coli* *Bacillus cereus* *Bacillus cereus* (S)	Gaseous ozone 1 ppm 9 ppm 360 min for spores	1.5 log reduction 2.0 log reduction 1.5 log reduction	(Akbas and Ozdemir, 2008a)
Ground black pepper	*Escherichia coli* *Salmonella* spp. *Aspergillus* spp. *Penicillium* spp.	Ozone and Ozonated gas 6.7 ppm 60 min	>3 log reduction >3 log reduction >4 log reduction >3 log reduction	(Zhao and Cranston, 1995)
Ground black pepper	*Escherichia coli*	Gaseous ozone 1 ppm 120 min	6.5 log reduction	(Emer et al., 2008)
Peanut flour	Aflatoxin B1, B2, G1, and G2	Gaseous ozone 15 min @ 25°C	48% reduction (B1) 43% reduction (B2) 42% reduction (G1) 21% reduction (G2)	(Proctor et al., 2004)
Peanut kernels	Aflatoxin B1, B2, G1, and G2	Gaseous ozone 15 min @ 25°C	69% reduction (B1) 75% reduction (B2) 74% reduction (G1) 40% reduction (G2)	(Proctor et al., 2004)
Pistachio kernels	Aflatoxin (B_1, B_2, G_1, and G_2)	Gaseous ozone 9 ppm	5% reduction	(Akbas and Ozdemir, 2006)
Rice	*Penicillium* spp.	Gaseous ozone	100% reduction	(Santos et

	Aspergillus spp.	1.0 $L \cdot min^{-1}$ 48 h (*Penicillium*) 60 h (*Aspergillus*)	or 3.8 log reduction	al., 2016)
Wheat	Fungi	Gaseous ozone 0.33 mg/g wheat/min 5 min	96.9% reduction	(Wu et al., 2006)
Whole black pepper	*Escherichia coli*	Gaseous ozone 1 ppm 120 min	7 log reduction	(Emer et al., 2008)

(S) denotes spores

Since ozone is a strong oxidizer, it mechanistically acts on microorganisms by oxidizing cell membranes, cell envelopes, cytoplasmic membrane, spore coats, and the capsid of viruses. Ozone disrupts the cell membrane by oxidizing sulfhydryl groups of enzymes and unsaturated fatty acids (Khadre et al., 2001), disrupting regular cellular activity and leading to rapid cellular death by impacting cell permeability (Kim et al., 1999). Damage to spores does not occur by damaging DNA, but by impacting the spores' ability to germinate to a vegetative state by damaging the inner membrane (Young and Setlow, 2004). Ozone damages viral capsid proteins by breaking them apart into subunits. Following the destruction of the viral capsid, nucleic acid (DNA or RNA) then becomes inactivated by inducing mutations (Kim et al., 1980).

1.4.6 Hurdle Technologies

Nonthermal technologies are combined with other technologies to synergistically increase microbial inactivation and hurdle technologies (Ramesh, 2003). Hurdle technologies such as thermal treatments, water assistance, ultrasound, antimicrobial chemical additives, modified atmospheric packaging, titanium dioxide (TiO_2), high

hydrostatic pressure, antimicrobials (oils, extracts or bacteriocins) are utilized in conjuncture with PL (Artíguez and Martínez de Marañón, 2015b; Bradley et al., 2012; Ferrario et al., 2015; Green et al., 2003; Huang and Chen, 2014; Marquenie et al., 2003; Tuema et al., 2000; Uesugi and Moraru, 2009). When combining different nonthermal technologies, some factors to consider are whether the properties of the food will be altered and if multiple technologies can be implemented on preexisting production lines without significant changes.

1.5 Select Pathogens of Concern in LMFs

1.5.1 *Cronobacter sakazakii*

Cronobacter spp. are Gram-negative, facultatively anaerobic, non-spore-forming bacilli from the family *Enterobacteriaceae*. The Centers for Disease Control and Prevention (CDC) reports that ~4-6 infections occur with infants per year in the United States and with fatality rates of 40-80% (CDC, 2011b). In 2011, the CDC reported that the number of *Cronobacter* infection in infants had tripled since 2010 (CDC, 2012b). *Cronobacter* is a cause of neonatal meningitis and is often associated with diarrhea and necrotizing enterocolitis (Farmer, 2015). Not only is neonatal infection severe, but it can be severe or fatal for susceptible demographics of the population, such as the elderly or immunocompromised. In the United States, Minnesota is the only state with mandatory reporting of *Cronobacter* infections (CDC, 2018b) and the lack of reporting elsewhere resulting in the underreporting of *Cronobacter* infections caused worldwide (Tóthová et al., 2011). In 2006, Bowen et al. analyzed 46 cases of reported *Enterobacter* (*Cronobacter*) *sakazakii* infections and determined that 12 sickened patients had

bacteremia, 33 meningitis, and one a urinary tract infection. Among the 33 meningitis patients, 33% (11) experienced seizures, 21% (7) developed a brain abscess, and 42% (14) died (Bowen and Braden, 2006).

Before 2008, *Cronobacter* nomenclature was singularly referred to in the literature as *Enterobacter sakazakii*. Medical literature reported that there was significant variation amongst *E. sakazakii* isolates, which lead to their eventual reclassification to the *Cronobacter* genus (Osaili and Forsythe, 2009). Currently, there are seven species of *Cronobacter*, 1.) *C. sakazakii*; 2.) *C. malonaticus*; 3.) *C. turicensis*; 4.) *C. muytjensii*; 5.) *C. dublinensis*; 6.) *C. condimenti*; and 7.) *C. universalis* (Song, et al., 2018). Before 2014, there were three more species of *Cronobacter*; however, Stephan et al. reclassified *C. zurichensis* to *Siccibacter turicensis*, *C. pulveris* to *Franconibacter pulveris* and *C. helveticus* to *Franconibacter helveticus* (Stephan et al., 2014).

Intrinsic properties of *Cronobacter* permits survival for extended periods in the environment, or in a host to cause illness. These properties include resistance to heat, oxygen radicals, pasteurization, stomach acids, and bile, desiccation, and UV radiation (Kalyantanda et al., 2015; Osaili and Forsythe, 2009). One of the reasons that *Cronobacter* is desiccation resistant is due to the presence of a heteropolysaccharide capsule. The presence of this capsule allows *Cronobacter* to remain viable for greater than two years, and cells without the capsule are reported to have shorter viability and less pathogenicity (Osaili and Forsythe, 2009). Acid tolerance allows *Cronobacter* to survive acidic conditions such as the low pH of the human stomach, where they can continue to infect the host. Evasion of the host immune system, growth inside macrophages, adherence, and cytotoxicity are thought to be possible through the presence

of the Type VI secretion systems utilized by *Cronobacter* (Holý and Forsythe, 2014; Kent et al., 2015). Some *Cronobacter* spp. produce heat-stable endotoxins and enterotoxins. The *Cronobacter*-produced enterotoxin can survive PIF pasteurization (Jaradat et al., 2014), and the endotoxin lipopolysaccharide facilitates translocation across the host's intestinal wall and eventually the BBB (blood-brain barrier) through the production of hemolysins (Holý and Forsythe, 2014). Jaradat et al. found that some *Cronobacter* spp. readily attach to hydrophobic surfaces such as Teflon and plastics (like those used in feeding tubes) and, if given proper nutrients, can proliferate on these surfaces (Jaradat et al., 2014). In the clinical setting as neonates, infants born prematurely, and those of low birth weight (< 2,500 g) more often than not are fed reconstituted infant formula through feeding tubes (Bowen and Braden, 2006). Once bacteria are attached to feeding tubes, they proliferate and form biofilms (Kim et al., 2006), allowing passage through the stomach of neonates resulting in severe illness and the potential for irreversible damage.

Cronobacter is ubiquitous in the environment, with natural habitats being predominantly plant and organic material. In 1979, there was a case report of a 7-day old infant who had bacteremia from "yellow-pigmented *Enterobacter cloacae,*" later reclassified to *Enterobacter sakazakii* (Monroe and Tift, 1979). This yellow pigment is a carotenoid produced by *C. sakazakii,* and a seven gene cluster (*crtE-idi-crtXYIBZ*) is responsible for the biosynthesis of carotenoids in *Cronobacter* (Lehner et al., 2006). Pigment production is not unique to *Cronobacter,* and other bacterial pathogens use them to survive in harmful environments and increase virulence. *Staphylococcus aureus* can produce golden staphyloxanthin, *Pseudomonas* spp. produce blue-green pyocyanin, and

Cryptococcus neoformans and *Aspergillus* spp. produce dark brown or black melanin (Liu and Nizet, 2009). Carotenoids are known to be able to act as antioxidants by scavenging reactive oxygen species, further increasing the microbes' survival in harsh environments (Mathews and Sistrom, 1959). Another benefit to bacteria by the production of carotenoids is the ability to stabilize cellular membrane and change membrane fluidity (Chattopadhyay et al., 1997; Gruszecki and Strzałka, 2005; Sedkova et al., 2005)

Cronobacter is known as a cause of contamination in powdered infant formula (PIF) (see Table 1.10 for clinical cases linked to PIF), and has been isolated from various food and environmental sources such as milk powder, whey powder, chocolate, dried herbs and spices, iced tea, dried cereals, starches, rice seed, grains, preparation equipment (blenders and spoons), and hospital air (Bar-Oz et al., 2007; Block et al., 2002; Clark et al., 1990; Cottyn et al., 2001; Iversen and Forsythe, 2004; Jung and Park, 2006; Kandhai et al., 2004; Masaki et al., 2001; Muytjens et al., 1988; Postupa and Aldova, 1984). A reason that *Cronobacter sakazakii* is of concern in PIF is that it is the only species of *Cronobacter* that can metabolize sialic acid, which is added to PIF to promote the brain development in infants (Joseph et al., 2013). *Cronobacter* can enter PIF through post-process contamination, from the production facility, the hospital, or attached to common household items such as spoons or in blenders.

Table 1.10: Clinical cases of *Cronobacter* spp. linked/presumably linked to powdered infant formula

Year	Country	Illnesses (deaths)	Reference
1979	United States (Macon, GA)	1	(Monroe and Tift, 1979)
1983[a]	Netherlands	8 (6)	(Muytjens et al., 1983)
1986-1987	Iceland	3 (1)[b]	(Biering et al., 1989)
1989	United States (Memphis, TN)	4[d]	(Simmons et al., 1989)
1990	United States (Baltimore, MD)	1[d]	(Noriega et al., 1990)
1991	New Zealand	2 (0)	(Norberg et al., 2012)
1990-1992	Canada	3[d]	(Pagotto and Farber, 2009)
1994	France	13 (3)	(Caubilla-Barron et al., 2007)
1998	Belgium	12 (2)[c]	(van Acker et al., 2001)
2001	Israel	5[d]	(Bar-Oz et al., 2007; Block et al., 2002)
2001	United States (Tennessee)	1 (1)[e] 9	(Weir, 2002)
2004	New Zealand	5 (1)	(Norberg et al., 2012)
2007	Canada	1[d]	(Pagotto and Farber, 2009)
2007	India	2 (1)	(Ray et al., 2007)
2008	United States (New Mexico)	2 (1)	(CDC, 2009)
2010	Mexico	2[d]	(Jackson et al., 2015)
2011-2012	United States (Florida, Illinois, Missouri, and Oklahoma)	4 (2)	(CDC, 2012b)
2017	China	2[d]	(Cui et al., 2017)

[a] These 8 cases were reported over six years.
[b] Even though two survived, one was left severely mentally disabled and quadriplegic while the other developed a seizure disorder.
[c] The two that died were twin brothers.
[d] There were no mentions of if the children affected recovered or died.
[e] Upon the child's death, enhanced surveillance was performed and found nine additional cases.

1.5.2 *Salmonella* spp.

Salmonella spp. are facultatively anaerobic, non-spore-forming bacillus (0.7-1.5 x 2.0-5.0 µm) that are usually motile through peritrichous flagella (Bergey et al., 1984).

There are more than 2,500 serotypes described for *Salmonella. S. enterica* and *S. bongori*

are the two recognized species of *Salmonella, S. enterica* represent 2,557 serovars, and *S. bongori* represents 22 serovars. Of these 2,500+ serotypes, >100 serotypes for many of the reported human infections. The Centers for Disease Control and Prevention (CDC) reports that of these human disease-causing serotypes, the five most common serotypes exhibiting antibiotic resistance are: Enteritidis, Typhimurium, Newport, Heidelberg, and I 4,[5],12:i:- (CDC, 2015b). The Kauffmann-White scheme helps with the classification of *Salmonella* into serotypes by utilizing a series of numbers and letters given to somatic O antigen (based on oligosaccharides), Vi antigen (virulence) and H antigen (flagellar proteins). O, Vi, and H were chosen due to being of primary diagnostic importance and in no way is representative of the complete record of the antigenic complement (Kauffmann, 1966). Refer to Table 1.11 to see examples of this scheme for the top four most common serotypes with antibiotic resistance.

Table 1.11: List of five common foodborne illness related *Salmonella* serotypes following the Kauffmann-White scheme

O group	**Serovar**	**Somatic (O) antigen**	**Flagellar (H) antigens**		
			Phase 1	**Phase 2**	**Other**
Group O:4 (B)	*S.* Typhimurium	1,4,[5],12	i	1,2	
Group O:9 (D_1)	*S.* Enteritidis	1,9,12	g,m	-	
Group O:6, O:8 (C_2)	*S.* Newport	6,8,20	e,h	1,2	[Z_{67}], [Z_{78}]
Group O:4 (B)	*S.* Heidelberg	1, 4,[5],12	r	1,2	
Group O:7 (C_1)	*S.* Oranienburg	6, 7, 14	m,t	[z_{57}]	

Modified from (Bergey et al., 1984).
Underlined represents the presence of these somatic factors relates to phage conversion.
[ ] may be present or absent without relation to phage conversion.
Z antigens (other) are still H antigens; however, they are coded neither by *fliC* nor *fliB*.

Salmonella belongs to the family *Enterobacteriaceae*, which also contains other well-known foodborne pathogens such as *Cronobacter* spp., *Escherichia coli*, *Shigella* spp., and *Yersinia enterocolitica*. The 2018 FoodNet Surveillance Data places *Salmonella* as the second most common cause of foodborne illness in the USA, with the number of incidences not declining in the previous three years (FoodNet, 2018) (Figure 1.7). The European Food Safety Authority (EFSA) reported in 2017 that *Salmonella* cases are no longer leveled off or declining. Since 2014, the EFSA reports that cases of *Salmonella* Enteritidis in the EU have increased by 3% (EFSA, 2017).

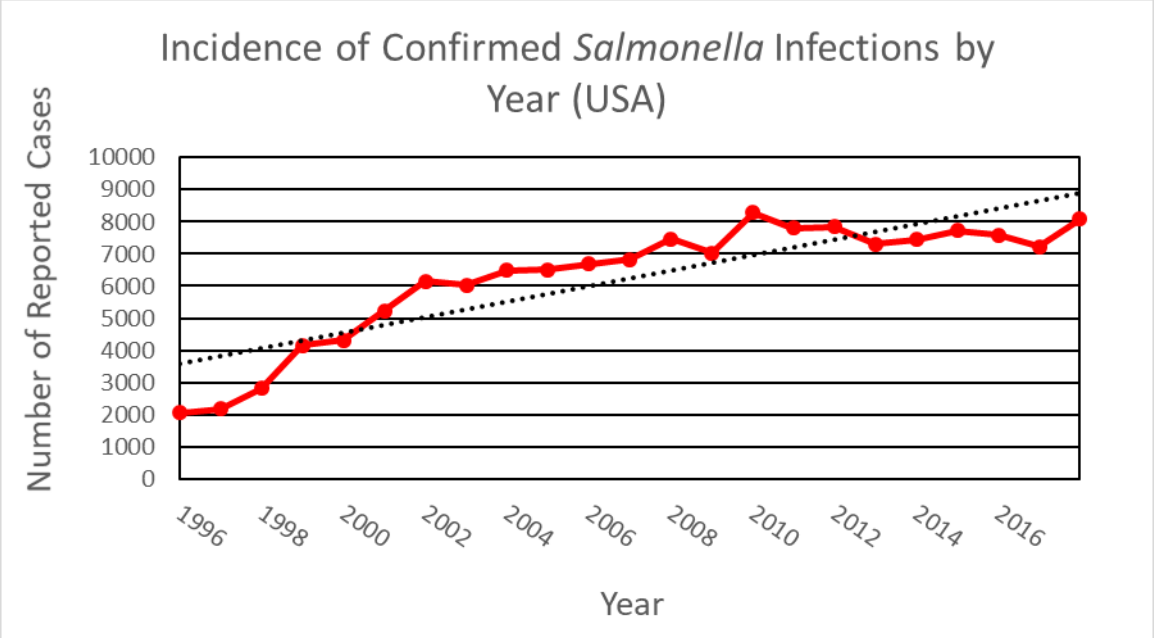


Figure 1.7: Incidence of confirmed *Salmonella* infections by year in the USA 1996-2018. Source of data (CDC, 2019b)

Salmonella is estimated to cause 1,000,000 foodborne illnesses every year, and worldwide, it is the cause of 1 in 4 diarrheal diseases. While most cases of salmonellosis are considered relatively mild, they can be life-threatening with the severity of the disease

depending on factors such as serotype and host factors. The onset of disease usually occurs 6-72 hours after consumption with the illness lasting anywhere from 2-7 days. Although most patients recover without hospital intervention, sometimes dehydration can become severe and life-threating to require hospitalization. One thing to note is that 60-80% of all known salmonellosis cases are not linked as a part of a known outbreak and are classified as sporadic cases sickening only one individual (WHO, 2018). *Salmonella* has a low infective dose, compared to other pathogens, which the Bad Bug Book estimates can be as low as one cell for some *Salmonella* spp. and Lehmacher et al. 1995 found the infective dose in a potato chip outbreak in Germany to be between 4-45 organisms (Lehmacher et al., 1995). The mere presence of a few cells is a risk for human health, especially in those with weakened immune systems (Finn et al., 2013). Between 2007-2012, *Salmonella* was responsible for 94% of low-moisture food recalls (Table 1.12) (Santillana Farakos and Frank, 2014).

Table 1.12: *Salmonella* outbreaks linked to LMFs

Year	***Salmonella* serotype(s)**	**Low-moisture food**	**Location(s)**	**Number of people affected**	**Reference**
1953	Senftenberg and possibly others	Desiccated coconut	Australia	>50	(Wilson and Mackenzie, 1955)
1960-61	Paratyphi B	Desiccated coconut	England	3	(Semple et al., 1961)
1970	Durham	Chocolate	Sweden	-	(Gästrin et al., 1972)
1972	Agona	Fishmeal	US	17	(Clark et al., 1973)
1973	Derby	Milk powder	Trinidad	3000	(Weissman et al., 1977)
1974	Eastbourne	Chocolate	Canada	95	(D'Aoust et al., 1975)

1982-83	Napoli	Chocolate	UK	245	(Gill et al., 1983)
1985	Ealing	PIF	UK	76	(Rowe et al., 1987)
1985-86	Nima	Chocolate	Canada, US	29 Canada 4 US	(Hockin et al., 1989)
1987	Typhimurium	Chocolate	Norway, Finland	349 Norway 12 Finland	(Kapperud et al., 1990)
1993	Saintpaul, Javiana, Rubislaw	Paprika-seasoned potato chips	Germany	1000	(Lehmacher et al., 1995)
1993	Tennessee	PIF	Canada, US	48	(CDC, 1993)
1995	Senftenberg	Infant cereals	UK	5	(Rushdy et al., 1998)
1996	Enteritidis PT4	Marshmallow	UK	45	(Lewis et al., 1996)
1996	Mbandaka	Peanut butter	Australia	15	(Scheil et al., 1998)
1996	Agona	Peanut-flavored maize snack	UK, US, Canada, and Israel	27	(Killalea et al., 1996)
1998	Agona	Toasted oats cereals	US	209	(CDC, 1998)
1999	Java PT Dundee	Desiccated Coconut	UK	18	(Ward et al., 1999)
2000-01	Enteritidis PT30	Raw almonds	US, Canada	157 Canada 11 US	(Isaacs et al., 2005)
2001	Oranienburg	Chocolate	Austria, Belgium, Denmark, Finland, Germany, Netherlands, Sweden, Canada, Croatia, and the Czech Republic	500+	(HPA, 2008; Werber et al., 2005)
2001	Typhimurium DT 104	Halva	Australia, Germany, Norway, Sweden, and UK	17 Australia 27 Sweden 18 Norway	(Brockmann et al., 2004)
2001	Stanley	Peanuts	Australia,	55	(Kirk et al.,

	Newport		Canada, UK	Australia 44 Canada 10 UK	2004)
2002	Montevideo	Tahini and Halva	Australia	55	(Tauxe and Kirk, 2008; Unicomb et al., 2005)
2002	Braenderup	Curry powder	UK	20	(Dennis et al., 2013)
2003	Agona	Tea	Germany	42	(Koch et al., 2005)
2003	Montevideo	Tahini	Australia	3	(Unicomb et al., 2005)
2003	Montevideo	Tahini and halva	New Zealand	10	(Unicomb et al., 2005)
2003-04	Enteritidis PT 9c	Raw almonds	UK, Canada	47	(CDC, 2004)
2005	Agona	PIF	France	141	(Brouard et al., 2007)
2005-06	Enteritidis NST 3+	Raw almonds	Sweden	15	(Müller et al., 2007)
2006-07	Tennessee	Peanut butter	US	715	(Sheth et al., 2011)
2007	Wandsworth Typhimurium	Seasoning/snack puff	US	65	(CDC, 2007)
2007-08	Senftenberg	Fennel seed	Serbia	14	(Ilić et al., 2010)
2008	Agona	Cereal	US	28	(CDC, 2008)
2008	Kedougou	PIF	Spain	31	(Rodríguez-Urrego et al., 2010)
2008	Typhimurium PT 42	Raw flour	New Zealand	67	(Mccallum et al., 2013)
2008-09	Typhimurium	Peanut butter and products containing peanut butter	US, Canada	713 US 1 Canada	(Cavallaro et al., 2011)
2008-09	Rissen	White pepper	US	87	(Dennis et al., 2013)
2009-10	Montevideo Senftenberg	Red and black pepper	US	272	(Gieraltowski et al., 2013)
2010	4,12:i:-	Dried pork	France	110	(Bone et al.,

		sausage			2010)
2010	Typhimurium PT 170	Peanut/cashew mix	Australia	19	(OzFoodNet, 2010)
2011	Enteritidis	Pine nut	US	43	(CDC, 2011a)
2011	Bovismorbificans	Tahini and hummus	US	23	(Blackwell et al., 2012)
2012	Bredeney	Peanut butter	US	42	(CDC, 2012a)
2012	Typhimurium	Raw almonds	Australia	27	(Whitworth, 2012)
2012	Montevideo Mbandaka Maastricht	Tahini and hummus	New Zealand	16	(Paine et al., 2014)
2013	Montevideo Mbandaka	Tahini	US	16	(CDC, 2013a)
2014	Newport Hartford Oranienburg	Sprouted chia powder	US	31	(CDC, 2014a)
2014	Braenderup	Almond and/or peanut butter	US	6	(CDC, 2014c)
2014	Stanley	Cashews (cashew cheese)	US	17	(CDC, 2014b)
2014-15	Enteritidis PT 13a	Dried vegetable spice mix	Sweden	174	(Jernberg et al., 2015)
2015	Paratyphi B (*S.* Java)	Sprouted almond and cashew-almond spreads	US	11	(CDC, 2016a)
2015-16	Montevideo Senftenberg	Pistachio	US	11	(FDA, 2016)
2016	Typhimurium	Hazelnut	US	5	(Modie, 2017)
2016	Virchow	Organic shake and Meal product	US	33	(CDC, 2013c)
2018	Mbandaka	Honey	US	135	(CDC,

		smacks			2018e)
2018	Typhimurium	Dried coconut	US	14	(Center for Disease Control and Prevention, 2012)
2018	Concord	Tahini	US	8	(CDC, 2018a)
2018	I 4,[5],12:b:- Heidelberg Javiana Okatie Weltevreden Thompson	Kratom	US	199	(CDC, 2013f)
2019	*Salmonella* spp.	Tahini	US	6	(CDC, 2019d)

Of concern to the food industry and for global health is that *Salmonella* is known to be a hardy bacterium that can survive extremely low water activity for several weeks to years. These extremes can be everywhere from desiccated dry environments to water (WHO, 2018), and this is especially concerning in low-moisture food production as thermal resistance of *Salmonella* spp. increases exponentially at reduced a_w (Liu et al., 2018). Miller et al. spray-dried milk, and compared the survival of *S.* Typhimurium at two different concentrations of total solids (20 and 40%), two inlet air temperatures of 165 and 225°C, and two outlet temperatures of 67 and 93°C, and found that the higher fat content (total solids) equated to greater bacterial survival at higher temperatures (Miller et al., 1972). The food we consume plays a role in the survival of *Salmonella* when exposed to our stomach acid. Two studies have shown that under a typical Western diet that upon ingestion of a meal high in fat, our gastric pH immediately rises to ~6.0 (Konturek et al., 1994; Snepar et al., 1982). *Salmonella* can grow in a broad pH range

(3.8-9.5), and upon ingestion of high-fat foods, *Salmonella* can survive passage through our stomach ~pH 2. Not only does our food intake affect our stomach pH, but the use of proton pump inhibitors (e.g., Nexium, Prilosec, and Prevacid) in patients who suffer from acid reflux also plays a role in the survival of *Salmonella*. Proton pump inhibitors impair the gastrointestinal host defenses, i.e., lowering stomach acid pH, potentially increasing the risk for *Salmonella*, as well as other pathogens such as *Campylobacter* and *Clostridiodes difficile* (García Rodríguez et al., 2007; Kwok et al., 2012).

1.5.3 *Bacillus cereus*

Bacillus cereus is a Gram-positive, toxin-producing, spore-forming, facultatively anaerobic bacilli (1 x 3-4 μm) that belongs to the phylum *Firmicutes* and is ubiquitous in the environment. Although optimal growth occurs between 28-35°C, *B. cereus* can grow between 5-50°C (Sutherland et al., 1996). Routine testing employed by food poisoning laboratories may not be able to differentiate between the *B. cereus* group species. This group consists of five species, *B. cereus*, *B. thuringiensis*, *B. anthracis*, *B. mycoides,* and *B. weihenstephanensis*, and *B. weihenstephanensis* is a psychrotolerant species of the group that can grow between 4-7°C (Lechner et al., 1998). *B. anthracis* causes anthrax in mammals and is famous for the bioterrorism act of 2001 that resulted in 11 cases of inhalation anthrax from letters mailed to unsuspecting victims (Spencer, 2003). *B. thuringiensis* is well known for its insecticidal properties; however, there have been reports of foodborne disease and human infections (Schoeni and Lee Wong, 2005). *B. mycoides* promotes plant growth and induces systemic resistance (Ambrosini et al., 2015).

Even the *B. cereus* group is often associated with food poisoning, and they also can cause soft tissue infections such as ocular infections (Drobniewski, 1993).

There are two forms of illness associated with *B. cereus* infection: diarrheal and intoxication due to an emetic toxin produced. During intoxication, the onset of symptoms typically occurs between 1-5 hours after consumption of the contaminated food and include nausea and vomiting. While the emetic form is often associated with rice products, other starchy foods such as potatoes, pasta, and cheese products may also harbor conditions for the bacterium to produce the emetic toxin. The diarrheal form of foodborne disease starts 8-16 hours after consumption, typically fish, meats, milk, and vegetables, and symptoms include abdominal cramps, nausea, and watery diarrhea. The Bad Bug Book and the Scallan et al. both estimate that there are 63,400 *B. cereus* related illnesses in the United States annually with 20 hospitalizations (Bennet and Tallent, 2012; Scallan et al., 2011).

Mortality from *B. cereus* is rare; however, all people are susceptible to infection, not just the immunocompromised. *B. cereus* has an infectious dose (ID50) of between 10^5-10^8 in order to produce a sufficient amount of toxin to cause illness (Canadian Public Health, 2012; Bennet and Tallent, 2012). Cooking food at their recommended temperatures will destroy vegetative *B. cereus* cells; however, spores are heat-resistant and will germinate/grow when the food is cooled gradually over time (Schneider et al., 2004.). When foods are allowed to sit in the danger zone (5-60°C) for an extended period, even if cooked, can permit *B. cereus* growth, albeit slowly, and produce toxins (NSW Food Authority, 2010). Rajkovic et al. determined that the emetic toxin, if present in food

before cooking, is heat stable up to 121 °C for 80 minutes (Rajkovic, 2014) and even if the food is reheated, can cause still illness.

Spores exist in a dormant state that is resistant to all manner of environmental stressors such as desiccation, heat, pH, freezing, UV, pressure, and organic solvents (Johnson and Setlow, 2014; Stewart, 2015). *B. cereus* is known to readily form biofilms on all manner of surfaces, plastic, glass wool, soil, and stainless steel (Auger et al., 2006; Oosthuizen et al., 2002; Vilain et al., 2006). *B. cereus* produces a wide variety of biofilms depending on the environment they find themselves in and exist as either immersed or floating biofilms. Once established, within the biofilm itself, *B. cereus* can enter different physiological states and generate adhesive and highly resistant spores (Majed et al., 2016). Once established, biofilms cause problems in the food processing environment as their elimination can prove difficult due to their resistance to biocides (Peng et al., 2002).

When compared to vegetative bacterial cells, spores have a different structure and contain seven layers: 1.) an exosporangium; 2.) coat; 3.) outer membrane; 4.) cortex; 5.) germ cell wall; 6.) inner membrane; 7.) core (Figures 1.8 and 1.9). Spores are more resistant when compared to their vegetative counterparts and are thought to be between 7-50 times more resistant to UV than vegetative cells. When *B. cereus* cells begin sporulation, UV resistance is acquired two hours before heat resistance. It occurs at the same time as the synthesis of small, acid-soluble spore proteins (SASP) that comprise between 10-20% of total spore proteins. SASPs are essential to the spore's UV resistance, and most SASPs are bound to DNA. Researchers have found that the spore's coat, cortex, and dehydration of the core are not involved in the UV resistance of the spore (Johnson and Setlow, 2014; Setlow, 2001). UV damage to spores is different than to vegetative or

proliferating bacterial cells. Exposure to UV generates photoproducts instead of cyclobutane dimers, and the repair of damaged DNA does not occur until germination (Setlow, 2001).

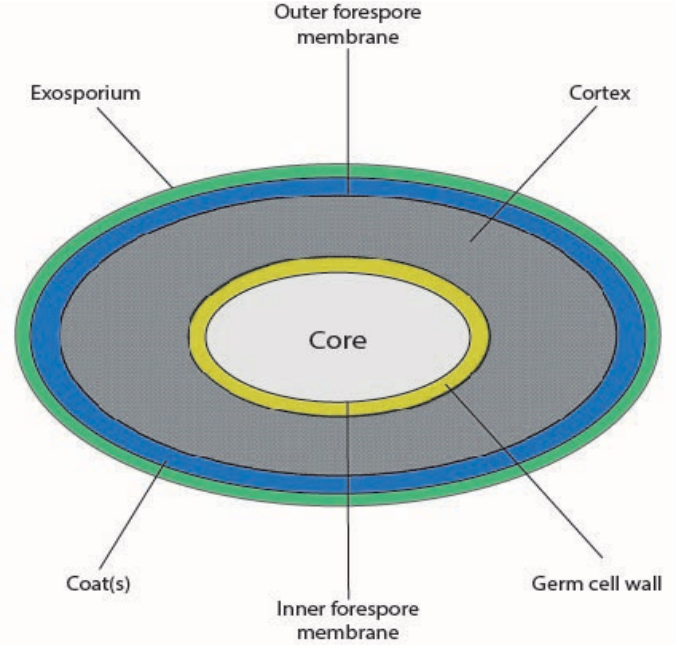


Figure 1.8: Structure of a bacterial spore
Adapted from (Johnson and Setlow, 2014)

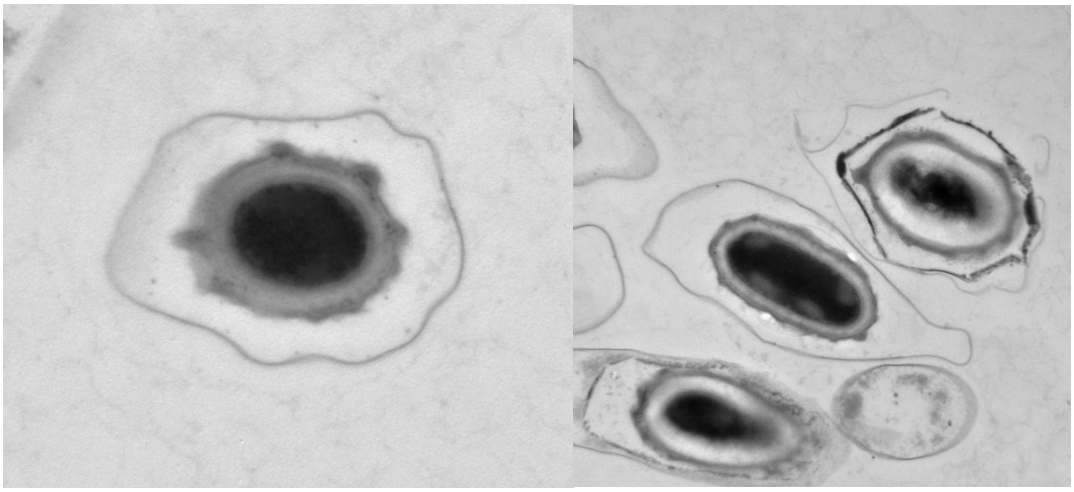


Figure 1.9: Transmission Electron Microscopy of *Bacillus cereus* spores

Once exposed to unfavorable conditions (ex. starvation, overcrowding), *B. cereus* cells begin the process to convert themselves into a spore. During Stage I, *Bacillus* forms a polar Z-ring, which establishes cellular asymmetry (Margolin, 2002). Stage II results in the formation of a septum separating the forespore from the mother cell. In this stage, both the forespore and the mother cell get a copy of the genomic DNA. In Stage III that the polar septum wraps around the forespore and the mother cell engulfs the forespore resulting in a double-membraned forespore inside of the cytosol of the mother cell (Abanes-De Mello et al., 2002). Stages IV and V result in a mature spore containing an outer shell coat, and an inner shell cortex. Spore coats contain up to seventy different proteins (which equates to ~30% of the total spore protein). Henriques et al. found that roughly 20 of the coat proteins in *B. subtilis* demonstrate enzymatic function with some involved in germination, and some affect spore protection while others posttranslationally modify proteins at the surface of the spore to assist in the assembly process (Henriques and Moran, Jr., 2007). McKenney et al. describe the coat as not just as a single layer but rather, as four distinct layers: basement layer, inner coat, outer coat, and crust (McKenney et al., 2010). The cortex is composed of specialized peptidoglycan that imparts heat and desiccation tolerance for the spore, and this layer of peptidoglycan lies between the two membrane layers of the forespore, inner germ cell wall, and outer cortex (Tipper and Linnett, 1976). Stage VI results in a mature spore that contains a tightly condensed, toroidal chromosome. Finally, Stage VII sees the mother cell lysing and releasing the dormant, mature spore into the environment (Tan and Ramamurthi, 2014). Refer to Figure 1.10 for the complete sporulation process.

Spores remain dormant in the environment and food matrices until favorable conditions return for their proliferation, which will initiate germination and subsequent outgrowth. First, spores start taking up water, releases Ca2+ ions, resume metabolic activity, and finally degrade their cortex resulting in a vegetative cell. Small molecule germinants are involved by diffusing across the outer spore layer where they bind to receptors found in the cytoplasmic membrane triggering germination. There is a lag phase between the addition of nutrient germinants and commitment. This lag phase is known to be extremely variable amongst spores of the same species and within the same population (Setlow, 2013). Although the precise events that take place in the activation step are still unknown, upon the addition of a nutrient germinant, the spore enters the commitment stage in germination where it releases H^+, K^+, and Na^+. Following commitment, spores enter stage I in which the spore releases CaDPA (Ca^+-dipicolinic acid), and the core undergoes partial hydration. It is during this point that spores lose some of their hallmark resistances. Stage II results in hydrolysis of the cortex and full hydration of the core resulting in its expansion. By stage II, the spore has lost its dormancy and almost all stress resistances. Outgrowth follows stage II (Setlow, 2014). Refer to Figure 1.10 for the full germination cycle.

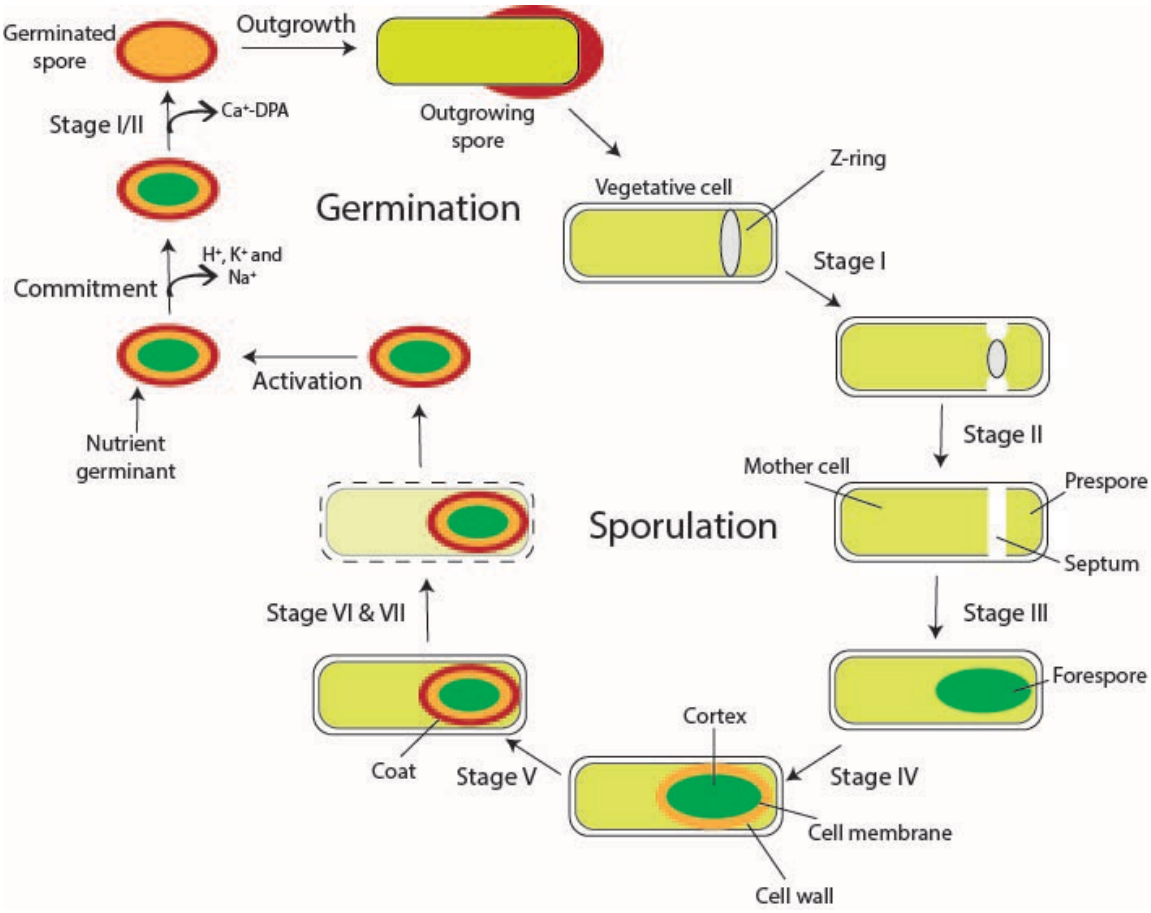


Figure 1.10: Sporulation and germination cycle of *Bacillus* spores
Adapted from (Sella et al., 2014; Setlow, 2014)

Spores remain a problem in the dairy industry, impacting products ranging from fluid milk to nonfat dry milk. Once pasteurized, spores, especially those that are psychrotolerant, can grow in the nutrient rich environment of liquid milk. Milk is an ideal environment to support microbial growth due to an abundance of nutrients, near neutral pH, and high a_w (Quigley et al., 2013). Spore growth has become an issue in high-temperature short-time (HTST) pasteurized milk, and these spore-forming psychrotrophs remain a hurdle in manufacturers trying to extend the shelf life of milk past 14 days (Durak et al., 2006; Fromm and Boor, 2004; Huck et al., 2007a; Huck et al., 2007b). Spores are not only a problem in fluid milk and milk products but also various types of

low-moisture foods (LMFs) (Table 1.13). Heat activates spores that survive pasteurization and have the potential to form biofilms before additional processing, such as spray drying, to produce milk powder. Presence of spores is problematic when trying to export powdered milk as the US Dairy Export Council has strict tolerances for the presence of mesophilic and thermophilic spores in milk powders, <500 CFU/g (thermophilic) and <1000 CFU/g (mesophilic) (Watterson et al., 2014).

B. cereus accounts for <2% of foodborne cases in the United States, and in Europe, *B. cereus* related illnesses account for 30% of the total foodborne illnesses. In France, *B. cereus* is the 2nd leading cause of foodborne outbreaks behind *Staphylococcus aureus* (Glasset et al., 2016; Glasset et al., 2018). Even though there are not many outbreaks linked to *B. cereus,* it is still frequently isolated from a wide variety of LMFs such as infant foods, chocolate, rice, cereal grains, dessert mixes, and spices (Becker et al., 1994; Choo et al., 2007; Sarrías et al., 2002; Te Giffel et al., 1997; Te Giffel et al., 1995; Warburton et al., 1987).

Table 1.13: *B. cereus* outbreaks linked to LMFs

Year	Low-moisture food	Location(s)	Number of people affected	Reference
1980	Powdered milk	US	4	(Holmes et al., 1981)
1999	Rice	US (Nebraska)	4	(CDC, 2013c)
2000	Rice	US (Washington)	4	(CDC, 2013c)
2000	Rice	US (Washington)	2	(CDC, 2013c)
2000	Rice	US (Washington)	4	(CDC, 2013c)
2005	Rice	US (New York)	16	(CDC, 2013c)
2007	Semolina	France	5	(Glasset et al., 2016)
2007	Spice blend	France	146	(Dennis et al., 2013)
2008	Semolina	France	61	(Glasset et al., 2016)
2008	Semolina and lamb	France	4	(Glasset et al., 2016)
2008	Wheat	France	3	(Glasset et al., 2016)
2009	Semolina and peas	France	7	(Glasset et al., 2016)
2009	Rice	US (Alabama)	13	(CDC, 2013c)
2010	White pepper	Denmark	112	(Dennis et al., 2013)
2010	Rice	US (Florida)	2	(CDC, 2013c)
2010	Rice	US (Pennsylvania)	6	(CDC, 2013c)
2010	Rice	US (Tennessee)	22	(CDC, 2013c)
2010	Rice	US (Florida)	103	(CDC, 2013c)
2011	Rice	US (Florida)	3	(CDC. 201c)
2012	Rice	US (North Carolina)	10	(CDC, 2013c)
2013	Semolina	France	3	(Glasset et al., 2016)
2013	Rice	US (Pennsylvania)	32	(CDC, 2013c)
2014	Semolina and ginger (spice)	France	11	(Glasset et al., 2016)

1.6 Other Microorganisms that Impact LMFs

1.6.1 Non-pathogenic Spore-forming Bacteria

Non-pathogenic spore-forming bacteria, such as thermophilic Bacilli, are a persistent problem in milk powder for the dairy and food industry (Daelman et al., 2013; Murphy et al., 1999). Kent et al. surveyed eleven United States dairy powder processing plants and found *Geobacillus*, *Anoxybacillus,* and *Bacillus licheniformis* as the major spore-forming contaminants in dairy powder with *Bacillus licheniformis* as most

prevalent (Kent et al., 2016). Thermophilic bacterial spores survive processing conditions, such as heat, and can remain viable for long periods in dry environments (Burgess et al., 2010; Janštová and Lukášová, 2001). Janštová and Lukášová reported that *Bacillus licheniformis* had a D_{100}-value of 2.37 minutes in ultra-high temperature (UHT) milk. The mere presence of higher levels of spore contamination (even if not pathogenic) in milk powders or other food products can result in product rejection and affect the export of spore-containing foods (Janštová and Lukášová, 2001).

Currently, there is no adopted single standard method to estimate the presence of all the microorganisms present in dried milk powders or other LMF, due to different specifications for each country. Even if milk powder ends up not being exported, spores are also problematic due to their ability to produce hydrolytic enzymes that break down food nutrients and lead to a loss of nutritional value and consumer acceptability (Hauschild, 1990; Sakaguchi, 1982).

1.6.2 Halophilic Bacteria and Osmophilic Yeasts

Food spoilage organisms may not cause human disease but are still a significant economic impact on the food industry due to product spoilage. Microorganisms, such as halophilic bacteria, osmophilic yeasts, and xerophilic molds, all contribute to spoilage of low-moisture foods (LMFs). Halophilic bacteria, such as *Bacillus* species, can grow in soy sauce at 0.80 a_w, and *Halobacterium salinarium* and *Salinicoccus roseus* cause spoilage called "pinking" of salted fish at even ~0.70 a_w (Hayman and Podolak, 2017).

Osmophilic yeasts, such as *Zygosaccharomyces rouxii* and *Zygosaccharomyces bailii*, can proliferate in foods with a_w ranging from 0.60-0.70 (Hayman and Podolak,

2017; Thompson, 2009). Sugar confectionery products are particularly susceptible to spoilage from osmophilic yeasts, which can lead to product bursting/fracturing, slime formation, and the formation of off-flavors and aromas. In sugar syrups, yeasts can grow slowly with lag times and generation times influence by a_w, inoculum size, and availability of non-sucrose nutrients. Osmophilic yeasts are of concern in honey due to their ability to ferment available sugars forming alcohol and carbon dioxide. Fourteen genera of yeasts have been isolated from honey, including *Zygosaccharomyces*, *Schwanniomyces*, and *Hansenula* (Thompson, 2009).

1.6.3 Xerophilic Fungi

Xerophilic fungi are common contaminants of low a_w foods such as cereals, nuts, dried meat, dried milk, and spices, and show rapid growth at >0.77 a_w and slow growth between 0.68-0.75 a_w. *Wallemiasebi* is present in cereals and spices; *Aspergillus* spp. in nuts and *Penicillium* spp. in cereals, and two xerophilic fungi have shown growth in foods as low as 0.61 a_w. *Chrysposporium* spp. are commonly associated with foods containing high sugar, such as confectionaries and dried fruits (Pitt and Hocking, 2009), and *Xeromyces bisporus* in sugary foods with low a_w (Leong et al., 2011). Molds, such as *Aspergillus flavus* and *A. parasiticus*, can cause spoilage of cashews, maize, peanuts, and spices, and produce aflatoxins. Other mycotoxins, such as ochratoxins and fumonisin, have been found in coffee, dried fruits, peanuts, rice, seeds, and spices (Hayman and Podolak, 2017).

1.7 Summary and Objectives

In the last five years, LMF recalls and outbreaks have been on the rise. Traditionally, LMFs never entered the discussion in terms of food safety due to the inherent low a_w of the products. While LMFs do not directly support the growth of pathogens, this does not mean that they are missing from the final product. Pathogens, such as *Salmonella* and *Cronobacter,* have been found to survive for up to two years in products such as flour and milk products. Most foodborne pathogens are well adapted to the stressors of the food industry, which makes it difficult for food companies to eliminate in their final product. Part of the challenge of detecting pathogens in LMFs is that they are often not homogenously distributed throughout the product lot and are often clumped together. What happens is those food companies could entirely miss the fact that one of their product lots contains *Salmonella* and accept the lot. One way to overcome this is for companies to increase their sampling size. This is often not realistic due to the cost-prohibitive nature of increased product testing.

Thermal processing used to be the go-to processing technique to eliminate microorganisms from food products. However, studies have shown that at low a_w, pathogens such as *Salmonella* increase in thermal resistance resulting in higher temperatures needing to be applied to reduce the presence of pathogens. The downside with increasing temperature is that a lot of LMFs are sensitive to heat and rapidly lose organoleptic properties. Still, something needs to be done to protect public health and reduce the number of recalls in LMFs. To meet this need, nonthermal techniques such as pulsed light, ozone, and microwave treatment have garnered a lot of interest in the food

industry due to the ability to eliminate pathogens while leaving the products relatively untouched.

The goal of this dissertation was to develop a continuous intense pulsed light (IPL) system to bridge the gap between academia and the food industry. One of the biggest challenges that nonthermal processing techniques face are factors such as scale-up, cost, feasibility, and consumer acceptance. Past research on pulsed light occurred on lab-scale systems using either a liquid matrix or on small quantities of products with little to no throughput. The intense pulsed light system developed here will serve as a stepping stone to implementation in the food industry by testing different LMF products ranging from powdered foods to whole grains.

As part of this research, the following objectives were accomplished:

- Define the need for nonthermal processing in the food industry and understand how IPL meet that need
- Evaluate various methods to inoculate powdered products and understand the impact each has on final sample homogeneity and bacterial concentration (CFU/g)
- Develop a continuous IPL system to be used for the rapid decontamination of various LMFs
- Assess how different processing parameters such as fluence, feed rate, residence time, water activity, and temperature impact log reductions
- Compare the effect IPL has on the organoleptic properties of LMFs with traditional thermal treatment

- Apply the knowledge gained to continuously improve the design of the system for eventual scale-up and implementation into the food industry

Chapter Two

EVALUATION OF METHODS FOR INOCULATING DRY POWDER FOODS WITH *SALMONELLA ENTERICA*, *ENTEROCOCCUS FAECIUM,* OR *CRONOBACTER SAKAZAKII*

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2.1 Introduction

Low-moisture foods have been the source of multiple recalls in the past few years. For food to be referred to as low moisture, it must have a water activity (a_w) below 0.70 (Beuchat et al., 2013; Blessington et al., 2013). Although foodborne pathogenic bacteria typically cannot grow and divide in low a_w environments (such as powdered foods), the bacteria may remain viable for long durations of time (Gurtler et al., 2005; Santillana Farakos et al., 2013). Powdered foods such as whole wheat flour, soy flour, milk powder, and powdered infant formula are not completely sterile foods; pathogens that have low infectious doses persist and may cause foodborne diseases upon consumption (Beuchat et al., 2013; Cahill et al., 2008; Caubilla Barron and Forsythe, 2007). Some studies have shown that *Cronobacter* spp. can remain viable in dehydrated powdered infant formula (0.25 – 0.30 a_w) for up to two years (Caubilla Barron and Forsythe, 2007; Edelson-Mammel et al., 2005). Caubilla Barron and Forsythe determined that *Salmonella* Enteriditis persisted up to 15 months in dehydrated powdered infant formula (PIF) (Caubilla Barron and Forsythe, 2007).

Cronobacter spp. (formerly known as *Enterobacter sakazakii*) is an opportunistic pathogen that is of concern in powdered foods due to its resistance to drying (Breeuwer et al., 2003) and its ability to survive the spray drying process (Arku et al., 2008), both

standard practices in the production of milk powder (Abdalla et al., 2017). It is ubiquitous in the environment leading to postprocessing contamination before packaging or once the package is opened by the consumers (CDC, 2015a; Gurtler et al., 2005; Kandhai et al., 2004; Mullane et al., 2007). Powdered infant formula is not the only food that can become contaminated with *Cronobacter* spp.; other powdered foods such as herbs, spices, flours, grains, milk powder and dehydrated rice powder have all been shown to be contaminated with *Cronobacter* spp. (Baumgartner et al., 2009; CDC, 2015a; Hochel et al., 2012; Huang et al., 2015; Iverson and Forsythe, 2004; Lou et al., 2014). The Centers for Disease Control and Prevention (CDC) reported about 4-6 infections in infants per year in the United States, and *Cronobacter* illness may be fatal for 40-80% of infected infants (Bowen and Braden, 2006). In 2011, the CDC reported that the number of *Cronobacter* infection in infants had tripled, and the trend continues to grow today (Bowen and Braden, 2006). *Cronobacter* infection can also be very serious or fatal for older people and for immunocompromised people such as those unable to fight pathogens, people infected with HIV, those with organ transplants, and those with cancer. In the United States, reporting is only required in the state of Minnesota (CDC, 2015a), and it is believed that the number of infections caused by *Cronobacter* is underreported worldwide (Tóthová et al., 2011).

Every year, *Salmonella* spp. cause over one million illnesses in the United States, with 19,000 hospitalizations and 380 deaths (CDC, 2017c). During the years 2007 to 2012, *Salmonella* was responsible for a clear majority (94%) of US low-a_w food recalls (Santillana Farakos and Frank, 2014). Even though pasteurization quickly destroys *Salmonella* in high-moisture ingredients (Miller et al., 1972), it is still of concern in

powdered foods. In 2014, there was a large multistate outbreak of *Salmonella* infections linked to the consumption of organic sprouted chia powder, which sickened 31 persons with the outbreak strains of *Salmonella* Newport (20 persons), *Salmonella* Hartford (7 persons), or *Salmonella* Oranienburg (4 persons). In addition to dried chia powder, over the past five years, there have been numerous *Salmonella* outbreaks in other dried food items such as dried dog food, pine nuts, and other dry powdered ingredients (CDC, 2014). It is thought that cross-contamination occurs through poor sanitation practices, poor equipment and facility design, inadequate ingredient and pest control, and poor operational and manufacturing practices (Podolak et al., 2010). This cross-contamination can lead to *Salmonella* being introduced at many stages of food production, including right before packaging.

Bacteria possess the ability to enter a desiccated physiological state, where metabolism is greatly reduced, allowing them to persist and remain viable for extended periods (Beuchat et al., 2013). All it takes is for a few cells to survive and infect the host upon ingestion, as most pathogens have low infectious doses. *Cronobacter sakazakii* has an infective dose of approximately 1000 colony forming units (CFU) (Parra-Flores et al., 2015), and according to the Bad Bug Book (2012), the infective dose for *Salmonella spp.* can be as low as one cell dependent on age, the health of the host, and strain variation (Hammack, 2012).

Powdered food inoculation procedures have been reported, but each have their differences. These differences being: types of food matrices tested, the requirement of carriers such as sand or talc, and the utilization of a dry or wet microbial inoculum (Blessington et al., 2013; Choi et al., 2009; De Roin et al., 2003; Enache et al., 2015;

Gurtler and Beuchat, 2007; Hildebrandt et al., 2016, 2017; Hoffmans and Fung, 1992; Rachon et al., 2016; Uesugi et al., 2006). A study by Hildebrandt et al. 2016 elucidated the differences between broth grown and lawn grown *Salmonella* bacterial cells inoculated onto wheat flour and their impact on thermal resistance and desiccation tolerance (Hildebrandt et al., 2016).

The objective of this study was to assess the effects of three different inoculation methodologies, utilizing three different bacteria (*C. sakazakii*, *E. faecium*, and *Salmonella* Typhimurium LT2) inoculated onto three different powdered foods (soy flour, nonfat dry milk, and all-purpose flour) using three different protocols, and evaluate their effects on homogeneity and survivability post-equilibration.

2.2 Materials and Methods

2.2.1 Bacterial Strains Used in the Study

Cronobacter sakazakii strain ATCC 29544, *Salmonella enterica* serovar Typhimurium LT2ATCC 700720, and *Enterococcus faecium* strain NRRL B-2354 were used in this study. The nonpathogenic *E. faecium* strain was chosen since it is a common surrogate organism for *Salmonella* spp in thermal process validation (Bianchini et al., 2014; Enache et al., 2015; Kopit et al., 2014; Xu et al., 2018). *C. sakazakii* was obtained from the American Type Culture Collection (ATCC) as a lyophilized culture and was revived following ATCC protocol for reviving freeze-dried microorganisms and maintained on OK agar medium (Oh and Kang, 2004). OK agar was made using: tryptone (20 g), bile salts no. 3 (1.5 g), sodium thiosulfate (1 g), ferric citrate (1 g), agar (15 g) per liter, and was made without supplementing 4-methylumbelliferyl-α-D-glucoside, as it was not crucial for growth and required only during differentiation. Both

E. faecium and ST LT2 were revived from frozen culture (-80 °C) and maintained on Tryptic soy agar supplemented with 0.6% (wt/vol) yeast extract (Sigma-Aldrich, St. Louis, MO) (TSAYE). Plates were stored at 4 ± 2 °C.

2.2.2 Bacterial Inoculum Preparation

Three inoculation methodologies previously reported for powdered foods were evaluated (Enache et al., 2015; Gurtler and Beuchat, 2007; Hildebrandt et al., 2016): (i) broth-grown pelletized inoculation (BGPI), which consisted of broth grown, centrifuged, and inoculated using a sterile wooden stick; (ii) broth-grown spray inoculation (BGSI), which consisted of broth grown, centrifuged, resuspended, and inoculated using a TLC reagent sprayer; (iii) lawn-grown liquid inoculation (LGLI) bacterial lawn grown on TSAYE, harvested, centrifuged, and inoculated using a pipette. To prepare a fresh inoculum for broth grown methodologies**,** a single bacterial colony from TSAYE or OK agar medium stored at 4 °C was re-streaked on fresh TSAYE or OK agar medium and incubated for 24 hours at 37 °C. A single isolated colony was then transferred from the streak plate and used to inoculate 500 ml Tryptic soy broth (TSB; Sigma-Aldrich, St. Louis, MO) supplemented with 0.6% (wt/vol) yeast extract (Sigma-Aldrich, St. Louis, MO) (TSAYE)) in 1-L Erlenmeyer flasks with screw tops. Flasks were then incubated at 37 °C for 24 hours using a rotary shaker (150 rpm) to reach stationary phase. 400 ml of each culture were then centrifuged at 4,000 rpm (1860 x *g*) (Sorvall Legend XT/XF Centrifuge, ThermoFisher) for 20 minutes to pellet cellular suspensions. For BGPI, the supernatant was decanted to leave behind only the bacterial cell pellet (total 2.5-3 ml). In BGSI, the bacterial cell pellet was re-suspended with 0.9% saline, made up to 3 ml, and

vortexed to get a homogenous culture, so that it did not block the nozzle of the thin layer chromatography (TLC) sprayer flask. LGLI inoculum was prepared by utilizing two consecutive transfers in 9 ml of TSBYE for 24 hours each at 37°C with rotary shaking (150 rpm). Finally, 1 m was spread evenly over a plate (150 x 15 mm) of TSBYE. After incubation at 37°C for 24 hours, the bacterial lawn was harvested using 20 ml of 0.1% peptone (Fisher Scientific, Fair Lawn, NJ) and lifted using an 18 cm Corning cell lifter (Corning Life Sciences, Tewksbury, MA) to dislodge cells from the plate surface. Dislodged cell suspensions were pipetted into 50 ml centrifuge tubes and centrifuged at 4,000 rpm (1860 x *g*) for 20 minutes. The supernatant was decanted and resuspended in 0.1% peptone to a final volume of 3 ml.

2.2.3 Powder Testing

Powders utilized in this study were: Gold medal all-purpose flour (General Mills, Minneapolis, MN), organic soy flour (Bob's Red Mill, Milwaukie, OR), and Nonfat dry milk (NFDM) (Land O'Lakes, Arden Hills, MN). For each batch of powder, a_w was determined using a water activity meter before microbial inoculation (Aqualab Pa_wkit, Decagon Devices, Inc., Pullman, WA). The a_w values for each powder upon arrival were assessed before storage in desiccators. NFDM (a_w 0.30 ± 0.05), soy flour (a_w 0.44 ± 0.04) and all-purpose flour (a_w 0.46 ± 0.04). Uninoculated NFDM, organic soy flour, and all-purpose flour were diluted 1:10 in 0.1% [w/v] sterile peptone broth and spread plated onto TSAYE. TSAYE medium plates were incubated at 37 °C for 24 hours and enumerated (CFU/g). In the uninoculated controls, the native bacteria (standard plate count) were lower than the limit of quantification (≤ 2 log CFU/g) for soy flour (3.2 log

CFU/g), for NFDM (< 2 log CFU/g) and for all-purpose flour (3.1 log CFU/g). Each powder was placed in an autoclaved Round Wide-Mouth Polypropylene (PPCO) Nalgene bottles (Thermo Fisher Scientific, Waltham, MA) and wrapped with Parafilm (Sigma-Aldrich, St. Louis, MO) to reduce moisture migration.

Each powder was also assessed for the presence of naturally occurring *Salmonella* and *Cronobacter*. TSAYE modified with 0.05% ferric citrate (Sigma-Aldrich, St. Louis, MO) and 0.03% sodium thiosulfate (Sigma-Aldrich, St. Louis, MO) was used to detect *Salmonella* following 48 h incubation at 37°C. A characteristic black center to colonies were counted as *Salmonella*. OK agar supplemented with 50 mg 4-methylumbelliferyl-α-D-glucoside (Sigma-Aldrich, St. Louis, MO) was used to detect *Cronobacter* following 48 h incubation at 37°C. Following incubation, OK agar plates were held under UV light and colonies that fluoresce were counted as *Cronobacter* (Oh and Kang, 2004).

2.2.4 BGPI

In a biological safety cabinet, 30 g of each powder was weighed out in a sterile Pyrex crystallizing dish with a diameter of 150 mm (Corning Life Sciences, Tewksbury, MA). Sterile wooden plain-tipped applicators (Fischer Scientific, Fair Lawn, NJ) were used to inoculate powders with each bacterial pellet and stirred continuously for 1-2 minutes to eliminate clumping and to disperse/mix the microbial cells homogeneously in the powdered samples. To break up any remaining clumps during inoculation, a Waring Commercial Spice Grinder (Grainger, Lake Forest, IL) was used. Each bacterial inoculated powder (30 g) was spread into a thin layer (1 cm) and placed in a desiccator

with saturated salt solutions to equilibrate to the desired a_w. This methodology can be visualized in Figure 2.1a.

2.2.5 BGSI

In a biological safety cabinet, 100 g powder of each powdered food was weighed in a 30 cm-diameter stainless steel mixing bowl. Using a 174 x 50 mm 50 ml Kimble Kontes TLC reagent sprayer (Fisher Scientific, Waltham, MA) with 3 psi and nitrogen gas, the bacterial cell mixture was sprayed on and dispersed into each powder sample. To ensure an even coating of inoculated powder, a sterile stainless-steel whisk was used every 0.1–0.2 ml of culture sprayed (1 ml total) to homogeneously mix the sample and prevent powdered ingredients from clumping. Inoculated powders were then spread into a uniform layer (2.5 cm) and placed in a desiccator with saturated salt solutions to equilibrate to the desired a_w. This methodology can be visualized in Figure 2.1b.

2.2.6 LGLI

In a biological safety cabinet, 10 g of each powdered food was weighed in a stomacher bag, inoculated with 1 ml of the LGLI prepared cell suspension, and hand-mixed for 1 minute. Using a Seward Stomacher 400 Paddle Blender (Seward Laboratory Systems Inc, Bohemia, NY), homogenize the 10 g sample at 260 rpm for 2 minutes. Add 90 g uninoculated powder into a stomacher bag containing inoculated powder and mix by hand to break up clumps. Return to Seward Stomacher and homogenize at 260 rpm for 2 minutes. Store samples in desiccators with saturated salt solutions to equilibrate to desired a_w. This methodology can be visualized in Figure 2.1c.

Figure 2.1. Flow chart for comparing log CFU/g data from broth-grown liquid inoculation (a), broth-grown spray inoculation (b), and lawn-grown liquid inoculation (c). *a_w denotes when water activity measurements were taken.

2.2.7 Sample Conditioning and Storage

Inoculated powders were stored at 20 °C in a biosafety cabinet for 24 hours. Clumping, if occurred during the drying process, was removed with an autoclaved whisk, and the Waring Commercial Spice Grinder was used for final sample homogenization. Samples were equilibrated for 4 to 6 days using saturated salt solutions and 262 mm by 246 mm Nalgene desiccators (Thermo Fisher Scientific, Waltham, VA) to adjust the final a_w to 0.25 for NFDM and 0.43 a_w for both soy flour and all-purpose flour. Relative humidity in each desiccator was maintained within ±2% of each saturated salt

solution %RH value and assessed using an a_w meter after preparation. Saturated salt solutions utilized were: Lithium chloride (25% RH) (Sigma-Aldrich, St. Louis, MO) and Potassium carbonate (43% RH) (Sigma-Aldrich, St. Louis, MO), both stored at 25°C.

2.2.8 Enumeration procedure

10 1-g samples of each inoculated NFDM, soy flour, and all-purpose flour were taken pre- and post-equilibration to test for sample homogeneity. Samples were enumerated by diluting 1:10 in 0.1% [w/v] sterile peptone broth and serially diluted up to 10^{-8} and surface plated (100 µL) in triplicate onto OK agar medium for *C. sakazakii* or TSAYE for *E. faecium* and *S. enterica*. OK agar and TSAYE media plates were incubated for 48-72 hours at 37 °C prior to enumeration. CFU/g concentration was determined by averaging the results from each triplicate.

2.2.9 Statistical analysis

The experimental design consisted of three different bacteria (*C. sakazakii*, *S. enterica*, and *E. faecium*), three inoculation procedures (BGPI, BGSI, and LGLI), three different powdered foods (organic soy flour, Nonfat dry milk, and all-purpose flour), and two testing times (pre and post-equilibration). For analysis, each experiment was conducted in triplicate, and data were converted to log CFU per gram and then averaged. The log values from three individual replicate experiments (n=3) were averaged, and standard deviation calculated for each bacterium across both methods on each powder. Using Excel (Microsoft, Redmond, Wash.), tables were assembled for each inoculation method, and analysis of variance ($\alpha = 0.05$) and Duncan's multiple range tests were

performed to determine significance. Differences between mean values were considered significant at *P* value less than 0.05.

2.3 Results and Discussion

A comparison among different methods revealed that broth-grown pelletized inoculation (BGPI) achieved significantly higher inoculation levels (log CFU/g) ($P < 0.05$) for *S. enterica, C. sakazakii,* and *E. faecium* on all powders in contrast to the other two methods (BGSI and LGLI). This result was expected since bacterial cell pellet was diluted using 0.1% peptone for both BGPI and LGLI methods, whereas BGPI utilized just the bacterial cell pellet. Not only was the starting inoculum concentration different, but BGPI was also used to inoculate only 30 g of powder instead of the 100 g inoculated with BGSI and LGLI. However, diluting the pellet with 0.1% peptone does not account for the ~ 2-log difference observed in organic soy flour and NFDM inoculated with *C. sakazakii* by the BGPI method when compared to BGSI and LGLI. One explanation for this difference may be that not all the powder was scraped off the 30-cm-diam stainless steel mixing bowl before homogenizing with the spice grinder in BGSI. Another explanation may be that although the TLC sprayer sprays bacterial culture uniformly, not all the culture reached the powder and instead stuck to the side of the mixing bowl, thus resulting in a lower log CFU/g for BGSI. As for the difference with LGLI, organic soy flour and NFDM tended to stick to the sides of the bag during the stomaching process and even after final stomaching making it hard to recover all the powder. All-purpose flour in all three methods with all three organisms was the easiest to homogenize, as shown in

Tables 2.1-2.3, where the greatest variation of ~1-log can be observed between LGLI and BGPI.

Comparing the three methods (BGPI, BGSI, and LGLI) post-equilibration homogeneity across all three bacteria and all three powders resulted in ±0.25, ±0.20, and ±0.18 log CFU/g, respectively. Lawn grown bacteria were more tolerant to desiccation, except for *C. sakazakii*, as seen in Tables 2.1-2.3. Average log reductions amongst the three bacteria that were lawn grown were: ~1-log for *C. sakazakii*, ~0.2-log reduction for *E, faecium*, and ~0.37 for *S. enterica*. BGPI log reductions were ~0.85 for *C. sakazakii*, ~0.64 for *E. faecium*, and ~0.88 for *S. enterica*. BGSI displayed some of the highest log reductions with ~0.94 for *C. sakazakii* and ~1.12 for both *E. faecium* and *S. enterica*. These findings confirm that lawn grown bacteria display greater desiccation tolerance than broth grown, which agrees with previously reported findings (Hildebrandt et al., 2016; Keller et al., 2012; Komitopoulou and Penaloza, 2009; Uesugi et al., 2006).

Due to the use of liquid inoculum for both the inoculation methods, the water activity increased as compared to the uninoculated control, usually between 0.10–0.15 a_w for BGSI and 0.02–0.05 a_w for both BGPI and LGLI. Samples were re-equilibrated using saturated salt solutions and desiccators to achieve final a_w values of 0.25 for NFDM and 0.43 for soy flour and all-purpose flour. These bacteria undergo severe stress from liquid culture to a dry powder matrix. During this transition from liquid to dry state, each bacterium undergoes between 0.2 to ~1.4--log reduction in cell viability depending on the inoculation method. Desiccation tolerance signals/pathways play a critical role in cellular adaptation during low a_w. *C. sakazakii* has been shown to have higher desiccation tolerance among the *Enterobacteriaceae* family (Breeuwer et al., 2003). In this study,

however, *C. sakazakii* showed the largest log-reduction across all three powders. One reason for this discrepancy could be that this study only describes desiccation tolerance over 4-6 days and previously reported studies tested for longer durations of time (Breeuwer et al., 2003; Kandhai et al., 2010; Koseki and Shiina, 2015). Another reason is that it was previously shown that *Cronobacter* strain MC10 exhibited higher desiccation tolerance than *C. sakazakii* ATCC 29544, proving that the strain also plays a significant role in desiccation tolerance (Kandhai et al., 2010). However, when the mean was taken, *C. sakazakii* exhibited ~0.93 log-reduction, *E. faecium* ~0.65 log-reduction, and *S. enterica* ~0.79 log-reduction. *E. faecium* has been reported to be highly tolerant to desiccation and other cellular stresses than most microbes, and the data in Table 2.2, especially the LGLI grown cells, corroborates these findings (Rachon et al., 2016; Santillana Farakos et al., 2013).

2.4 Conclusions

After comparing three inoculation methodologies, with three different types of bacteria on three different powdered food matrices, it is found that inoculation methodologies impact repeatability and survival of each bacteria. *C. sakazakii* was found to be the most susceptible to log-reductions during equilibration regardless of the inoculation method employed. When comparing the inoculation methodologies with *S. enterica* and *E. faecium*, the inoculation method is chosen impacts the survivability of each bacteria no matter the powder inoculated. LGLI yielded the most stable populations out of the three inoculation methods evaluated and indicates that the methodology chosen can have an impact on low-moisture food studies.

Table 2.1. Effect of various inoculation methods on *Cronobacter sakazakii* ATCC 29544 pre- and post-equilibration on various powdered foods and log reduction[a]

Inoculation method	Food matrix	Initial population (log CFU/g)[b]	Post-equilibration population (log CFU/g)[c]	Reduction[d]
BGPI	Soy flour	9.77 (0.10) A	8.75 (0.14) A	1.02 (0.17) AB
	Nonfat dry milk	9.58 (0.39) A	8.81 (0.12) A	0.84 (0.21) BC
	All-purpose flour	8.65 (0.21) B	7.98 (0.40) B	0.67 (0.24) CD
BGSI	Soy flour	7.90 (0.06) CD	6.78 (0.31) CD	1.12 (0.10) AB
	Nonfat dry milk	8.14 (0.14) C	7.72 (0.15) B	0.42 (0.16) D
	All-purpose flour	7.83 (0.12) D	6.55 (0.26) D	1.28 (0.15) A
LGLI	Soy flour	7.46 (0.22) E	6.61 (0.18) CD	0.85 (0.17) BC
	Nonfat dry milk	7.32 (0.28) E	6.02 (0.26) E	1.30 (0.26) A
	All-purpose flour	7.80 (0.12) D	6.95 (0.12) C	0.86 (0.09) BC

[a] BGPI, Broth-grown pelletized inoculation; BGSI, Broth-grown spray inoculation; LGLI, Lawn-grown liquid inoculation. Population values reported as the mean (standard deviation). Within columns, values that share a common letter are considered not to be significantly different ($\alpha = 0.05$).

[b] Initial population log CFU/g tested immediately following inoculation.

[c] Post-equilibration population log CFU/g tested following a 4-6-day equilibration period in a desiccator with saturated salt solutions.

[d] Reduction (log CFU/g) refers to the bacteria that were lost during the equilibration period. Calculated by Initial population minus post-equilibration population.

Table 2.2. Effect of various inoculation methods on *Enterococcus faecium* NRRL B-2354 pre- and post-equilibration on various powdered foods and log reduction[a]

Inoculation method	Food matrix	Initial population (log CFU/g)[b]	Post-equilibration population (log CFU/g)[c]	Reduction[d]
BGPI	Soy flour	9.21 (0.22) A	8.71 (0.23) A	0.50 (0.24) BC
	Nonfat dry milk	9.30 (0.25) A	8.58 (0.24) A	0.72 (0.16) B
	All-purpose flour	8.46 (0.24) CD	7.76 (0.23) BC	0.70 (0.06) B
BGSI	Soy flour	8.81 (0.12) B	7.67 (0.13) BC	1.13 (0.09) A
	Nonfat dry milk	8.39 (0.17) CD	7.29 (0.20) D	1.11 (0.18) A
	All-purpose flour	8.65 (0.12) BC	7.54 (0.24) CD	1.11 (0.24) A
LGLI	Soy flour	7.66 (0.15) F	7.52 (0.14) CD	0.14 (0.10) D
	Nonfat dry milk	8.30 (0.10) DE	7.96 (0.06) B	0.34 (0.10) CD
	All-purpose flour	8.06 (0.11) E	7.94 (0.19) B	0.12 (0.11) D

[a] BGPI, Broth-grown pelletized inoculation; BGSI, Broth-grown spray inoculation; LGLI, Lawn-grown liquid inoculation. Population values reported as the mean (standard deviation). Within columns, values that share a common letter are considered not to be significantly different ($\alpha = 0.05$).

[b] Initial population log CFU/g tested immediately following inoculation.

[c] Post-equilibration population log CFU/g tested following a 4-6-day equilibration period in a desiccator with saturated salt solutions.

[d] Reduction (log CFU/g) refers to the bacteria that were lost during the equilibration period. Calculated by Initial population minus post-equilibration population.

Table 2.3. Effect of various inoculation methods on *Salmonella enterica* ATCC 700720 pre- and post-equilibration on various powdered foods and log reduction[a]

Inoculation method	Food matrix	Initial population (log CFU/g)[b]	Post-equilibration population (log CFU/g)[c]	Reduction[d]
BGPI	Soy flour	9.32 (0.36) A	8.40 (0.30) A	0.92 (0.05) B
	Nonfat dry milk	8.87 (0.13) B	8.09 (0.22) AB	0.78 (0.11) BC
	All-purpose flour	8.62 (0.15) B	7.69 (0.31) B	0.93 (0.40) B
BGSI	Soy flour	8.11 (0.21) C	7.12 (0.23) C	1.00 (0.40) AB
	Nonfat dry milk	7.95 (0.05) CD	6.90 (0.06) C	1.05 (0.05) AB
	All-purpose flour	8.19 (0.43) C	6.83 (0.21) C	1.36 (0.23) A
LGLI	Soy flour	7.45 (0.13) E	7.03 (0.15) C	0.42 (0.11) CD
	Nonfat dry milk	7.53 (0.28) E	7.16 (0.25) C	0.33 (0.06) D
	All-purpose flour	7.61 (0.22) DE	7.26 (0.29) C	0.35 (0.18) D

[a] BGPI, Broth-grown pelletized inoculation; BGSI, Broth-grown spray inoculation; LGLI, Lawn-grown liquid inoculation. Population values reported as the mean (standard deviation). Within columns, values that share a common letter are considered not to be significantly different ($\alpha = 0.05$).

[b] Initial population log CFU/g tested immediately following inoculation.

[c] Post-equilibration population log CFU/g tested following a 4-6-day equilibration period in a desiccator with saturated salt solutions.

[d] Reduction (log CFU/g) refers to bacteria that were lost during the equilibration period. Calculated by Initial population minus post-equilibration population.

Chapter Three

EFFECTS OF INTENSE PULSED LIGHT ON *CRONOBACTER SAKAZAKII* INOCULATED IN NONFAT DRY MILK

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3.1 Introduction

Cronobacter sakazakii, formerly known as *Enterobacter sakazakii*, is associated with a severe but rare form of life-threatening neonatal meningitis, resulting in a mortality rate as high as 40-80%. Not only are neonates at risk, but *C. sakazakii* can also cause disease in humans of all ages, including the elderly and those who are immunocompromised (Nazarowec-White and Farber, 1997). Low-moisture foods, including PIF, herbal teas, and starches, are a major source of *C. sakazakii* infection. Contaminated PIF has been particularly associated with cases of *C. sakazakii* infections in premature infants. In 2011, the Centers for Disease Control and Prevention (CDC) reported that the number of infant cases of *C. sakazakii* has tripled and continues today to rise (Feeney, 2014). *C. sakazakii* can survive in extremely low water activity foods such as powders and cereals for extended periods and has more thermal resistance than other members of the *Enterobacteriaceae* family (Asakura et al., 2007). For example, a study demonstrated that the decimal reduction times of *C. sakazakii* strains in saline solution were 12–16 and 3–5 min at 52 °C and 56 °C, respectively (Kim and Park, 2007). However, when translating decimal reduction times from saline solutions to food matrices such as skim milk powder, it is essential to understand the glass transition temperature (T_g). The T_g for skim milk powder is ~58 °C (Ozmen and Langrish, 2006),

and the difference between the powder temperature (T_1) and T_g are directly associated with the caking rate and caking mechanism (Hartmann and Palzer, 2011). No caking can be observed on a relevant time scale in free-flowing glassy powders when T1 is smaller than T_g (Aguilera et al., 1995). However, severe unwanted agglomeration will be induced when the condition of T_1 is larger than Tg (Karel et al., 1994). Therefore, it is difficult to rapidly conduct conventional thermal decontamination of *C. sakazakii* in PIF without affecting the functional attributes and quality of the milk powder.

Various non-thermal techniques have been developed to inactivate *C. sakazakii* in PIF; however, these techniques are not always practical, quick, or feasible in application. For example, Pina-Perez et al. reported that the maximum inactivation levels for *C. sakazakii* in PIF was around 1.22 $\log_{10}$ CFU/g using pulsed electric field treatments (Pina-Perez et al., 2007). For the ultraviolet radiation treatment, the time required to ensure ∼3 log10 CFU/g reductions in PIF was 20 min UV radiation combined with hot water treatment (Liu et al., 2012). Gonzalez et al. (2006) treated *C. sakazakii* with hydrostatic pressure processing (HPP), achieving a maximum inactivation level of 3.11 $\log_{10}$ CFU/g for *C. sakazakii* ATCC 29544 under 600 MPa. One thing to note is that the HPP process was used one liquid PIF before spray drying.

Intense pulsed light (IPL) is being investigated to kill *C. sakazakii* in NFDM prior to packaging. IPL has the potential to rapidly inactivate microorganisms on food surfaces using short-duration pulses of the intense broad-spectrum (100–1100 nm), which is rich in UV light (Oms-Oliu et al., 2008). A previous study reported that approximately a 1.5-log reduction of *C. sakazakii* in PIF on a petri dish was achieved with IPL treatment (Choi et al., 2009). In terms of the nutrient change in milk powder after IPL treatment,

Elmnasser et al. found that short time pulsed light treatment produced no significant change in the amino acid composition of proteins and lipid oxidation in milk powder (Elmnasser et al., 2008). Based on these findings, utilizing IPL as a nonthermal processing technique shows promise for milk powder. However, undesirable agglomeration of milk powder particles can be caused by various inter-particle forces developed due to the moisture absorption and elevated temperature that occurred during IPL treatment (Peleg, 1983). Increased particle clumping leads to a lower inactivation efficiency of microorganisms during IPL treatment. In order to apply IPL on a commercial scale in the food industry, a variety of processing parameters affecting the bactericidal efficacy of IPL treatment needs to be controlled through light intensity, vibration frequency, water activity, the temperature of milk powder samples, and environmental relative humidity. The penetration depth also limits the IPL efficacy on solid foods (Elmnasser et al., 2007), to ensure uniform (360°) exposure of IPL to the surface of food particles must be ensured for efficient IPL decontamination.

A vibratory feeder was employed in this study to convey the milk powder during IPL treatment. The vibration was expected to create tumbling of the milk powder particles during IPL treatment to facilitate exposure of the entire surface of individual milk powder particles to the IPL. Many types of foods have been tested with IPL technology; however, currently, there is no continuous IPL system commercially available for powder foods. Therefore, through the use of a vibratory feeder, the data presented here will start to reduce the knowledge gap preventing IPL from commercialization. The goal of this study was to measure various processing conditions and parameters and how they impact the inactivation of *C. sakazakii* by IPL.

3.2 Materials and Methods

3.2.1 Bacterial Inoculum Preparation and Adjustment of Water Activity (a_w)

NFDM was provided by Land O'Lakes, Inc., Arden Hills, Minnesota. The product was made from pasteurized milk, and all samples were vacuum-sealed and transported to the laboratory at the University of Minnesota. The inoculation procedure was as follows: Tryptic Soy Broth (TSB) was aseptically added to tubes, which were inoculated with *C. sakazakii* ATCC 29544 from isolated colonies on streak plates. *C. sakazakii* was incubated at 37 °C for 24 ± 2 h in TSB on a rotary shaker (150 rpm). Suspensions were then centrifuged (4000 rpm, 20 min at 25 °C) to pellet cellular suspensions. Then the supernatant was decanted, and cell pellets were combined and washed twice with 0.9% saline (w/v). The bacterial pellet was then suspended into 20 ml w of 0.9% saline. The pellet mixture was sprayed onto NFDM in a 50-cm diam sterile stainless-steel bowl using a 50-ml Kimble Kontes chromatography reagent sprayer (Fisher Scientific, Waltham, MA) and nitrogen gas (Gurtler and Beuchat, 2007). A sterile stainless-steel whisk was used to prevent clumping and to homogenously mix the sample after every 1–2 ml of cell pellet mixture sprayed (20 ml total). Approximately 10^7 CFU/g *C. sakazakii* was then inoculated into the NFDM sample. The inoculated NFDM samples were spread in a thin layer (7 mm) into sterile 150mm×15mm glass petri dishes (Fisher Scientific, Waltham, MA) and placed in Nalgene Desiccators (Thermo Fisher Scientific, Waltham, MA). Each desiccator contained 250 ml of saturated salt solutions: 35 °C potassium fluoride (0.20 a_w), 45 °C potassium fluoride (0.25 a_w), 40 °C magnesium chloride (0.30 a_w), and 35 °C sodium iodide (0.35 a_w) (Greenspan, 1977). The inoculated samples equilibrated for

7–10 days, and then the a_w was tested with a Decagon Pa_wkit (METER Group, Pullman, WA).

3.2.2 Intense Pulsed Light Treatment

The IPL treatment system included an IPL source, a vibratory, a humidity controller, and a temperature controller. The IPL source was provided by a lab-scale Z-1000 steripulse- XL system (Xenon Corporation, Woburn, MA) consisting of a xenon flash lamp and an RC- 800 power/control module. The Z-1000 steripulse- XL system generated polychromatic radiation in the wavelength range of 190 nm–1100 nm. An air-cooling device was attached to the lamp housing to prevent the lamp from overheating during the process (Figure 3.1).

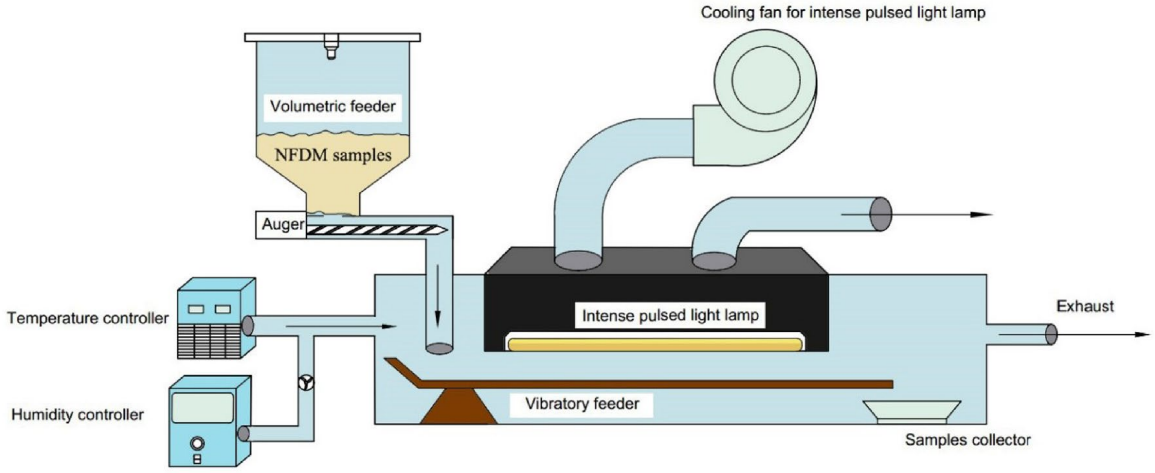

Figure 3.1. A schematic diagram of the IPL system

High-intensity pulses at a rate of 3 pulses per second and a pulse width of 360 µs were generated and applied to the NFDM. Each pulse delivered 1.27 J/cm^2 at an input of 3800 voltage, and distance from the quartz window of 8 cm. Residence time was 18, 25, and 28 s by adjusting the vibratory frequency; the feeding rate of the milk samples was

~100 g/min, to maintain a 2mm thickness of NFDM. Three initial temperatures, 25, 30, 35 °C, were achieved by preheating the samples to maintain the final temperature of ~57 °C after the IPL process. Table 3.1 shows the factorial experiment design. All of the samples were inoculated with approximately 6.8 ± 0.2 $\log_{10}$ CFU/g *C. sakazakii* prior to the treatments. There was a 1-min interval after each treatment to cool the IPL system down. Each treatment was performed in triplicate with the initial temperature at around 25 °C and relative humidity of 35-45% in a temperature and humidity controlled chamber.

Table 3.1. Processing parameters of each treatment

Code	Initial a_w of NFDM	Treatment conditions
1	0.20	T = 28 s, Tem = 25 °C
2	0.20	T = 25 s, Tem = 30 °C
3	0.20	T = 18 s, Tem = 35 °C
4	0.25	T = 28 s, Tem = 25 °C
5	0.25	T = 25 s, Tem = 30 °C
6	0.25	T = 18 s, Tem = 35 °C
7	0.30	T = 28 s, Tem = 25 °C
8	0.30	T = 25 s, Tem = 30 °C
9	0.30	T = 18 s, Tem = 35 °C
10	0.35	T = 28 s, Tem = 25 °C
11	0.35	T = 25 s, Tem = 30 °C
12	0.35	T = 18 s, Tem = 35 °C

Initial temperature and residence time were labeled as Tem and T, respectively.

3.2.3 Measurements of the IPL Fluence and Temperature

The broadband energy of each pulse, expressed in J/cm^2, was measured using a Vega laser power meter (Ophir Optronics Inc., Wilmington, MA) equipped with a PE-50C pyroelectric energy sensor (Ophir Optronics Inc., Wilmington, MA). To measure the fluence received by the NFDM samples during IPL treatments, the pyroelectric sensor

was placed in the center of the starting end of the vibratory feeder and moved through the vibratory feeder in 28, 25, and 18 s. It should be noted that the height of the energy sensor was approximately 2 cm. To measure the energy received at 8 cm in height, the lamp was raised to 10 cm. Precautions were taken to allow about 30 s pauses during each measurement to prevent the sensor from overheating. All fluence measurements were performed at least in triplicate. The temperature profiles of the NFDM linked to the IPL treatment were monitored using a non-contact infrared thermometer with laser targeting (Cen-Tech, Montessori, NV). The temperatures of the milk samples were immediately measured before and after each IPL treatment.

3.2.4 Particle Size Measurement

The particle size of the treated and untreated NFDM samples were measured using a LS 13 320 laser diffraction particle size analyzer (Beckman Coulter, Inc, Brea, CA). The process was referred to the method described by Pisecky (1997). The measurements were taken from five grams of the NFDM particles from each IPL treatment. The particle size was recorded every 2 min for each IPL untreated and treated samples until successive readings became constant. The NFDM particle size is expressed as the diameter (μm).

3.2.5 Bacterial Enumeration

Following the IPL treatment, the NFDM was diluted 1:10 in 0.1% (w/v) sterile peptone broth (Fisher Scientific, Fair Lawn, NJ). Each sample was serially diluted and spread plated in triplicate onto HiCromeTM *Cronobacter* spp. agar, Modified (Sigma-

Aldrich, St. Louis, MO). Spread plates were incubated for 24 ± 2 h at 44 °C (Garbowska et al., 2015). Samples were also plated on Tryptic soy agar (TSA) to assess the effect of non-selective media for the enumeration of *C. sakazakii* under the IPL. Spread plates were inoculated for 24 ± 2 h at 37 °C. Each spread plate was counted manually, and CFU/g concentrations were determined by averaging the results from each triplicate.

3.2.6 Statistical Analysis

At least three independent trials were conducted for all experiments. Colony counts were converted to $\log_{10}$ CFU/g reductions, and the standard deviations (SD) were determined. Statistical analyses were conducted using JMP Pro 13 (SAS Cary, NC). As for the analysis of variance (ANOVA) procedure for balanced data, T-test analysis was used to access significant differences among the treatments ($p < 0.05$), and correlation analysis was conducted to verify the correlation between the different factors.

3.3 Results and Discussion

3.3.1 Effect of IPL on *C. sakazakii* Inoculated in NFDM

NFDM was treated using IPL under different conditions to determine the bactericidal efficacy on *C. sakazakii* in NFDM. The total fluences received after 18, 25, and 28 s IPL treatments were 15.12, 22.35, and 29.36 J/cm^2, respectively (Figure 3.2). For the residence time at each water activity level from 0.20 to 0.35, there was a significant time-dependent increase of inactivation in the NFDM from 18 to 28 s ($p < 0.05$ using two-way ANOVA), which resulted in a 0.27–3.18 $\log_{10}$ CFU/g reduction of *C. sakazakii* in the NFDM. A higher dose of pulsed light or long duration was required to

achieve higher pathogenic inactivation at each water activity level (Figure 3.3). Comparing the inactivation between water activity levels of 0.20 and 0.25, it was observed that inactivation at the water activity level of 0.25 was significantly higher than the water activity level of 0.20 ($p < 0.05$ using two-way ANOVA). This result indicated that *C. sakazakii* exhibited less resistance in relatively high moisture conditions than in a dry environment. Beuchat et al. reported that there is an inactivation effect associated with the water activity level on *C. sakazakii* in PIF (Beuchat et al., 2009). *C. sakazakii* survival in PIF was better at a water activity level of 0.20 than in PIF at other water activity levels.

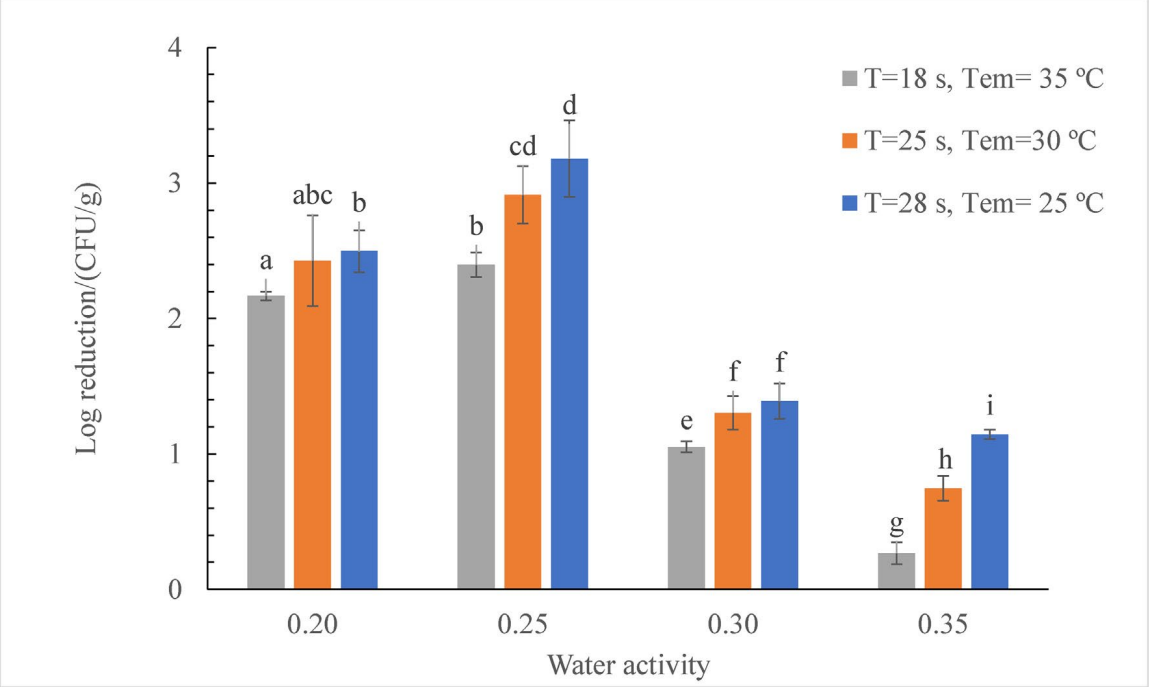

Figure 3.2. Log reduction (CFU/g) of *Cronobacter sakazakii* inoculated NFDM treated with IPL at different water activity levels, initial temperatures, and residence time. Data in the figure followed by the same lowercase letter are not significantly different ($p > 0.05$). Values are expressed as the mean ± standard deviation of measurements made in triplicate.

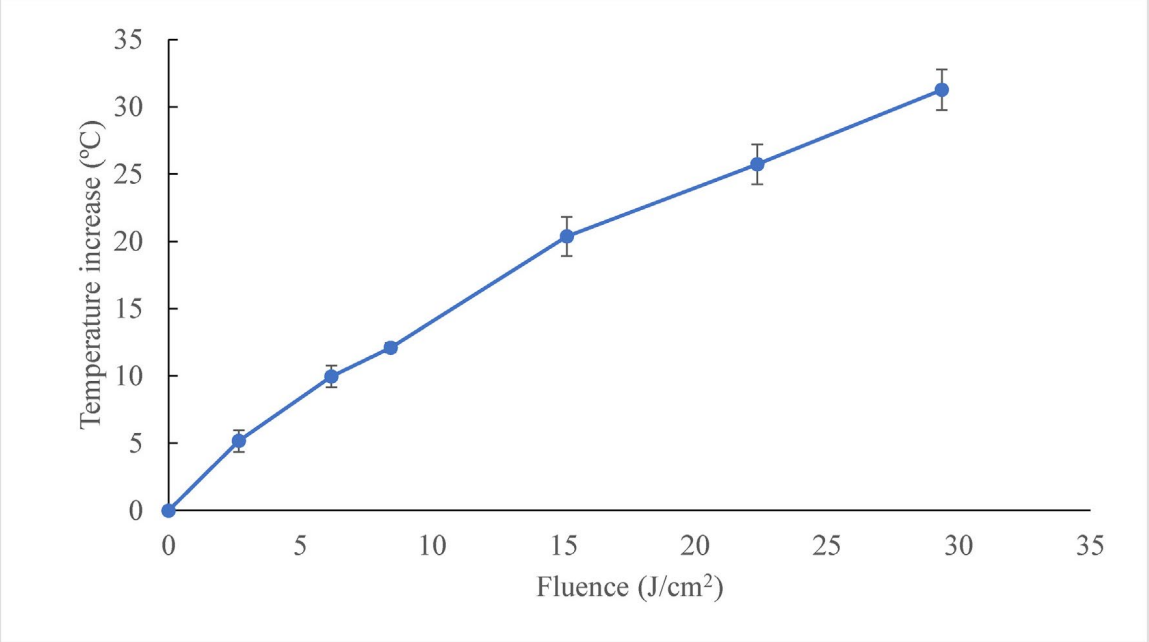

Figure 3.3. Surface temperature increase of NFDM at fluences from 0 to 29.36 J/cm^2. Data are expressed as the mean ± standard deviation of measurements made in triplicate.

However, inactivation decreased with increasing the water activity levels of NFDM from 0.25 to 0.35 ($p < 0.05$ using two-way ANOVA). High water activity levels and undesirable agglomeration significantly reduced the inactivation of IPL (pairwise correlations= −0.8036, and $p < 0.0001$, Figure 3.4). This agglomeration might provide a shield for the inner bacteria against IPL treatment. Due to the limitation of IPL penetration depth, the IPL fluence decreased as the thickness of the particles increased. Uesugi and Moraru (2009) found that the penetration of pulsed light through sausage slices was a function of thickness, and the fluence at a thickness of 0 mm was 1.10 J/cm^2, while the fluence at 2.5 mm was ~0.10 J/cm^2. The result indicates that IPL treatment is more effective for the inactivation of bacteria on particle exterior surfaces than bacteria embedded inside particles.

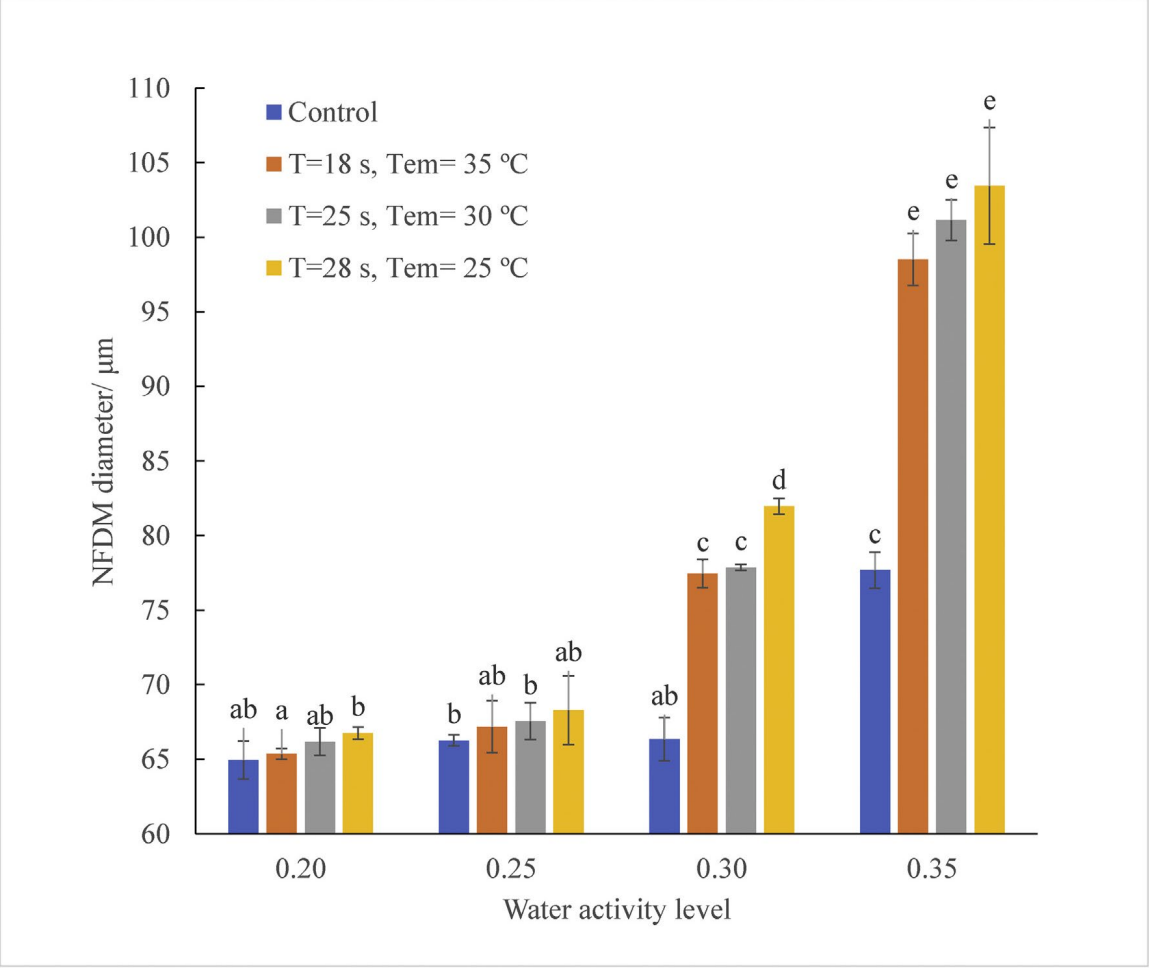

Figure 3.4. NFDM mean particle size as a function of water activity and initial temperature. Control at each water activity level is the NFDM samples without IPL treatment. Data in the Figure followed by the same lowercase letter are not significantly different ($p > 0.05$). The values were expressed as the mean ± standard deviation of measurements made in triplicate.

The temperature and water activity profiles of the NFDM samples during IPL treatments are shown in Table 3.2. In this study, the surface temperatures of NFDM at different initial temperatures were monitored. Throughout the study, the temperature never exceeded 58 °C. Numerous studies support that many types of amorphous lactose in NFDM, such as glassy lactose, are quite stable below the glass transition temperature (Yrjo, 2002; Ozmen and Langrish, 2006). However, powder stability is lost above the glass transition temperature. Therefore, the stickiness of powders could be controlled

using the glass transition temperature (Ozmen and Langrish, 2006; Yrjo, 2002). During the treatments, the NFDM samples absorbed the light energy, increasing temperature. Figure 3 shows the increase of temperature profiles of the NFDM that are linked to the fluence of IPL treatments. The highest temperature increase of 31.6 °C on the surface of the NFDM was obtained after receiving 29.36 J/cm^2 fluence with IPL treatment.

Table 3.2. Water activity (a_w) levels and temperature profiles of NFDM before and after IPL treatment

Code	Initial a_w	Treatment conditions	a_w after IPL	Temperature after IPL/°C
1	0.20	T = 28 s, Tem = 25 °C	0.18 ± 0.01	57.7 ± 1.5
2	0.20	T = 25 s, Tem = 30 °C	0.18 ± 0.01	57.7 ± 1.4
3	0.20	T = 18 s, Tem = 35 °C	0.19 ± 0.01	56.1 ± 1.1
4	0.25	T = 28 s, Tem = 25 °C	0.21 ± 0.01	57.5 ± 0.7
5	0.25	T = 25 s, Tem = 30 °C	0.20 ± 0.01	57.2 ± 1.4
6	0.25	T = 18 s, Tem = 35 °C	0.20 ± 0.01	56.7 ± 0.3
7	0.30	T = 28 s, Tem = 25 °C	0.20 ± 0.01	57.9 ± 0.9
8	0.30	T = 25 s, Tem = 30 °C	0.20 ± 0.01	57.7 ± 0.6
9	0.30	T = 18 s, Tem = 35 °C	0.20 ± 0.01	56.3 ± 1.9
10	0.35	T = 28 s, Tem = 25 °C	0.22 ± 0.01	57.3 ± 0.7
11	0.35	T = 25 s, Tem = 30 °C	0.22 ± 0.01	57.3 ± 2.1
12	0.35	T = 18 s, Tem = 35 °C	0.22 ± 0.01	56.4 ± 0.8

Values of temperature and a_w are expressed as the mean ± standard deviation of measurements made in triplicate.

3.3.2 Particle Size Measurement

The NFDM samples were treated with IPL at the initial water activity levels of 0.20–0.35, initial temperatures from 25 to 35 °C, and residence times from 18 to 28 s. As shown in Figure 4, for the residence time at each water activity level of 0.20 and 0.25, there was no significant increase in particle size from 18 to 28 s ($p > 0.05$). In comparing the diameters of different water activity levels, the mean particle diameter increased with the increasing of water activity level from 0.20 to 0.35 at each residence time,

respectively ($p < 0.05$ using two-way ANOVA). A maximum particle diameter of 103.45 μm was observed at the initial temperature of 25 °C, a residence time of 28 s, and a water activity level of 0.35. In this study, a significantly larger particle than the control was observed at the water activity levels of 0.30 and 0.35 ($p < 0.05$). Higher water activity could decrease the glass transition temperature of the amorphous lactose, which resulted in a more severe agglomeration, producing larger particles. These results were consistent with previous studies that indicate interactions among water activity, residence time, and elevated temperature. A longer residence time provided higher energy to the particles and allowed longer contact time and collision among the milk particles during IPL treatment, which was aided by higher water activity and elevated temperatures, all of which might increase agglomeration of milk powder particles (Ozmen and Langrish, 2006).

Milk powder is a high-risk food due to possible bacterial contamination. IPL is a promising nonthermal technology to establish efficacy in inactivating various microorganisms in transparent suspensions and some food contact surfaces (Oms-Oliu et al., 2008). However, few studies have focused on improving the applicability and efficacy of this technology in powdered food, such as milk powder. In particular, the application of IPL on milk powder has been a challenge since sample heating is a crucial factor limiting commercial application. Most other studies investigating the effects of IPL on bacteria have focused only on one or two processing factors (Fine and Gervais, 2004; Marianne et al., 2005; Karel et al., 1994). For the operation of IPL in the processing of NFDM, a balance is required among a treatment with sufficient residence time/fluence, proper initial water activity, and initial temperature to eliminate *C. sakazakii* in NFDM. Ideally, the process should have limited adverse effects on the quality of NFDM.

Additionally, it also needs to be economically and commercially viable for the processor (i.e., minimal residence times). Thus, the research was designed to acquire IPL inactivation data over a range of attributes that are most applicable to the related powdered food industry at a water activity level of 0.25, an initial temperature of 25 °C, and a residence time of 28 s (29.36 J/cm^2).

3.3.3 Analysis of IPL Inactivation Corresponded with Temperature Profiles of NFDM

In this study, NFDM samples were treated at the initial temperatures of 10–25 °C using vibratory and stationary methods. As shown in Table 3.3, comparing treatments among different initial/final temperatures using a vibratory method, the inactivation increased with increasing the initial temperature ($p < 0.05$ using one-way ANOVA).

Table 3.3. Effect of IPL treatments on inactivating *C. sakazakii* in NFDM at different initial temperatures or treated by IPL with (V) or without (S) a vibratory feeder

Treatment	Final temperature °C	Log reduction (CFU/g)
IPL-V-10 °C	41.8 ± 0.7	0.87 ± 0.06a
IPL-V-15 °C	47.6 ± 0.6	1.14 ± 0.05b
IPL-V-20 °C	52.1 ± 0.7	2.08 ± 0.12c
IPL-V-25 °C	57.5 ± 0.7	3.18 ± 0.28d
IPL-S-25 °C	57.2 ± 0.2	0.84 ± 0.08a

For a simplified description of the different IPL initial temperatures, IPL-V-10, 15, 20, and 25 °C was defined as the NFDM powder samples that were treated with IPL using vibratory feeder at the initial temperature from 10 to 25 °C. IPLS- 25 was defined as the sample that was treated with IPL at the initial temperature of 25 °C without using the vibratory feeder. All samples were initially adjusted to water activity levels of 0.25 and had a residence time of 28 s. All IPL treatments received the same amount of fluence. Five grams of NFDM was inoculated with 6.4 ± 0.6 log10 CFU/g. Data in the same column, followed by the same lowercase letter are not significantly different ($p > 0.05$). All the experiments were performed in triplicate.

Osaili et al. reported that only 1 $\log_{10}$ CFU/g reduction was achieved for *C. sakazakii* in skim milk after 1.49 ± 0.05 min at a temperature of 56 °C (Osaili et al.

2009). The average temperature was substantially lower than 56 °C in the current study. Thus, this study indicates that limited inactivation is due to the elevated temperature generated by IPL alone. Results indicate that elevated temperatures are very relevant for practical applications of IPL since there are potentially synergistic effects between mild temperature (~57.5 ± 0.7 °C) and IPL treatments were observed. In this study, the bactericidal effects of IPL treatment on bacterial cells are similar to previous studies using pulsed light to inactivate bacteria without a food matrix. The synergistic effects between mild heat and continuous UV were used to inactivate 5.71 ± 0.60 $\log_{10}$ CFU/g and 4.01 $\log_{10}$ CFU/g for *E. coli* and *Salmonella*, respectively, when the final temperature was 57.5 °C, but killed significantly fewer pathogens when the final temperature was 50 °C (Gayan et al., 2011, 2012). However, more research is needed to determine whether a combination of IPL and a mild temperature area are effective for the inactivation of bacteria on other types of powdered food.

This study utilized a new strategy by combining a vibratory feeder with IPL illumination for the decontamination of NFDM. Comparing between the inactivation of vibratory and stationary IPL treatments (Table 3.3), a significantly higher inactivation effect was achieved ($p < 0.05$) for the vibratory IPL treatment than the stationary IPL treatment. It seems that stationary IPL treatments result in only partial decontamination. Choi et al. (2009) also found that the low reduction level of IPL treatment indicated that the opaqueness and the viscosity of powdered milk led to lower penetration of pulsed light than a milk powder beverage. In the current study, a solution to this problem would be to agitate the particles to increase the probability of them being exposed to IPL. An appropriate design of combining a vibratory feeder with IPL may lead to better

penetration and inactivation for commercial success since this type of vibratory feeder combined with the non-linear nature of Coulomb friction causes the particles to move along a straight line (Chandravanshi and Mukhopadhyay, 2017). During the process of vibratory IPL treatment, the vibratory feeder not only acts as a conveying tool, but also helps the NFDM tumble, and thus allows more uniform IPL exposure of the entire surface of individual NFDM particles to IPL.

In addition, organic matter can prevent microorganisms in NFDM from absorbing IPL. The maximum inactivation of 3.18 $\log_{10}$ CFU/g was achieved on *C. sakazakii* with IPL treatment in this study. In contrast, higher log reductions can be attained in media without organic matter. Choi et al. (2009) reported that it could produce a 5 $\log_{10}$ CFU/g reduction of *C. sakazakii* suspended in media in a petri dish after 4.6 ms of treatment at 10 kV. UV wavelengths (180–380 nm) in pulsed light play a vital role in the inactivation of pathogens by forming dimers in bacterial DNA, resulting in impaired replication and gene transcription (Giese and Darby, 2000). NFDM has a relatively low concentration of lipids (~1.5%), but a high concentration of protein (~34%) in NFDM can absorb a UV wavelength spectrum at 190 nm, 280 nm as well as the UV-B region. Conjugated double bonds also absorb UV (Elmnasser, 2008; Hollosy, 2002). So these proteins may limit the inactivation effect of IPL on *C. sakazakii* in milk samples. The result is consistent with a study of pulsed UV light inactivation of the hepatitis A virus and norovirus surrogate in suspension and on food-contact surfaces, indicating that the presence of organic matter inhibits the efficiency of pulsed light significantly on these conditions (Jean et al., 2011).

3.4 Conclusions

This study evaluated IPL treatment as an alternative method for the inactivation of *C. sakazakii* in NFDM. Factors such as fluence, residence time, temperature, and particle were examined. This study revealed that IPL effectiveness on the reduction of *C. sakazakii* on NFDM was significantly affected by the water activity levels and residence time. Water activity levels significantly impacted IPL treatment on *C. sakazakii* in NFDM. Higher water activity resulted in the severe agglomeration of NFDM particles after IPL treatment, directly impacting log reductions when compared to lower water activity levels.

The inactivation method used in this study also significantly reduced *C. sakazakii* in the NFDM, thus demonstrating a promising new technology for foodborne pathogen reduction in powdered foods and ingredients. Utilizing vibratory equipment was particularly effective during the IPL treatment, especially when the powder samples were at the relatively low water activity level and under appropriate environmental conditions. When the conditions are controlled, and a vibratory feeder is used, the results indicate that higher microbial inactivation can be achieved. This new control strategy eliminates the disadvantages of partial decontamination of commonly used IPL treatment methods. The choice of this innovative method provides a feasible alternative to scale-up this non-thermal technology.

Finally, this study reveals that the synergistic effect of intense pulsed light and mild temperature (~57 °C) exhibit an inactivation effect of *C. sakazakii* at the water activity level of 0.25 and residence time of 28 s with IPL treatment while maintaining the quality of NFDM. Before the commercialization of the IPL system in the food industry,

complementary studies are needed to verify whether significant changes in other nutrients and sensory factors of IPL-treated NFDM occur.

3.5 Acknowledgements

Special thanks to Dongjie Chen in Dr. Ruan's Lab for operating the IPL system, testing particle size, temperature, and for the statistical analysis. Without your help, the microbiology data would be numbers in an Excel document without much meaning behind them. Data not shown here was the testing of volatile compounds and color changes that were performed by Qingqing Mao from Dr. Chi Chen's lab.

Chapter Four

MICROBIAL DECONTAMINATION OF HARD RED WHEAT UTILIZING INTENSE PULSED LIGHT

4.1. Introduction

Worldwide, cereal grains yield account for 60% of the cultivated fields and are a vital crop to humans and as a large portion of animal feed (Cordain, 1999; Koehler and Wieser, 2013). Within the cereal grains, wheat is the third most common cereal grain cultivated, with 736.1 million metric tonnes produced worldwide in 2018 (Ekpei et al., 2018). As a food source for humans, wheat provides 19% of our total available calories (FAO, 2014). With grain being as crucial as it is for human consumption, it is also susceptible to spoilage, particularly by molds, and to contamination with pathogenic microorganisms. Unfortunately, for the food industry, most spoilage organisms are part of the standard flora of wheat. These organisms possess the ability to proliferate on the surface of grains themselves during storage when temperature abuse occurs during storage. Environmental factors leading to microbial proliferation are water availability and temperature. Typically, good manufacturing practices (GMPs) are used to control the bioburden of pathogenic and spoilage organisms during processing and storage. This adherence to GMPs has the potential to result in microbial reduction over time and undermine their ability to proliferate (Hood and Moorman, 2015). However, if GMPs fail, the growth of spoilage organisms results in the loss of dry matter, alters the nutritional value, decreases germination rate, causes discoloration, and leads to other deteriorative changes (Magan et al., 2003).

Grains are a product of the environment they grow in and contain a multitude of different microorganisms in the microflora. Environmental factors such as air and dust particles, temperature, sunlight, rainfall, agricultural practices, bird, insects, rodents, and the condition of the soil all play a role in the heterogeneity of grain flora (Laca et al., 2006). Grains typically do not support the growth of microorganisms; however, their water activity (a_w) can vary from 0.50-0.70 depending on storage conditions (Berk, 2009). Most bacteria, including foodborne pathogens, require a_w to be >0.91 for growth, and most molds experience decreased proliferation at a_w <0.70 (Sperber, 1983). However, some osmophilic yeasts can grow at a_w between 0.60-0.65. While some xerophilic molds and *Saccharomyces bisporus* have a minimum a_w between 0.65-0.70. Growth of *Aspergillus* on grains produces black mold rot and aflatoxin production, and *Claviceps purpura* results in ear rot and ergotism. Both are detrimental to the overall quality of the grain and pose a risk to human health.

Particularly of concern are the mycotoxin producing mold genera *Fusarium*, *Penicillium*, and *Aspergillus* (Magan et al., 2010). Even though mycotoxins are naturally occurring chemical compounds, they still pose a severe threat to human health (Jha, 2016). *Fusarium* spp. are responsible for high economic impact by causing worldwide yield losses and resulting in reduced grain quality (Schaarschmidt and Fauhl-Hassek, 2018). In the Northern hemisphere, especially in the temperate regions, ochratoxin A (OTA) can be produced postharvest by *Penicillium verrucosum* at temperatures below 30°C (Limay-Rios et al., 2017). Also, non-pathogenic fungi may be present on wheat grains and are a significant cause of spoilage of grains during storage. Wheat diseases that are caused by a fungus such as black tip, common smut or bunt, and dwarf bunt are

detrimental and lead to decreased yields, stunted plants, poor grain quality, seedling blight, and rotten roots (Bockus, 2010; Burnett et al., 1986).

Bacteria are common microorganisms found on wheat, with some studies even reporting the presence of foodborne pathogens. Between 2012-2014, Myoda et al. (2019) analyzed 3,891 samples across four types of wheat (hard red spring, hard red winter, soft red winter early, and soft red winter late) for the presence of foodborne pathogens (Myoda et al., 2019). In this recent study, 17 (0.44%) samples tested positive for EHEC, 48 (1.23%) were positive for *Salmonella*, and 1 (0.08%) was positive for *Listeria* spp. Even though, when combined, only ~1.7% of the 3,891 samples tested positive for the presence of foodborne pathogens, this still poses a significant health risk to consumers once grains are milled into flour. Wheat flour has been linked to two foodborne outbreaks since 2016. In 2016, contaminated flour produced at a Missouri General Mills plant resulted in 63 illnesses and 17 hospitalizations from 24 states. Upon investigation, the flour tested positive for two strains of Shiga toxin-producing *Escherichia coli* (STEC O121 and STEC O26) (CDC, 2016b). Another multistate outbreak of flour contaminated with STEC O26 occurred in 2019 with a reported 21 cases across nine states resulting in three hospitalizations (CDC, 2019c). Consumers are not aware that flour is a raw product and needs to be cooked before consumption due to a lack of a control step during the milling of grains (FDA, 2017). To eliminate foodborne outbreaks and recalls associated with wheat flour, researchers have investigated multiple sterilization techniques for grains ranging from gamma irradiation (Azzeh and Amr, 2009; Köksel et al., 1998) to high-pressure processing (Bárcenas et al., 2010; McCann et al., 2013). To date, these

studies have yet to present a continuous processing method that can decontaminate grains efficiently and retain functional properties.

In a previous study, our research group demonstrated that a 3.18 log-reduction CFU/g of *Cronobacter sakazakii* could be achieved in nonfat dry milk (NFDM) in one pass (28 s) of intense pulsed light (IPL) (Chen et al., 2018). A further study investigated multiple IPL passes and extended treatment times, which resulted in up to a 5 log-reduction of *C. sakazakii* and a 2.7 log-reduction of *Enterococcus faecium* on NFDM, wheat flour, and egg white powder (Chen et al., 2019a). Although both studies demonstrated effective decontamination of powdered foods, research is needed to determine the effect of IPL on grains using a continuous system. IPL, a non-thermal treatment method, makes use of a high-intensity radiation source that uses a flashlamp to produce short-duration pulses of broad-spectrum light (typically between 100-1,100 nm) to inactivate microorganisms from the surface of foods. Aron et al. used IPL to evaluate decontamination of grains and were able to achieve up to a 4 log-reduction of native molds on wheat in a stationary sample when wheat grains were placed in bags, and 40 pulsed light flashes were applied to both sides of the bag (Aron et al., 2014). In this current study, evaluation of IPL treatment to grains in a continuous IPL process was evaluated using a vibratory feeder to tumble the product and ensure uniform IPL exposure. The first objective of this study is to investigate the effects of a continuous IPL system on the microbial decontamination of grains, whether native or artificially inoculated. Also, the effects of IPL on the functional properties of hard red wheat were determined. Findings from this study provide new insight into the use of IPL technology for surface decontamination of wheat grains.

4.2. Materials and methods

4.2.1. Bacterial Strains

The bacterial strains utilized in this study are *Cronobacter sakazakii* strain ATCC 29544, *Enterococcus faecium* strain NRRL B-2354, and *Bacillus cereus* strain ATCC 14579. *E. faecium* is a well-studied, nonpathogenic surrogate used in place of *Salmonella* spp. for thermal process validation (Bianchini et al., 2014; Enache et al., 2015; Kopit et al., 2014; Xu et al., 2018). *C. sakazakii*, *E. faecium*, and *B. cereus* were revived from frozen culture (-80 °C) onto tryptic soy agar (TSA; Neogen, Lansing, MI) supplemented with 0.6% (wt/vol) yeast extract (TSAYE; Sigma-Aldrich, St. Louis, MO), and stored at 4 °C ± 2 °C.

4.2.2. Hard Red Wheat

Ardent Mills (Hastings, MN) supplied the hard-red wheat (harvested 2018), and grains were assessed for naturally occurring *Cronobacter sakazakii*, mesophilic spore formers, and mesophilic bacteria. HiCrome^{TM} *Cronobacter* spp. agar (Sigma-Aldrich) was used to detect *Cronobacter* following 48 h of incubation at 37 °C. TSAYE was used to detect native mesophilic bacteria following 48 h incubation at 37 °C. Mesophilic spore formers were detected following heat shock and plating onto skim milk agar (SMA; Sigma-Aldrich).

4.2.3. Inoculum Preparation

Preparation of microbial inoculums were conducted by transfer of a single isolated bacterial colony from TSAYE plates into 9 ml tryptic soy broth supplemented

with 0.6% (wt/vol) yeast extract (TSBYE; Neogen, Lansing, MI) on a rotary shaker set to 200 rpm. Cultures were incubated to stationary growth phase for 24 h at 37 °C for *C. sakazakii* and *E. faecium* and 24 h at 30 °C for *B. cereus.* One ml of *C. sakazakii* and *E. faecium* grown cultures were used to inoculate 150 mm x 15 mm Petri plates containing TSAYE, spread uniformly using sterile spreaders (Fisher Scientific, Waltham, VA), and incubated for 24 h at 37°C. One ml from *B. cereus* cultures were used to inoculate four 150 x 15 mm Petri plates containing AK agar No. 2 (sporulation agar) (HiMedia Laboratories, LLC, Kennett Square, PA) and incubated at 30 °C for 7-10 days to ensure sporulation. Sporulation was assessed using the Wirtz-Conklin method for the rapid detection of bacterial spores (Hamouda et al., 2002).

Bacterial lawns from *C. sakazakii* and *E. faecium*, as well as spores from *B. cereus,* were harvested by first flooding the plates with 25 ml of 0.1% (wt/vol) peptone (*C. sakazakii* and *E. faecium*) or 25 ml DI water (*B. cereus* spores) then dislodged from the surface of the plate with a Corning cell lifter (Corning Life Sciences, Corning, NY) and filtered through a 40 µm cell strainer (Corning Life Sciences, Corning, NY) to collect any remaining agar. Filtered suspensions of *C. sakazakii* and *E. faecium* were centrifuged at 5000 x *g* for 10 minutes, supernatant poured off, and resuspended in 4 ml 0.1% peptone. Filtered spore suspensions were centrifuged at 10000 x *g* for 15 minutes, supernatant poured off, and washed three more times with DI water. After the final wash, spores were resuspended in 10 ml 50% ethanol and stored at 4 °C overnight to lyse the remaining vegetative bacteria, washed three more times with DI water, and resuspended in 4 ml DI water (Coroller et al., 2001).

4.2.4. Sample Inoculation

In a biological safety cabinet, 100 g of hard red wheat was placed in stomacher bags (Seward Laboratory Systems Inc., Bohemia, NY). Four ml of each inoculum were pipetted into the stomacher bag containing hard red wheat, mixed by hand/shaken for 1 minute, and air-dried overnight in a biological safety cabinet. Once dry, samples were shaken once more for 1 minute and transferred to PPCO Nalgene bottles (ThermoFisher Scientific, Waltham, MA) until needed.

4.2.5. IPL Treatment

The continuous IPL apparatus utilized in this study consists of an X-1100 steripulse- XL system (Xenon Corporation, Woburn, MA) with a 76-cm linear xenon flash lamp, X-1100 power/control module, a Model-105 volumetric feed (Tecnetics Industries, 168 Inc., St. Paul, MN), two 6-inch 390 CFM inline duct mixed flow fans, sealed model-66C vibratory feeder (Eriez Manufacturing Co., Erie, PA), ultrasonic humidifier/dehumidifier, nitrogen gas tank, and an infrared heater. Our IPL lamp has an output spectrum between 190-1100 nm. Inoculated grains were added to an environment-controlled volumetric feeder with paddles where the grains were heated to 55 °C. Once starting sample temperature was attained, the IPL lamp, vibratory feeder, and auger were turned on in that order. Grains were then fed under the IPL lamp and exposed for 30s and 120s until they were finally collected at the end in a sanitized container. Parameters for this study were as follows, pulse rate of 1 Hz, 342 µs pulse duration, 3000 V, and a feed rate of 1800 g/h. Sample temperature was monitored and never exceeded ~58 °C for each test. The same parameters were used for the exposure of spores minus the feed rate.

4.2.6. Enumeration

To enumerate, 5 g of hard red wheat was transferred to 750 ml (24 oz) Whirl-Pak® filtered blender bags (Nasco, Fort Atkinson, WI) with 45 ml of 0.1% (wt/vol) peptone and homogenized in a stomacher for 3 minutes at 260 RPM (Seward Laboratory Systems Inc., Bohemia, NY). One ml of stomached samples was transferred to 9 ml of 0.1% peptone, serially diluted, and plated in duplicate onto TSAYE for *C. sakazakii*, *E. faecium*, and uninoculated grains (native mesophilic bacteria). Plates were incubated at 37°C for 48-72 hours to allow for injured cells to resuscitate. *B. cereus* samples were heat-activated in a water bath set to 80 °C for 10 minutes and allowed to cool before serially diluting, plating onto SMA, and incubated at 30 °C for 24-48 hours. 3M Petrifilm Yeast and Mold plates (3M, Maplewood, MN) were used to assess native yeasts and molds and were used following product instructions with incubation at room temperature for 3-5 days. CFU/g concentrations were determined by averaging the results from each duplicate.

4.2.7. Functional Properties of Hard Red Wheat

In order to investigate the influence of intense pulsed light on wheat grain properties, samples were treated for 120 s (3000 V, 342 μs, IPL frequency of 1 Hz, and feed rate of 1800 g/h) and analyzed by following standard methods. Falling number (on a 14% moisture basis) for characterizing starch damage was evaluated following AACC 56-81.04 (AACC, 1999b). Moisture was calculated using the force draft oven method (130°C for 2 h) following AOCS Ba 2a-38 (AOCS, 2017), and gluten content was determined by the Hand Washing Method AACC 38-10 (AACC, 1999a).

4.2.8. Statistical Analysis

Tests were conducted in triplicate (n=3) with microbial data converted to log CFU per gram, averaged, and error bars representing standard deviation were generated for each microorganism using SigmaPlot v14.0 (Systat Software, San Jose, CA). Functional properties were only generated for the maximum treatment time of 120s. Results were averaged, standard deviations generated, and Student's t-test (p <0.05) was performed using Excel (Microsoft, Redmond, WA).

4.3. Results and Discussion

4.3.1 Influence of Grain Shape

Grain poses a unique challenge for IPL treatment due to its overall geometry and surface structure. Not only does the geometry and structure of wheat grains have the potential to undermine the effectiveness of IPL, but the ventral furrow and loose pieces of bran contribute to lower log-reduction values. Microorganisms in the ventral furrow or covered by the uneven surface and loose bran lead to a shadowing effect limiting the effectiveness of IPL (Butscher et al., 2016). Hwang *et al*. observed this loss of efficacy while trying to sterilize sesame seeds with IPL (Hwang et al., 2017). Other studies have also reported that protein or fat composition of food can absorb UV emitted from IPL and therefore impair the effectiveness of microbial decontamination (Gómez-López et al., 2007; Nicorescu et al., 2013).

When compared with previous findings in wheat flour (Chen et al., 2019a), there is a stark contrast between the log-reductions achieved in whole grain versus milled flour. Significantly higher microbial inactivation could be achieved (~5 logs CFU/g) with the

help of a vibratory feeder. However, in grains, there still exists the ventral furrow and loose bran, which prevent microorganisms from IPL exposure even with the use of a vibratory feeder. Furthermore, the uneven shape of grains makes it a challenge to tumble and expose to IPL uniformly. As a result, some of the microbes remain viable.

4.3.2 Effect of IPL on Artificially Inoculated Bacteria

Cronobacter sakazakii, *Enterococcus faecium*, and *Bacillus cereus* spores artificially inoculated on grain and exposed to IPL demonstrated variable effectiveness for decontamination. *C. sakazakii*, a Gram-negative bacterium, after 120 seconds, a ~2.7 (± 0.28) log-reduction (average energy density is ~0.285 J/pulse/cm^2) was observed. *E faecium*, which is Gram-positive, was less susceptible to IPL and with a reduction of ~1.5 (± 0.04) log CFU/g viable cells. Finally, the spores from *B. cereus* showed the most resistance to IPL treatment with <1 (0.09) log inactivation observed (Figure 4.1). These findings agree with other studies, determining that Gram-negative bacteria are more susceptible to IPL than Gram-positive bacteria (Anderson et al., 2000; Farrell et al., 2010; Rowan et al., 1999; Williams et al., 2007). Spores are known to be even more resistant to UV (especially 254 nm) than their vegetative counterparts (Setlow, 2001), and results showed that they were more resistant to IPL than the vegetative cells of *C. sakazakii or E. faecium*.

Gram-positive bacteria possess a thicker cell wall (20-80 nm) and increased amounts of peptidoglycan in comparison to the cell wall in Gram-negative bacteria (1.5-10 nm). This difference in cell wall thickness has been hypothesized to impede the penetration of UV radiation to the cytoplasmic contents (Williams et al., 2007). Besides

cell wall thickness, other differences in the cell envelope, such as an outer membrane, between Gram-negative and Gram-positive bacteria are thought to attribute to differences in responses to antibiotics, heat, and UV radiation (Mai-Prochnow et al., 2016).

Bacterial spores are estimated to be between 5-50 times more resistant than their growing cells. Whereas vegetative bacterial cells are susceptible to 254 nm UV radiation by the formation of thymine dimers and DNA damage, these detrimental effects are observed to a lesser extent in bacterial spores (Setlow, 2001; Slieman and Nicholson, 2000). Spores also contain small, acid-soluble spore proteins (SASP) and of interest to UV resistance are α/β-type SASP, and they impart resistances to not only UV radiation, but also heat, peroxides, and other extrinsic treatments deemed sporicidal (Moeller et al., 2009). With their increased resistance to UV radiation. Consequently, of the artificially inoculated foodborne pathogens exposed to IPL tested in this study, *C. sakazakii* was the most susceptible, followed by *E. faecium*, and *B. cereus* spores were not as susceptible to deactivation. While the type of cell damage to Gram-negative and Gram-positive cells due to IPL exposure are well known, there is a paucity of information on the effect of IPL to spore anatomy.

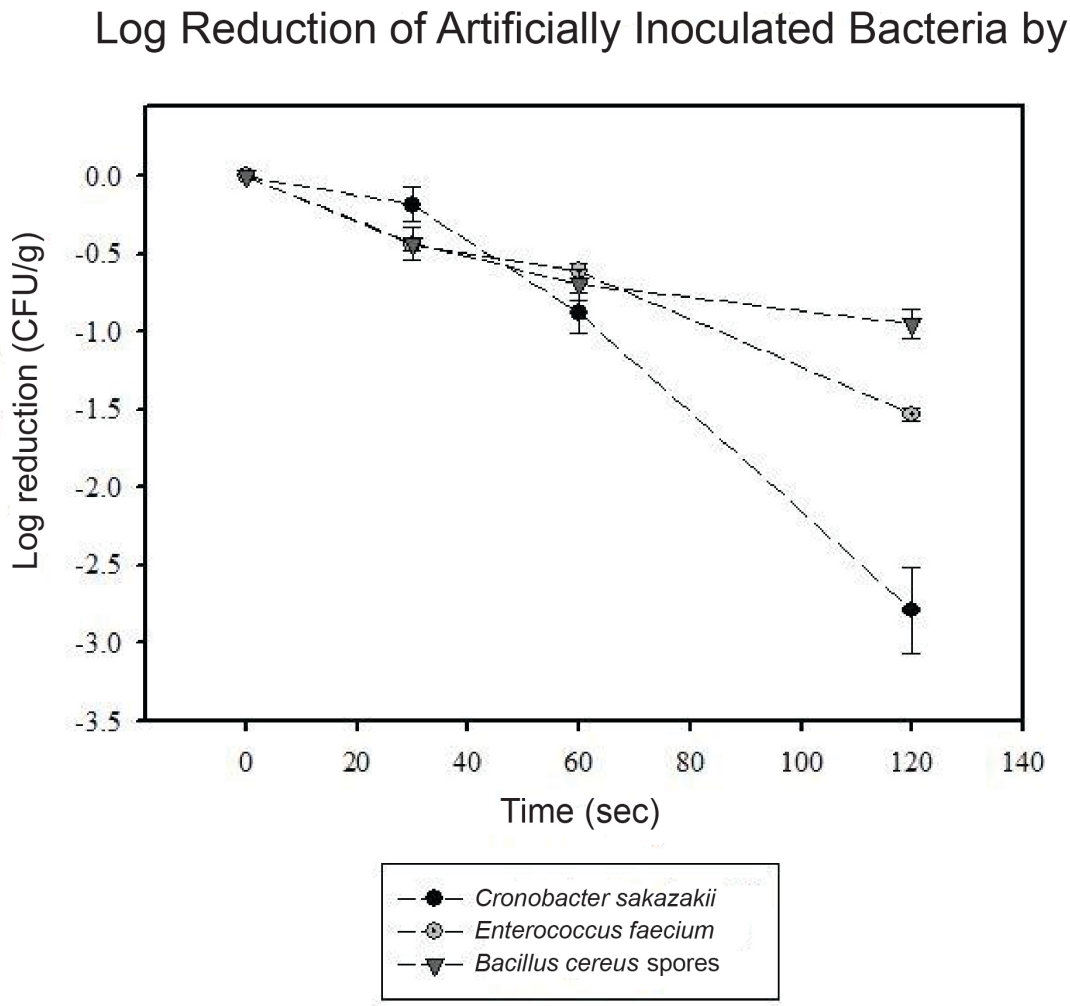

Figure 4.1. Log reduction of bacteria and bacterial spores inoculated onto hard red wheat by intense pulsed light. Error bars represent standard deviation (n=3).

4.3.3 Effect of IPL on Native Microorganisms

Native microorganisms present on grains may be uniquely adapted to handle UV exposure due to the amount of time they spend in the open air in the environment. Natively, the sun emits UV-radiation, and even though most of the peak germicidal portion (250-260 nm) will have been filtered by the upper and middle atmosphere (UVC and some of UVB), microorganisms are still exposed to UV radiation between 290 and 400 nm (Farmer et al., 1996). One hour of exposure to the sun's UV radiation leads to a microorganism exposure of a combined dose of 200 kJ $m^{-2,}$ which results in the formation

of damaging photoproducts. Without a way to repair their damaged DNA, microorganisms would surely perish with prolonged exposure (Bintsis et al., 2000). Therefore, yeasts, molds, and native mesophilic bacteria exhibited the ability to survive extended periods of IPL exposure in comparison to artificially inoculated microorganisms (Figure 4.2).

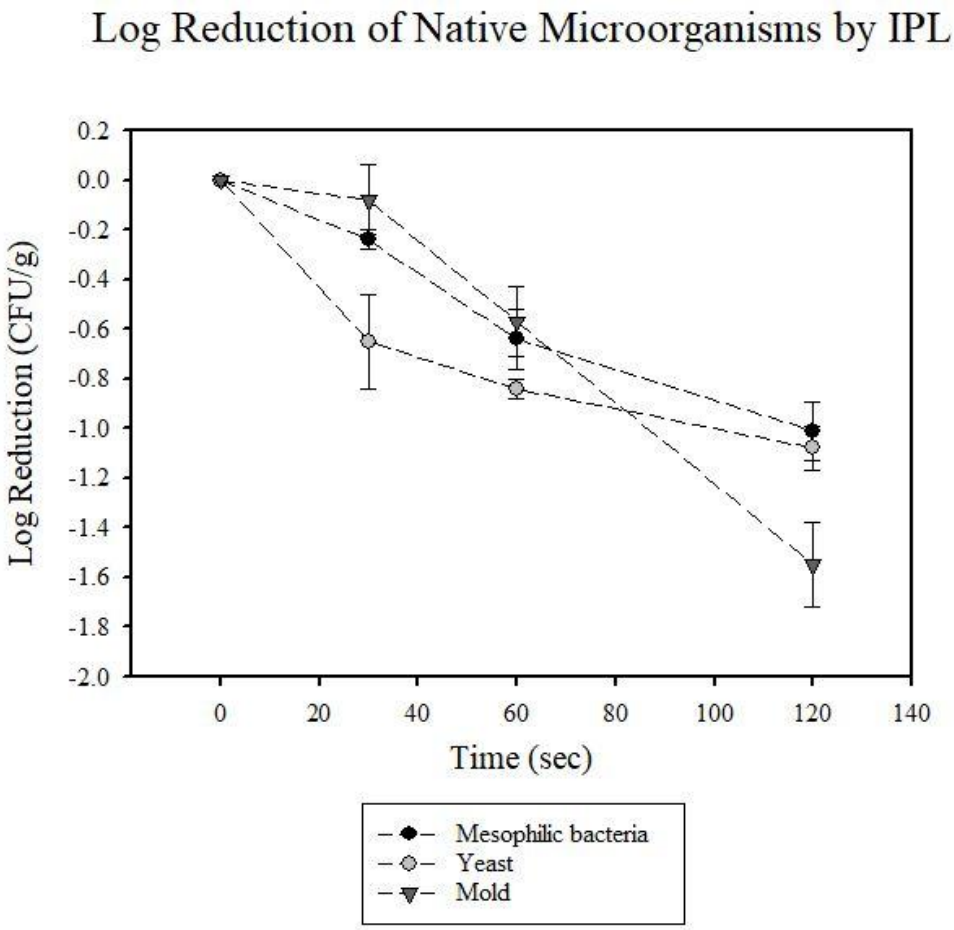

Figure 4.2. Log reduction of native microorganisms present on hard red wheat by intense pulsed light. Error bars represent standard deviation (n=3).

After 120 seconds of IPL exposure, both yeasts and mesophilic bacteria were reduced by a very similar amount (~1 log CFU/g). Yeast was reduced by 1.08 (± 0.09) CFU/g, and mesophilic bacteria were reduced by 1.01 (± 0.12) CFU/g (Figure 4.2).

Molds were initially more resistant to IPL in the first 30 seconds but saw a sharp decline after 60 seconds and 120 seconds (Figure 4.2), eventually resulting in a 1.55 (± 0.17) log-reduction. UV radiation has been shown to generate reactive oxygen species (ROS) through the photooxidation of oxygen (Song et al., 2016). ROS are detrimental to microorganisms unless they can eliminate their effect, and one of the ways they can achieve this is through carotenoids (if they contain them). Carotenoids act as protectants and have proven to protect against UVB in fungi, including *Fusarium* and *Penicillium* (Gmoser et al., 2017). Production of carotenoids has also been demonstrated to protect against photodynamic damage in bacteria as well, with protection against high fluences of UV between 320 to 400 nm. In a study by (Tuveson et al., 1988), genes that controlled carotenoid synthesis in *Erwinia herbicola* were cloned into *Escherichia coli*. Once expressed, significant protection was observed even after the addition of photosensitizing molecules.

4.3.4 Impact of IPL on Functional Properties

In order to test whether the use of IPL impacts the overall functional properties of grains, the IPL treatment of wheat samples occurred at the maximum exposure time of 120 seconds (3000 V, 342 μs, 1 Hz, and feed rate of 1800 g/h). Table 4.1 shows the effects of IPL exposure on the falling number, moisture content, and gluten content. Hagberg falling number is an indirect measure of the amount of α-amylase in the grain and is used to indicate starch damage. A low falling number correlates to a large amount of α-amylase present that breaks down the starch and results in a watery slurry, whereas a high falling number indicates very little enzyme activity and will result in a viscous,

gelled slurry (Number & For, 2013). After 120 seconds of IPL exposure, there was a significant (p = 0.005) increase in the falling number (454.33 s → 487.67), resulting in a decreased α-amylase activity. Even with temperature and humidity controlled in the IPL apparatus, moisture content decreased slightly (11.47% → 11.39%). Gluten content also remained unchanged (23.07% → 23.1%); however, this was to be expected as the bactericidal effect of IPL is known to have a low penetration depth (Bhavya and Umesh Hebbar, 2017; Cheigh et al., 2013). IPL is an effective control strategy for the surface of foods, while most of the functional components of the wheat grain are part of the endosperm, and little change occurs internally in wheat grains due to IPL exposure (Butscher et al., 2016).

Table 4.1. Physical properties of hard red wheat grains before and after IPL treatment

	Untreated HRW	120s IPL treated HRW
Falling number (s)	454.33 (6.11) a	487.67 (6.66) b
Moisture (%)	11.47 (0.04) a	11.39 (0.05) a
Gluten content (%)	23.07 (0.46) a	23.1 (0.71) a

Numbers and percentages reported as the mean (standard deviation). Within rows, values that share a common letter are considered not to be significantly different (α = 0.05).

4.4. Conclusion

In this study, IPL was used to evaluate the decontamination of grains, harboring both native microorganisms and artificially inoculated pathogens. We assessed a continuous IPL apparatus in the reduction of microorganisms on artificially inoculated grains (Gram-negative, Gram-positive, and spores). 120s of IPL exposure reduced *C.*

sakazakii by 2.7 log CFU/g; however, *E. faecium* and *B. cereus* spores were more resistant to IPL, achieving only a ~1.5 and <1 log reduction respectively. Microorganisms natively present on grains such as yeasts, molds, and mesophilic bacteria also were reduced by IPL treatment. Molds were reduced by 1.5 log CFU/g with yeasts and mesophilic bacteria experiencing around a 1 log reduction.

Our study demonstrates that the IPL apparatus can achieve at least a 1.5 log reduction on all microorganisms examined while the temperature of the wheat never exceeded ~58 °C. Future studies are warranted to examine if further decontamination is achievable with increased IPL exposure time beyond 120s, as the functional property results suggest that hard red wheat can endure prolonged IPL exposure. Overall, this study demonstrates that non-thermal IPL treatment of grains can reduce levels of microorganisms by >1.5 logs and that longer exposure time and refinement of the IPL apparatus, or the use of multiple control technologies in conjuncture with IPL may lead to higher levels of decontamination of hard red wheat.

Chapter Five
OVERALL SUMMARY, CONCLUSIONS, AND FUTURE DIRECTIONS

The goal of this dissertation was to develop a continuous intense pulsed light (IPL) system for the nonthermal decontamination of low-moisture foods (LMFs). In order to test the efficacy of IPL on the reduction of microorganisms, three inoculation protocols were evaluated following two criteria, homogeneity and survivability of the inoculated organisms. Once an inoculation method was determined, multiple treatment parameters were investigated, such as fluence, distance from Xenon lamp, feed rate, frequency, and residence time. Various food matrices were investigated with varying success, and factors such as fat content, protein content, water activity, and particle size impacted log reductions. These findings will start to bridge the gap between academia and the food industry to meet consumer's needs for safe, minimally processed foods.

Based on these findings, the following conclusions can be drawn:

- Inoculation methodologies directly impact the desiccation tolerance of microorganisms. Determining the log reduction following equilibration ensures a repeatable inoculation preparation. Moving to a “lawn-grown” method to inoculate LMFs resulted in only a ~0.2-log reduction for *Enterococcus faecium* and ~0.37-log reduction for *Salmonella enterica* following a 6-day equilibration period.
- As demonstrated by the addition of a vibratory feeder, tumbling a product to ensure uniform exposure to IPL achieved a significantly higher log reduction. A 3.18-log reduction (CFU/g) when using a vibratory feeder at 28 seconds compared to a 0.84-log reduction when stationary.

- The starting water activity (a_w) of NFDM directly impacted the efficacy of IPL on the reduction of *Cronobacter sakazakii*. With an increase in a_w comes undesirable agglomeration leading to increased particle size (from 68 μm to 103.45 μm), and this has the potential to shield bacteria on the inside of the particle from IPL exposure. As the a_w increased from 0.25 to 0.35, there was a dramatic decrease in log reductions over 28 seconds of IPL exposure, from ~3-log reduction (0.25 a_w) to a ~1-log reduction (0.35 a_w).
- There appears to be a synergistic effect between IPL treatment and mild heating of the product. An additional log reduction in 28 seconds was observed when the final product temperature went from 52.1 °C to 57 °C, from 2.08 to 3.18-log reduction.
- Understanding the food matrix is important to the efficacy of IPL because it is not a one size fits all technology. Certain factors should be taken into consideration when evaluating log reductions. These factors include hygroscopicity, nutrient composition (high protein and lipids), the ability of particles to scatter/reflect light, cracks/crevices, and how well the particle tumbles.
- Results demonstrate that IPL can be used for the rapid decontamination of LMFs with minimal impacts on organoleptic properties when compared to continuous UV and thermal treatment.

Opportunities for future research were identified as part of the research conducted in this dissertation:

- Build a larger scale model of our IPL system to be implemented onto a production line and understand the impacts of larger throughput on the efficacy of IPL. Flour mills and powdered milk production facilities process tons of products a day, and

implementing IPL onto those lines will need more improvements. These improvements could be a mirrored finish on the vibratory feeder to aid in uniform exposure, multiple lights in a row to extend treatment time, and to make the vibratory feeder out of a transparent material to pulse light from the bottom.

- Explore the combination of technologies to overcome some of the shortcomings of IPL. As part of this project, there was the start of the use of titanium dioxide (TiO_2) to increase the efficacy of IPL without extending treatment times. Preliminary results showed promise, and in just 60 seconds, the addition of TiO_2 reduced bacteria and spores by an additional 1-1.5-log.
- Continue to explore other types of food matrices and partner with industry to see what the demand is for various products. Certain companies value the idea of minimal processing and the ability to retain their organic certifications, especially the companies that handle high-value commodities such as spices and herbs.

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