Influence of Water Activity on Thermal Resistance of *Salmonella enterica* and Quality Changes in Low-moisture Foods

By

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ABSTRACT

Low-moisture foods (with $a_w < 0.85$) including pet foods and black pepper powder have been associated with major recalls due to contamination by *Salmonella spp*. Increased thermal resistance and prolonged survival at low-moisture conditions are major challenges to achieve effective inactivation of *Salmonella* in low-moisture foods. At low water activity (a_w) conditions, elevated temperatures or increased treatment times are generally required for achieving a required level of inactivation of *Salmonella*. The objective of this study was to evaluate the effect of a_w on the thermal resistance of *Salmonella enterica* in pet food pellets and black pepper powder quantitatively, and the effect of thermal treatments on selected quality parameters of these food products.

Non-contaminated pet food pellets were inoculated with two pure strains of heat resistant *S. enterica* and black pepper powder was inoculated with an additional three more strains of *Salmonella*. Both inoculated food samples were equilibrated at 0.33, 0.54, and 0.75 a_w in controlled humidity chambers. Inoculated pet food pellets and black pepper powder in closed aluminum cells were thermally treated at specific temperatures for selected times. The observed log reductions in *Salmonella* population after thermal treatments were used to analyze the inactivation rate in both samples. The results showed that the inactivation was well fitted with the Weibull model. At a specific temperature, the rate of inactivation increased with the increase in the a_w from 0.33 to 0.75, and the 5-log reduction times decreased for *Salmonella* in both food samples with higher a_w. Water adsorption isotherms of pet food pellets and black pepper powder at initial and treatment temperatures were developed to understand the change in a_w during thermal treatments. The similar TBARS values for control and treated samples for different a_w samples presented no significant change in lipid oxidation after the thermal treatments. But the piperine level in 0.75 a_w sample was significantly lower than any treated samples with 0.33 and 0.54 a_w, except for 0.54 a_w samples treated at 80ºC.

The quantitative analysis of thermal reduction of *Salmonella* with respect to a_w and quality analysis of heat-treated samples will help to select the appropriate initial a_w to develop effective thermal treatment protocols for adequate reduction of *Salmonella* in pet foods and black pepper powder with minimal changes in quality parameters.

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CHAPTER I
INTRODUCTION

1.1. Summary of related studies and problem statements

Food safety is a matter of global concern. Recalls and outbreaks due to contamination of low-moisture food (LMF) products by various foodborne pathogens like *Salmonella enterica, Escherichia coli, Cronobacter spp.* and other pathogens have affected manufacturers and consumers worldwide (Beuchat et al., 2013).

1.1.1. *Salmonella enterica* as foodborne pathogens

S. enterica is a foodborne pathogen responsible for many cases of gastrointestinal human infections worldwide (Lamas et al., 2018). *S. enterica* is a gram-negative, facultative anaerobic rod-shaped bacterium from the Enterobacteriaceae family (Graziani et al., 2017). Non-typhoidal *S. enterica* is the main foodborne diarrheal agent, causing 230,000 deaths in 2010 (WHO, 2015). Due to non-typhoidal *Salmonella*, more than 88,000 people suffer from foodborne illnesses each year in Canada (De Cesare, 2018). It was estimated that *Salmonella* causes approximately 1.2 million illnesses and 450 deaths every year in the United States (CDC, 2018). Among many sub-species of non-typhoidal *S. enterica*, human salmonellosis is caused by *S. enterica subsp. enterica* which consists of more than 2600 serotypes and are the main cause of foodborne illnesses and death globally (De Cesare, 2018; Graziani et al., 2017; Santos, 2015). *S. enterica* is identified as one of the major species of bacteria linked to outbreaks from a wide variety of food products, ranging from fresh produce to dry and processed food products and poultry-based food products (Jarvis et al., 2016; Riggio, Wang, Kniel, & Gibson, 2019).

1.1.2. Low-moisture foods

Low-moisture foods (LMFs) are either already low in moisture when they are harvested or they lose water content through drying or dehydration when they are processed from high-moisture food products (WHO, 2014). For LMFs, different researchers have considered different range of a_w in food such as 0-0.60 (Hu, 2016; Lang et al., 2017), 0-0.85 (Beuchat et al., 2013; Jarvis et al., 2016; WHO, 2014), and a_w<0.70 (Santillana Farakos & Frank, 2014). The LMFs include cereals and grains, spices and dried aromatic herbs (including teas), nuts and nut products, seeds for consumption, confections and snacks, honey, dried fruits and vegetables, dried protein products, animal feeds such as fishmeal and pet foods (Beuchat et al., 2013; Tadapaneni, Syamaladevi, Villa-Rojas, & Tang, 2017; WHO, 2014). As pathogenic bacteria grow at a_w above 0.85, LMFs do not support the growth of foodborne pathogens such as *Salmonella* (Jarvis et al., 2016). The LMFs have longer shelf life attributed by low a_w and were even considered to be microbiologically safe with insufficient validation (Jarvis et al., 2016).

1.1.3. Outbreaks and recalls due to *Salmonella* in food products

Outbreaks and product recall due to the occurrence of *Salmonella* in LMFs have drawn worldwide attention (Tadapaneni et al., 2017). From 2007 to 2012, there were 119 recalls involving LMFs such as pet food, powdered infant formula, peanut butter, spices, dry nuts, seeds, etc in the United States (CDC, 2012; Santillana Farakos, Frank, & Schaffner, 2013). Similarly, from 2007 to 2012, 22 cases of worldwide outbreaks due to the occurrence of *Salmonella* in LMFs was reported, resulting in 2293 foodborne illnesses and nine deaths (CDC, 2012; Santillana Farakos et al., 2013). In Canada, in 2017 and 2018, there were recalls of wide varieties of LMFs such as chili powder, cheese crackers, whole green cardamom seeds, various brand sesame seeds,

breading mix, sweet basil seed, potato chips of different brands, desiccated coconut, chocolate pretzels, three-ring pretzels, and cake mix due to *Salmonella* (PHAC, 2018). According to CDC (2018), *Salmonella* outbreaks were linked to different foods, for instance, *S.* Typhimurium in dried coconut, *S.* Senftenberg in pistachios, and *S.* Infantis in dry dog foods. S. Typhimurium is the major strain associated with numerous foodborne outbreaks and recalls including LMFs (Lang et al., 2017; Song & Kang, 2016). Hence, researchers are using this strain as a model foodborne pathogen in their studies (Gruzdev, Pinto, & Sela, 2012). Few outbreaks due to *S*. Senftenberg has been reported in past and it has been isolated from LMFs such as peanut butter, desiccated coconut, and infant food and cereal (Beuchat et al., 2013; Song & Kang, 2016). According to Public Health Agency Canada (PHAC) investigation, as of October 19, 2018, there have been 45 laboratory-confirmed cases of *Salmonella* illness investigated in five provinces (British Columbia, Alberta, Saskatchewan, Manitoba, and Quebec) with no case of death (PHAC, 2018). Also, *S*. Typhimurium and *S*. Senftenberg have been isolated from low-moisture pet food products (Lambertini et al., 2016).

1.1.4. Survival and persistence of *Salmonella* in LMFs

The increasing number of outbreaks of foodborne pathogens and recalls of LMFs in recent years indicate that though foodborne pathogens cannot grow in LMFs, they can survive in low-moisture environment for long period of time and can continue their metabolic activities and growth once they get exposed to favorable growth conditions (Santillana Farakos et al., 2013; Smith, Hildebrandt, Casulli, Dolan, & Marks, 2016; WHO, 2014). Even the presence of a very small number of low infectious dose pathogens such as *S. enterica* in food products can cause foodborne illness to the consumers (Beuchat et al., 2013; WHO, 2014). In low-moisture condition,

S. enterica may change into physiologically dormant state, also known as viable but non-culturable state, with decrease in genome transcription (<5% genome gets transcribed instead of 78%), degradation of ribosomal RNA molecules for nutrients, filamentation, biofilm formation, expression of two outer membrane porins (OmpF and OmpC), increase in osmoprotectants, and an increase in ratio of trans- and cis- unsaturated fatty acids in cell membrane to prevent the loss of water (Burgess et al., 2016; Deng, Zengxin, & Zhang, 2012; Finn, Condell, McClure, Amézquita, & Fanning, 2013). *S*. Typhimurium, *S*. Senftenberg, and other *Salmonella* strains were reported to respond differently to different stress conditions (Alvarez-Ordonez, Fernandez, Bernardo, & Lopez, 2009). Various studies suggested that *S*. Senftenberg were resistant to different stresses such as acidity, heating, and desiccation (Alvarez-Ordonez, et al., 2009; Andino & Hanning, 2015; Pedersen, Olsen, & Bisgaard, 2008). *S.* Typhimurium is more resistant to dry heat and *S*. Senftenberg is more resistant to moist heat (Lang et al., 2017; Podolak, Enache, Black, Stone, & Elliot, 2010). Previous studies reported that the exposure of *Salmonella* to one stress can increase the virulence potential and the cross-protection towards other stresses (Begley & Hill, 2015; Finn et al., 2013; Gruzdev, Pinto, & Sela, 2011). Dehydration rate affects the survival of *Salmonella* during drying (Gruzdev et al., 2012). When bacteria get exposed to stress conditions, they synthesize enzymes and compatible solutes to protect the cell from damage and shortening of the dehydration time affects the metabolic activity to adapt to drying (Gruzdev et al., 2012).

1.1.5. Negative impacts of *Salmonella* contamination in LMFs

LMFs cover wide varieties of food products with a very long shelf life at room temperature (Beuchat, et al., 2013; Santillana Farakos et al., 2013). They are used as ingredients in several ready-to-eat food products. Survival and persistence of foodborne pathogens like *Salmonella* in

LMFs and low-moisture food processing environments impose a great risk on consumer health due to outbreaks and recalls (Beuchat et al., 2013). Such outbreaks and recalls have negative impacts not only on consumer health but also on the finance and credibility of the manufacturing companies along with the finance of the country. Outbreaks will increase the cost of treatment, depending upon the number of consumers being affected, cause the loss in labor due to illnesses of workers. Outbreaks and recalls can cause potential legal trouble to the manufacturer, loss of market share, cost of production and negative impact in public opinion about the company. Hence, this issue has drawn the attention of food scientist, food manufacturing companies and food safety regulating bodies, globally (WHO, 2014).

1.1.6. Guidelines to control the microbiological hazard in LMFs

In response to various serious negative impacts of *Salmonella* contamination in LMFs, several regulatory bodies around the world have proposed recommendations and guidelines for food industries on how to prevent and manage potential risks of LMFs from microbiological hazards (Beuchat et al., 2011; Enache et al., 2015). Various studies suggested that the contamination of food products by pathogens is facilitated by various factors such as insufficient hygiene, cross-contamination, contaminated equipment, and contamination by personnel, where cross-contamination was found to play the major role (Beuchat et al., 2011; Podolak et al., 2010). The Codex Alimentarius Commission decided on developing a Codex Code of Hygienic Practice for LMFs (Beuchat et al., 2011; WHO, 2014). The critical control measures for LMF involve preventing contamination during pre-harvest, post-harvest, and processing through implementation of Good Agricultural Practices (GAPs), Good Manufacturing Practices (GMPs), Good Hygiene Practices (GHPs) and Hazard Analysis Critical Control Point (HACCP) programs

(Beuchat et al., 2013; WHO, 2014). But sometimes it is impossible to prevent the contamination in primary ingredients and during processing of food products at each step. Hence, there is a need for additional decontamination methods.

1.1.7. Treatment methods for foodborne pathogens in LMFs

There are different methods of treatment to inactivate the foodborne pathogens in LMFs. They can be categorized as thermal and non-thermal methods (Villa-Rojas et al., 2013). Thermal treatment involves the application of high heat for a definite time (Lang et al., 2017). It can be done by using hot oil, hot water/liquid, high-temperature forced air, super-heated steam (vapor heat), infrared pasteurization etc. (Atungulu & Pan, 2012; Villa-Rojas et al., 2013; Berk, 2013). In non-thermal treatments, food products are not exposed to high temperatures. Some non-thermal treatments are irradiation, ozone processing, fumigation by ethylene oxide or propylene oxide etc. (Molnar et al., 2018).

1.1.7.1. Thermal treatments

For thermal treatments, the extent of microbial inactivation depends upon the amount of heat or temperature, time of exposure and resistance of microorganisms to the high heat (Berk, 2013). Thermal treatments can destroy pathogenic microorganisms but also bring changes in the flavor, texture and other organoleptic quality of food products (Berk, 2013; George, Razali, Santhirasegaram, & Somasundram, 2015). In some foods, it is undesirable and is known as thermal damage (Berk, 2013).

1.1.7.2. Thermal treatment of *Salmonella* in LMFs

Thermal decontamination method is the conventional and most widely accepted process in food industries as there is no risk of the presence of chemical residue and is relatively easy to perform and considered as the most effective and reliable treatment method (Villa-Rojas et al., 2013). Liquid or high a_w food products contaminated with foodborne pathogens such as *Salmonella* can be decontaminated effectively by heating, subjecting the food products to specific time-temperature combinations (Lang et al., 2017). During thermal decontamination process, drying can happen in an open system, which leads to a further decrease in a_w. Previous reports suggest that the thermal resistance of foodborne pathogens such as *S. enterica*, *E. coli*, *Clostridium perfringes*, or *Cronobacter sakazaki* was significantly higher at low a_w compared to higher a_w (Beuchat et al., 2011; Lang et al., 2017; Smith et al., 2016). Exposure of *Salmonella* to the low a_w environment may lead to the development of pathogen tolerance to various other stresses such as resistance to sanitizers, UV irradiation, dry heat and bile salts (Chen et al., 2013; Finn et al., 2013; Gruzdev et al., 2011). Previous works reported that the exposure of the pathogens like *Salmonella* to low a_w environment leads to the development of resistance to thermal stress (Begley & Hill, 2015; Finn et al., 2013; Lang et al., 2017).

Several serovars of *S. enterica* have developed thermal resistance and it differs between strains which might be due to specific variations in gene composition (presence and absence of specific genetic loci) for each respective strain (Begley & Hill, 2015; Dawoud et al., 2017). According to the study by Ng et al. (1965), *S.* Senftenberg 775W was found to be most heat resistant than other strains of *Salmonella* in an aqueous solution while *S.* Typhimurium was found to be more heat resistant than *S.* Senftenberg in chocolate (Podolak et al., 2010). Inactivation trend was the same for both strains in peanut butter (Song & Kang, 2016). Some study suggested the *S.* Enteritidis PT30, the major strain responsible in outbreaks from raw almonds, was found to be

more resistant to dry heat than other strains of *Salmonella* evaluated (Podolak et al., 2010). The stages of growth, the age of the culture, and the condition of the bacterial growth are also responsible for different results in thermal treatments (Chung, Birla, & Tang, 2008; Dawoud et al., 2017). Stationary phase microbial cells are more resistant to heat than log phase cells (Dawoud et al., 2017). Similarly, pathogens exposed to a temperature slightly higher than the optimal growth temperature, grown on limited carbon sources, surviving in low a_w condition, and limited nutrients before thermal treatment have shown more thermal resistance (Chung et al., 2008; Dawoud et al., 2017). During heating also, some factors affect the thermal resistance of *Salmonella*, such as acidity, fat content, and the addition of solutes to the matrix (Chung et al., 2008; Podolak et al., 2010). Along with the a_w of the food products, the thermal resistance of *Salmonella* is also affected by other intrinsic and extrinsic properties of the foods which makes it important to do evaluation and comparison of the results from different research studies, considering similar food types, same bacterial strain, and method employed for the thermal inactivation of pathogens, etc. (Podolak et al., 2010). *Salmonella* has become the major problem in low-moisture food manufacturing industries due to its high prevalence and survival in low-moisture and other stress conditions, which makes it useful to be used as a model pathogen for thermal resistance studies in the case of LMF products (Finn et al., 2013).

1.1.7.3. Determination of thermal resistance of microorganisms

Despite the development of different new methods of decontamination for LMFs, thermal treatment has been proven by far the most effective and reliable way to decontaminate food to achieve safe food products (Atungulu & Pan, 2012). Consumers accept this method more easily than any other new microbial decontamination techniques (Atungulu & Pan., 2012). Understanding the amount of heat reaching every part of the food product in the treatment system

is important while developing a new thermal treatment process for the related product (Chung et al., 2008). The knowledge on thermal resistance of foodborne pathogens and the data on thermal kinetics for representative microorganisms obtained from the microbial inactivation kinetic study could help to develop new or validate traditional thermal processes (Kou et al., 2016). It can be done by calculating the most appropriate process parameters like temperature and time for inactivation of the target pathogens from the study (Chung et al., 2008).

Different types of equipment have been used by various researchers for thermal death kinetic tests in their studies depending on the type of food product used (Kou, et al., 2016). These include miniature thermal death time (TDT) cells, three-neck flask, sealed plastic pouches, and/or capillary tubes and their selection depends upon the product type to be tested, whether it is low acid, acidified, thick puree, solid or liquid (Chung et al., 2008; Kou et al., 2016). It is important to choose the test method appropriately for correct determination of a bacteria's thermal resistance.

Chung et al. (2008), developed an aluminum test cell and evaluated the performance for determining the thermal resistance of bacterial spores in foods. The result suggested that the developed test cell can be used as reliable experimental equipment for studying the inactivation of bacterial spores in semi-solid and solid food at high temperature (Chung et al., 2008). This test cell has a cavity with a holding capacity of 1 ml and allows easy loading and unloading of samples (Chung et al., 2008; Villa-Rojas et al., 2013). The design of the test cells allows hermetical sealing and rapid heating of dry samples in a water bath, providing close to ideal isothermal condition (Villa-Rojas et al., 2013). Many other inactivation studies were performed for different strains of bacteria and types of foods using the thermal death time (TDT) test cells. However, studies on *Salmonella* thermal inactivation kinetics on pet food pellets and black pepper powder are very limited.

1.1.8. Modeling of microbial inactivation in LMFs

Salmonella is taken as the target organism for environmental monitoring for LMFs as a suitable indicator for *Salmonella* has not been found (GMA, 2009). The outcome of the thermal process for LMFs can be predicted through the modeling of the inactivation of *Salmonella* in respective LMFs. The modeling helps to determine the critical parameters required for the thermal process to achieve the desired reduction levels in LMFs (FDA, 2018). There are different types of modeling to describe the inactivation curves and the thermal inactivation data are tested for the best fit in those models.

1.1.8.1. The log-linear model

Primary models (the first order kinetic or the log-linear model and the Weibull model) are used to fit the *Salmonella* inactivation at selected temperatures and a_w values in different types of foods. Microbial inactivation is assumed to follow the first order kinetics (Peleg, 2006).

The first order kinetics is based on the theory that, when exposed to an isothermal lethal temperature, there is an exponential decrease in the number of affected bacteria or bacterial spores (Peleg, 2006). It can also be stated as the rate of inactivation of bacteria when exposed to a constant temperature is proportional to the number of viable bacteria or bacterial spores (Peleg, 2006).

The log-linear model equation (Peleg, 2006) is written as:

$$\log\left(\frac{N}{N_0}\right) = -\frac{t}{D} \qquad (1)$$

Where N and N_0 are the populations (CFU/g) of *Salmonella* at time t and 0, respectively, t is the time of isothermal treatment. Thermal death time (D) is the time in minutes required to reduce bacteria by 90% or one log cycle at a given temperature (Villa-Rojas et al., 2013).

The relationship between $\log_{10}(N/N_0)$ and t in equation (1) must be a straight line and hence it is also referred to as a log-linear model (Peleg, 2006). For the log-linear model to be the best fit for inactivation data, the temperature should be ideally constant, and all targeted bacteria should be inactivated at the exact same time (Peleg, 2006). The first order kinetics assumes that the probability of inactivation of the target microorganism is independent of time, which means the exposure time has no effect on the probability of inactivation of the microorganisms and all microorganisms possess the same level of thermal resistance (Bevilacqua, Speranza, Sinigaglia, & Corbo, 2015; Peleg, 2006; van Boekel, 2002). This equation can be used to describe the inactivation kinetics of simple systems like laboratory media or for a single strain population (Bevilacqua et al., 2015). This cannot fulfill the purpose for best fitting of inactivation data from complex environments and for population showing a significant deviation from the linearity (van Boekel, 2002).

1.1.8.2. The Weibull model

The data that doesn't fit the log-linear model due to non-linear trend can be tested with the modified Weibull model (Peleg, 2006) which can be expressed in an equation as:

$$\log_e\left(\frac{N}{N_0}\right) = -\left(\frac{t}{\alpha}\right)^{\beta} \qquad (2)$$

Here, α is a scale parameter which gives the overall steepness of the survival curve (Villa-Rojas et al., 2013). β is the dimensionless or shape parameter determining the shape of the curve. $\beta > 1$ reflects convexity of the survivor curve or increases inactivation rate and $\beta < 1$ reflects concavity of the curve or decreasing inactivation rate with time (Bevilacqua et al., 2015; Peleg, 2006). The curve is log-linear when the $\beta = 1$ which means each cell is equally susceptible to heat regardless of treatment time (Peleg, 2006; van Boekel, 2002). Hence, the Weibull model can be

used for fitting the inactivation data with both linear and non-linear case (Peleg, 2006).

1.1.8.3. The 5-log reduction time

It is recommended to target 2 to 5-log reductions of *Salmonella* in LMF products based on a hazard analysis that consist of previous association of ingredients with *Salmonella*, the type of food product, prevalence and extent of contamination (the initial load of pathogens), and the purpose of the final product (GMA, 2009). The selected log reduction should include a margin of safety, that can be additional 2-log reductions beyond the extent or levels of contamination expected to occur in the ingredients (FDA, 2018; GMA, 2009). The time required for the specific number of log reductions can be obtained experimentally or by predicting it by the Weibull model using the equation:

$$t_d = \alpha \left(- \log_e \left(10^{-d} \right)^{\frac{1}{\beta}} \right) \qquad (3)$$

Where, d is the number of log reductions (van Boekel, 2002). For predicting 5-log reduction, d can be replaced by value 5 in equation (3) and calculate the 5-log reduction time.

1.1.9. Water activity

Water activity (a_w) of food is equivalent to the relative humidity of the air in equilibrium with the food sample, where they neither gain or lose moisture (Labuza, 1975). The a_w is the measure of the amount of free water which is not bound by solutes or macromolecules such as proteins and carbohydrates and is a measure of the energy status of the water in a system (Chen & Rogers, 2018; Labuza, 1975). The concept of a_w is of particularly important in determining product quality and safety (Labuza & Altunakar, 2007). It is a better indicator of food stability than water content and predicts the safety and stability of foods with respect to microbial growth, chemical

and biochemical reaction rates, and physical properties (Labuza & Altunakar, 2007; Syamaladevi et al., 2016b). The a_w measurably ranges from 0 (dry) to 1 (pure water). The a_w of a food system can be measured by equilibrating the liquid phase water in the sample with the vapor phase in the headspace and measuring the relative humidity of the headspace (Fontana, 2007).

1.1.10. Water sorption isotherms

With a decrease in a_w of a food system, the thermal resistance of *Salmonella* increases and an increase in a_w can make the *Salmonella* more sensitive to heat (Santillana Farakos et al., 2013; Tadapaneni et al., 2017; Villa-Rojas et al., 2013). The thermal resistance of *Salmonella* has been reported based on the a_w of food products measured at room temperature (Syamaladevi et al., 2016a). In a closed system, the a_w of food products changes with a change in temperature and the change depends on the chemical composition, physicochemical state, and physical structure of food product at a fixed water content (Syamaladevi et al., 2016b). Hence, it is necessary to determine the a_w of foods at the treatment temperatures and analyze the difference in the effectiveness of the thermal treatment based on the changes in a_w of food products during the treatment (Tadapaneni, Xu, Yang, & Tang, 2018). But it is also important to understand the change in the intrinsic properties of LMFs such as a_w due to rise in temperature during thermal treatments so that proper protocol can be developed for effective thermal processing to inactivate *Salmonella* (Syamaladevi et al., 2016b). However, it is difficult to directly measure the a_w of the LMFs during thermal treatment (Tadapaneni et al., 2017).

For modeling the thermal inactivation of *Salmonella* at different temperature and a_w, it is necessary to equilibrate the sample to specific a_w and treat the sample at high temperature (Villa-Rojas et al., 2013). At a specific temperature, once the sample reaches equilibrium, the

corresponding water activities and water contents are recorded and the relationship between water content and a_w or water sorption isotherm is developed (Labuza, 1975). This water sorption isotherm can help in studying the behavior and the structure of water inside the foods and at the surface as well (Saleh, Karim, Hensel, & Sturm, 2018). The nature of water sorption isotherms depends on the chemical composition and physicochemical state of foods constituents (Labuza & Altunakar, 2007).

The water sorption isotherm of different food products can be measured by the chilled-mirror dew-point technique (Fontana, 2007). A vapor Sorption Analyzer (VSA) (METER group Inc, Pullman, WA) can be used to generate sorption isotherms of food products. The VSA can generate dynamic isotherms using the Dynamic Dew Point Isotherm (DDI) method and static or equilibrium isotherms using Dynamic Vapor Sorption (DVS) method (VSA, 2018). Using the DVS method, sample weight change can be tracked as the sample gets exposed to different equilibrium RH to determine the water contents to generate water sorption isotherms (Mermelstein, 2009).

In this technique, the food sample is equilibrated with the air in the headspace of a sealed chamber with an attached mirror, optical sensor and a small fan (Fontana, 2007). During the measurement of a_w, the system cools the chamber forming dew (Fontana, 2007). When the water vapor equilibrium is attained in the headspace of the chamber, the dew point temperature and the relative humidity of the headspace are measured which can be expressed as a_w of the sample (Labuza & Altunakar, 2007).

The water sorption isotherm is different for different type of food product and temperature and provides information about product quality and safety (Labuza & Altunakar, 2007). It helps to evaluate the stability of a product at different water activity values after drying since the dried

product is exposed to different humidity and temperature fluctuations during storage and distribution (Saleh et al., 2018). At high a_w conditions, the quality of LMFs can change and may be more susceptible to quality deterioration, which includes the microbial spoilage, resulting in shelf life reduction (Labuza & Altunakar, 2007). Hence, the data obtained from water sorption isotherm will be helpful in determining the end point of drying process to a pre-defined moisture level that helps to keep product safe during storage conditions and increase the shelf life (Labuza, 1975; Saleh et al., 2018).

Most of the a_w measuring equipment available in the market cannot measure the a_w of foods above 60°C (Syamaladevi et al., 2016a). Syamaladevi et al. (2016a) in collaboration with Decagon Devices, developed a sealed thermal cell containing a commercial relative humidity sensor (HX15-W, Omega Engineering Inc.) to measure a_w of food sample above 60 °C. It determines the a_w of the food sample by measuring the relative humidity of headspace above the food sample after thermodynamic equilibrium is achieved (Syamaladevi et al., 2016b). At a fixed water content, the change in a_w with temperature is observed and water sorption isotherm is developed from the obtained data.

Water content is the amount of water present in the product (Berk, 2013). There are many methods to determine water content, but the two most commonly adopted methods of water content determination are 1). Loss on drying, 2). Karl Fischer titration method (Mermelstein, 2009). Loss on drying is very simple and generally followed method in which water content is determined gravimetrically by drying in a hot air oven until a constant final weight of the sample is achieved (Mermelstein, 2009).

Many mathematical models have been proposed to predict the water sorption isotherms, but complex nature of different types of foods makes it difficult for different models to represent

all the sorption isotherms of all the food products (Labuza & Altunakar, 2007; Saleh et al., 2018). Brunauer-Emmett-Teller (BET), Guggenheim, Anderson, and de Boer (GAB), Oswin, Halsey, Henderson, Langmuir, Curie, Peleg, Chung & Pfost are some of the common mathematical model used in many studies (Saleh et al., 2018). Among all these models, the GAB equation based on the monolayer and multilayer adsorption is widely accepted as an appropriate model to explain the moisture sorption isotherms of food products (Sormoli & Langrish, 2015; Staudt, Tessaro, Marczak, Soares, & Cardozo, 2013).

The GAB equation is expressed as:

$$\frac{X}{X_m} = \frac{CKa_w}{(1- Ka_w)(1- Ka_w+CKa_w)} \quad (4)$$

where X is the dry basis water content of the material, X_m is the monolayer water content (dry basis). The parameter C is a measure of the strength of binding water to the primary binding sites of the food, and the parameter K is a correction factor (Quirijns, van Boxtel, van Loon, & van Straten, 2005). X_m is the monolayer water content which is regarded as a constant. The parameters C, K, and X_m are thermodynamic in nature and the temperature dependence of these parameters can be expressed as:

$$C = C_0 \exp\left(\frac{\Delta H_c}{RT}\right) \quad (5)$$

$$K = K_0 \exp\left(\frac{\Delta H_K}{RT}\right) \quad (6)$$

$$X_m = X_{mo} \exp\left(\frac{\Delta H_K}{RT}\right) \quad (7)$$

where ΔH_C is the difference in enthalpy between monolayer and multilayer sorption. ΔH_K is the difference between the heat of condensation of water and the heat of sorption of a multi-molecular layer (Quirijns et al., 2005).

Sorption isotherms of food at selected temperatures can be predicted by incorporating the temperature dependence of the parameters X_m, C and K to the GAB model, where temperature (T) is expressed in Kelvin (K).

1.1.11. Selected LMF products related to previous recalls and outbreaks

1.1.11.1. Pet food products

Pet food industries have been growing rapidly in North America (Koppel, Gibson, Alavi, & Aldrich, 2014). There are three main types of commonly used pet foods: dry and semi-dried (low-moisture) shelf-stable extruded food; thermally processed low acid canned food; and other different forms known as treats (Carrion & Thompson, 2014). Low-moisture pet foods are popular because of ease in feeding and storage. The potential food safety hazard present in pet food ingredients are the same as human foods because both the food products share similar ingredients (Carrion & Thompson, 2014). Animal-based pet food products may contain rendered fish, egg, chicken, duck, goat, bison protein meal, animals and vegetable fats along with some vegetables and grain flour (Olatunde & Atungulu, 2018). The pet food processing involves the mixing of these different ingredients, steam heating and extrusion (Lambertini et al., 2016). The extruded mixture is cut into kibble, dried, coated with fat and flavorings, cooled to room temperature, and packaged (Lambertini et al., 2016). The microbiological safety, quality, and longer shelf life of food kibbles or pellets are ensured by maintaining a low a_w in the final products, that is approximately 0.5 a_w after extrusion of pellets at a temperature as high as 92°C (Lambertini et al., 2016). Presence of

Salmonella in primary ingredients and improper thermal processing steps might lead to the survival of *Salmonella* in the final product (Lambertini et al., 2016). Contamination of coating fat and flavoring can be another reason because they are added after extrusion and drying after which pet food pellets do not undergo any decontamination process (Lambertini et al., 2016). Pet food pellets contaminated with *Salmonella* can cause salmonellosis not only to animals but for humans also due to improper handling of foods during feeding their pets or through fomites or from ingestion of pet foods by children (Carrion & Thompson, 2014; Lambertini, et al., 2016). While some pets might be resistant to the clinical effects of infections by human food pathogens, they can still play a role as a carrier of foodborne pathogens (Carrion & Thompson, 2014).

The 45 serotypes of *Salmonella* were isolated from dry pet foods and treats in the U.S. survey in 2002-2009 (Li et al., 2012). Different serotypes of *S. enterica* including Senftenberg, Montevideo, Newport, Enteritidis, and Typhimurium have been isolated from dry pet food and treats (Lambertini et al., 2016). Although pet food processing may be carried out at relatively high temperatures (~92°C), lethal to *Salmonella*, post-process cross contamination may still take place (Lambertini et al., 2016). Hence, additional intervention processes such as thermal treatments may be necessary to eliminate the incidence of *Salmonella* in low-moisture pet food products. *Salmonella* cells can exhibit increased persistence at low a_w and high temperatures, and the presence of fat in the food matrix may provide an additional protective effect (Santillana Farakos et al., 2013). Low a_w conditions can enhance the ability of the surviving cells to withstand thermal treatments and other antimicrobial interventions (Lambertini et al., 2014). The pet food industries grew rapidly since 2007, and cases of recalls are also increasing every year (Olatunde & Atungulu, 2018). However, studies conducted on survival and inactivation of *Salmonella* in low-moisture pet foods are not available.

The quality analysis in pet foods

The components primarily present in pet foods are carbohydrates, protein, fats, fiber, vitamins, minerals, and water (Olatunde & Atungulu, 2018). Fat is a source of energy, taste, and flavor in pet food pellets and they are obtained from animal fats, and fish, plant and seed oil (Lin, Hsieh, & Huff, 1998; Olatunde & Atungulu, 2018). The unsaturated fats from canola oil, cashew nuts, soybean, sunflower, or corn oil used in pet foods might lead to fat rancidity, thereby degrading their quality (Angelo, Versellotti, Jacks, & Lengendre, 1996; Olatunde & Atungulu, 2018). When unsaturated fat gets exposed to oxygen, it forms peroxides (oxidative rancidity) which decomposes into a complex mixture of aldehydes, ketones, and other volatile products that generates an offensive odor (Labuza & Dugan, 1971). The fat rancidity can be accelerated by fluctuation or an increase in temperature, which accelerates the oxidative reaction in food samples (Lin, et al., 1998).

Most of the pet food products are meat-based and lipids are their important constituents. While lipids have an important role in food palatability and nutrition, their oxidation adversely affects the sensory and nutritional quality of pet food products (Barden & Decker, 2016; Gavahian, Chu, Khaneghah, Barba, & Misra, 2018). Heat reduces the oxidative stability of meat-based foods (Ma, Ledward, Zamri, Frazier, & Zhou, 2007) because lipid oxidation occurs when unsaturated fats in such food products react with oxygen. Lipid oxidation results in rancidity, giving undesirable color and flavor, possibly producing toxic compounds, and affecting the shelf life of pet food products (Chanadang, Koppel, & Aldrich, 2016).

Determination of lipid oxidation

Several methods are in use for measuring lipid oxidation in pet food industries such as peroxide value (PV), thiobarbituric acid reactive substances (TBARS), oxygen consumption and

gas chromatography-mass spectrometry (GC-MS) for the quantitative analysis of volatile compounds such as aldehydes and ketones (Angelo et al., 1996; Chanadang et al., 2016)

TBARS value: In TBARS assay, thiobarbituric acid reacts with thiobarbituric acid reactive substances (TBARS) which are produced as secondary products from lipid peroxidation in pet food (Mahmoodani, Perera, Abernethy, Fedrizzi, & Chen, 2018). Malonaldehyde is the final product which is measured by TBARS Assay. The amount of malonaldehyde is measured by fluorescence spectroscopy at an excitation wavelength of 515 nm, and an emission wavelength of 553 nm (Mahmoodani et al., 2018), or by spectrophotometer at 532 nm wavelength (Gomes, da Silva, do Nascimento, & Fukuma, 2003). The standard curve is prepared by acid hydrolysis of 1,1,3,3-tetramethoxypropane (TMP), an MDA precursor (Angulo et al., 1996; Gomes et al., 2003). The concentration of MDA in samples are obtained through calculation comparing the absorbance of the sample with the absorbance of the standard using the standard curve (Angulo et al., 1996; Mahmoodani et al., 2018).

1.1.11.2. Black pepper powder

Black pepper, also known as *Piper nigrum*, is one of the major cosmopolitan spices which is used in wide varieties of food products as seasoning due to its appealing flavor (Ban et al., 2018). Black pepper powder is commonly used in ready-to-eat food products which do not need further decontamination or cooking and might impose a risk to human health (Ban et al., 2018). From 1980 to 2000, 95% of U.S. food recalls related to spices is due to *Salmonella* contamination (Doren et al., 2013). The report on the multistate outbreak of *S.* Montevideo in the U.S. in 2009 suggested that the consumption of salami products seasoned with contaminated black and red pepper powder was the main reason for the outbreak (CDC, 2010). There were 272 cases of salmonellosis reported in 44 states and Washington DC of the USA in 2010 (CDC, 2010). Some serotypes of *S. enterica*

which were related to the spice-associated outbreaks of salmonellosis are Typhimurium, Senftenberg, Enteritidis, Montevideo, Weltevreden, Wandsworth, Oranienburg and few others (Doren et al., 2013). The contamination of black pepper and spices can occur at various steps starting from harvesting to processing and packaging (Wei et al., 2018). It is difficult to eliminate all the sources of contamination in spices, but improvement in methods for detection and decontamination, and validation of inactivation methods can help to improve the product safety (Bowman, Waterman, Williams, & Ponder, 2015).

There are many methods of decontamination for spices such as ethylene oxide fumigation, irradiation, and steam treatment (Kim, Sagong, Choi, Ryu, & Kang, 2012). Among these, steam treatment is widely used (Atungulu & Pan, 2012). The conventional heating methods of decontamination involve the transfer of heat from the exterior of the food product to the interior by conduction or convection (Kim et al., 2012). Detailed analysis of thermal decontamination of spice powders and validation would be useful in developing a suitable protocol for industrial application of these methods.

Quality of black pepper

The black pepper comprises of ash, fiber, starch, protein contents, distillable oil, solvent extractable oleoresin with the principal pungent substance, piperine (Ravindran & Kallupurackal, 2012). The quality of black pepper is mainly determined by two main components: volatile oil that is responsible for the aroma and flavor, oleoresins and piperine content that contributes the pungency (Ravindran & Kallupurackal, 2012). The chemical structure of piperine is known as piperoylpiperidine and the chemical formula is $C_{17}H_{19}NO_3$ (Gorgani, Mohammadi, Najafpour, & Nikzad, 2017). Piperine is considered one of the principal pungent compounds in black pepper which constitutes around 98% of the total alkaloids in the pepper (Gorgani et al., 2017). Herbs and

spices are heat sensitive products and application of heat can alter the color, flavor, and volatile components of the black pepper (Hertwig et al., 2015; Kim et al., 2012). Thus, after the thermal treatment, quality analysis of the treated sample should be conducted.

Piperine extraction and quantification

Piperine is pungent and non-volatile compound found in the oleoresin of the black pepper (Narayanan, Sree Kumar, & Sankarikutty, 2000). It constitutes around 6% to 13% of black pepper and can be extracted from ground black pepper by volatile organic solvents such as ethanol, acetone, ether, dichloroethane, or ethyl acetate and those solvents are removed by evaporation under reduced pressure (Gorgani et al., 2017; Narayanan, Sree Kumar, & Sankarikutty, 2000). Traditional solvent extraction methods such as soaking, maceration, and Soxhlet extraction are common practices, but they require long extraction time and, in some cases, high temperature to accelerate the process which can cause thermal degradation of heat-sensitive bioactive compounds (Azwanida, 2015). A conventional method of extraction might require a higher volume of solvent to extract piperine from a large amount of the black pepper and the solvents used are not selective towards piperine which might extract some amount of resins and gums as well (Gorgani et al., 2017). The amount of piperine in extracts from black pepper powder subjected to treatment condition is determined and compared.

Different methods are in use for the piperine assay and some of the major techniques include Kjeldahl nitrogen determination, spectrophotometric analysis, and high-performance liquid chromatography (Rathnawathie & Buckle, 1983). The Kjeldahl and UV-spectrophotometric methods are mostly used in different research work for piperine assay (Rathnawathie & Buckle, 1983). HPLC method is also popular as it is a rapid, sensitive and specific for quantitative determination of piperine (Rathnawathie & Buckle, 1983).

In the UV spectrophotometric method, the piperine assay is done by measuring the absorption maxima of the extracted piperine solution at 342-345 nm (Ravindran & Kallupurackal, 2012). The piperine solution to be estimated should not be exposed to direct light because piperine is highly sensitive to light (Ravindran & Kallupurackal, 2012). Irradiation of piperine in alcoholic solution produces a mixture of isopiperine and isochavicine (Ravindran & Kallupurackal, 2012). The piperine content is calculated with reference to the calibration curve obtained from the absorbance of the standard piperine solution at a definite range of concentration (Gupta & Jain, 2011; Rathnawathie & Buckle, 1983).

After compiling all the obtained data from the experiment, proper thermal treatment protocols can be developed, considering the inactivation of *Salmonella* at selected temperatures and the change in the a_w of the food product with rise in temperature that brings about change in the heat sensitivity of *Salmonella* (Syamaladevi et al., 2016b; Tadapaneni et al., 2018). Evaluating the change in quality parameters helps to determine the effects of treating pet food pellets and black pepper powder samples at specific time-temperature combinations and it helps in choosing appropriate thermal treatment with minimal damage to the quality.

1.2. Hypothesis and Objectives

Hypothesis:

The thermal resistance of *Salmonella* and quality changes in low-moisture food products are dependent on their initial a_w and thermal treatment temperature and the understanding of their functional relationship will provide necessary knowledge for selecting optimized time-temperature combinations to produce safe low-moisture food products.

General Objective:

The general objective of this study is to develop improved microbial inactivation models with maximum quality retention in selected low-moisture food products.

Specific Objectives:

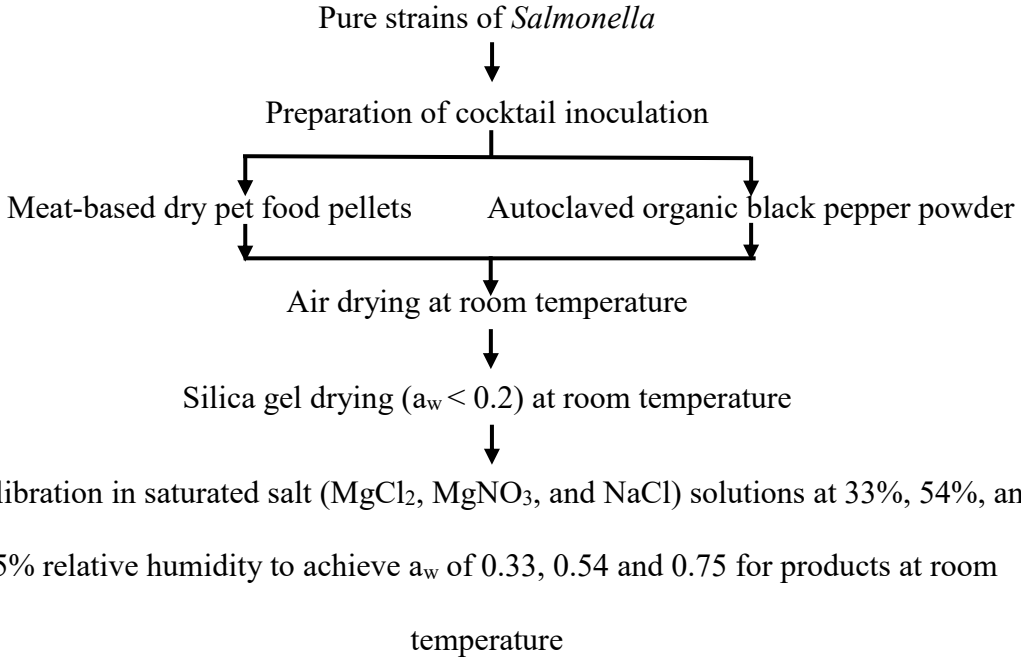
1. Determination of inactivation kinetics of *Salmonella* in pet food pellets and black pepper powder equilibrated to different a_w and treated at specific temperatures in a closed system.
2. The quantitative analysis of lipid oxidation in pet food pellets and piperine content in black pepper powder after the heat treatment.

CHAPTER II

MATERIALS AND METHODS

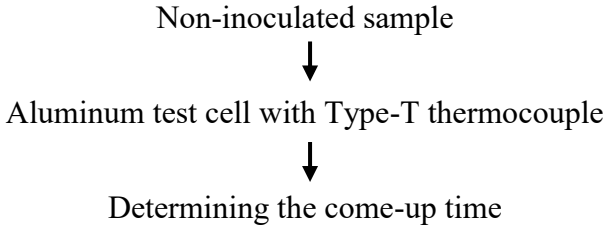
The laboratory analysis of the research work was conducted at the University of Alberta. The experimental plan for the thesis is as follows (Fig. 1).

1. *Sample preparation*



2. *Thermal treatment and modeling*

Come-up time:



Thermal treatment and statistical analysis:

Inoculated and equilibrated food sample

↓

Heat treatment in hot water bath (60, 65, 70, 75, 80, and 85ºC) in aluminum test cells

↓

Homogenization at 260 rpm for 3 min in a Stomacher

↓

Serial dilution in sterile 0.1% peptone water

↓

Plating on TSA agar plate and incubation at 37ºC for 24 h

↓

Enumeration

↓

Fitting the inactivation data in the Weibull model.

↓

Statistical analysis by SAS

3. Water adsorption isotherm

Storing the pet food pellets in desiccating (Silica gel) chamber for 1 week for drying

↓

Equilibration of dried pet food pellets and black pepper powder at 11.3%, 23%, 33%, 43%, 54%, 60%, and 75% relative humidity conditions using super-saturated salt (LiCl, CH_3COOK, $MgCl_2$, K_2CO_3, $NaNO_2$, NaBr, & NaCl) solutions in airtight containers for two weeks.

↓

Obtaining adsorption isotherm curve for equilibrated samples at 20, 23.5, 60 and 81°C.

4. Analyzing change in quality parameters

Pet food pellets: Lipid oxidation

Equilibration of non-inoculated pet food pellets at 33%, 54%, and 75% relative humidity conditions

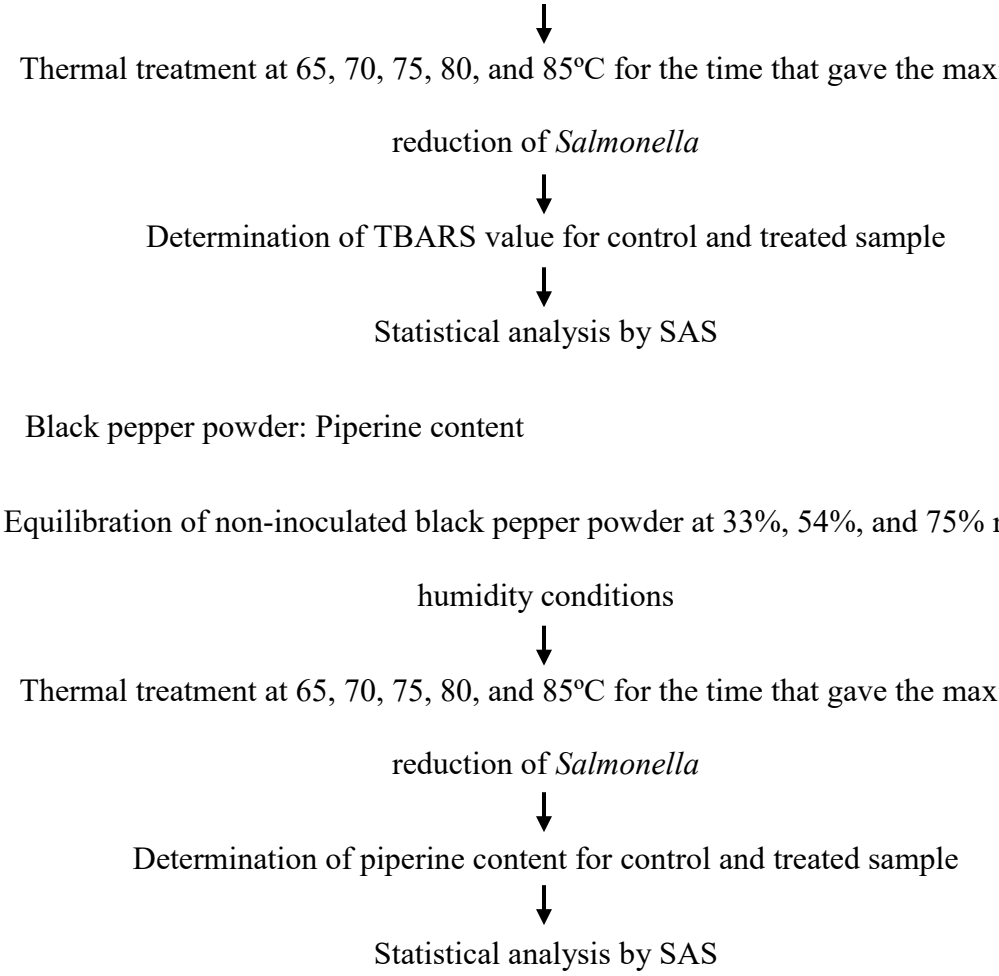


Fig. 1. Schematic diagram of the experimental plan.

All steps including inoculum preparation, inoculation of *Salmonella* on samples, air drying, equilibration, and plating of inoculated samples before and after thermal treatment were conducted at the room temperature inside a biosafety cabinet (Thermo Scientific, 1300 Series A_2, Fisher Scientific Company, Ottawa, ON).

2.1. Sample preparation

For the study, two different types of food products were selected: meat-based pet food pellets and black pepper powder. Small breed dog food pellets and organic whole black peppercorn

were bought from local superstore in Edmonton, AB. The a_w values of samples were measured with a water activity meter (Model 3TE, Decagon Devices, Pullman, WA) and same water activity meter was used to measure a_w of the sample in successive steps. All microbial experiments were carried out in the biosafety cabinet. Initially, black peppercorn was ground and stomached in stomacher bag (Fisherbrand™ Lab Blender Bag, Fisher Scientific Company, Ottawa, ON) using stomacher (Seward™ Stomacher™ Model 400C circulator Lab Blender, Fisher Scientific Company, Ottawa, ON) at 260 rpm for 3 min. The homogenized suspension was serially diluted in sterile 0.1% peptone water (Fisher Bioreagents, Fisher Scientific Company, Ottawa, ON) and the obtained dilutions were plated on tryptic soy agar (BD Difco Tryptic Soy Agar (Soybean-Casein Digest Agar), Becton, Dickinson and Company, Mississauga, ON) plates with 0.6% yeast extract (Fisher Bioreagents, Fisher Scientific Company, Ottawa, ON) (TSAYE) and incubated at 37ºC (Fisherbrand™ Isotemp™ Microbiological Incubator, Fisher Scientific Company, Ottawa, ON) for 24 h. Different types of colonies like blisters covering the agar plate were observed. Hence, the whole black peppercorn was autoclaved (Beta-Star, Life Science Equipment) at 121°C for 30 min to get rid of any background microflora. Approximately, 25 g of the autoclaved black peppercorn was ground in a coffee grinder (Krups coffee grinder) for 45 seconds, with pausing and shaking samples few times in between. The absence of *Salmonella* on the pet food pellets and black pepper powder was confirmed by homogenizing 0.3 g of samples in 100 ml of sterile 0.1% peptone water in a stomacher bag and plating on tryptic soy agar (BD Difco) plates with 0.6% yeast extract (TSAYE) and xylose lysine deoxycholate (XLD) (Thermo Scientific™ Oxoid™ XLD Agar-Dehydrated, Fisher Scientific Company, Ottawa, ON) agar plates, and incubating for 24 h at 37°C. The XLD media was used to confirm the presence of *Salmonella* in the inoculum and inoculated sample as it is the selective growth medium used in isolation of *Salmonella* spp.

2.2. Bacterial strain and inoculum preparation

The cocktail of two *Salmonella* strains (*S. enterica* subspecies *enterica* serovar Typhimurium (ATCC 13311) and *S. enterica* subspecies *enterica* serovar Senftenberg (ATCC 43845) were kindly provided by Dr. Michael Gänzle (Department of Agricultural, Food, and Nutritional Science, University of Alberta). *S.* Typhimurium has been linked to the outbreaks in pet foods and found to be highly heat resistant in a dry state (Beuchat et al., 2013). *S.* Senftenberg was reported to be more resistant to moist heat and desiccation (Andino & Hanning, 2015; Goepfret & Biggie, 1968; Pedersen et. al., 2008). As both strains, *S.* Typhimurium and *S.* Senftenberg, are more resistant to stresses than other serovars, these strains were taken as model microorganisms for thermal inactivation study. Other *Salmonella* strains isolated from the water treatment plant, named as FUA 1934, FUA 1946, and FUA 1955, were also used for the inactivation modeling in black pepper powder besides the other two major strains. The study was conducted involving these strains with variation in stress responses because in a real scenario, recalls and outbreaks from LMF products has been caused by different strains of *Salmonella* with varying adaptation ability in the different stress conditions (CDC, 2012).

Lawn-based liquid inoculum (LLI) of *Salmonella* was used in this study as LLI was reported to be more stable and repeatable (Hildebrandt et al., 2016). After obtaining pure culture isolates of two strains of *Salmonella* on TSAYE, they were sub-cultured in tryptic soy broth (BD Difco Tryptic Soy Broth (Soybean-Casein Digest Medium), Becton, Dickinson and Company, Mississauga, ON), with 0.6% yeast extract (TSBYE). Stock culture was prepared by mixing 500 µl of 24 h tryptic soy broth culture and 500 µl of sterile 70% glycerol (Fisher Bioreagents, Fisher Scientific Company, Ottawa, ON) and stored at -80°C freezer. *Salmonella* was recovered from the stock culture by streaking on TSAYE plates and incubating at 37°C for 24 h. The isolated single

colonies from agar plates were transferred to TSBYE and incubated in shaking incubator at 37°C for 24 h. The 100 µl of 24-hour TSBYE culture was transferred to fresh TSBYE and incubated at 37°C for 18 h. After 18 h of incubation 100 µl of broth culture of each strain were spread plated on TSAYE and incubated at 37°C for 24 h. Bacterial lawn obtained on agar plates were scrapped out in 2 ml of sterile 0.1% peptone water using a sterile scrapper and collected in sterile Eppendorf tubes. After centrifugation at 9700×g for 5 min in a microcentrifuge (Thermo Scientific™ Sorvall™ Legend™ Micro 21 Centrifuge, Fisher Scientific Company, Ottawa, ON), the supernatant was discarded, and pellet was washed in one ml fresh sterile 0.1% peptone water and homogenized in a vortex (Fisher Scientific, Fisher Scientific Company, Ottawa, ON). The resulting suspension was centrifuged at 9700×g for 5 min and the supernatant was discarded. The resulting pellet was resuspended in sterile 0.1% peptone water to make one ml inoculum. Equal volumes of inoculum of each strain of *Salmonella* were mixed together in a 15 ml falcon tube (Polypropylene conical tubes-sterile, Fisher Scientific Company, Ottawa, ON) to make a cocktail inoculum of *Salmonella*.

Stationary phase bacteria were used to get the lawn on agar plates because different studies suggested the better adaptation of stationary phase bacteria to multiple stresses than the log phase bacteria (Gruzdev et al., 2012). Also, *Salmonella* grown on solid media were more resistant to dehydration than broth-grown pathogens (Gruzdev et al., 2012).

2.3. Inoculation and drying of samples

Inoculation and drying of samples were conducted following the method adopted by Bowman et al. (2015) and Tadapaneni et al. (2018), but with some modifications.

From the homogenous suspension of cocktail inoculum of *Salmonella*, 50 µl was inoculated on the flat side of a pellet. Mixing of the inoculated sample before absorption made pet

food pellets moist and sticky, forming a clump. Hence, the pet food pellets were left on Petri plates inside the biosafety cabinet to absorb the inoculum for approximately 30 min. Inoculated pellets were transferred to a 50 ml falcon tube (Polypropylene conical tubes-sterile, Fisher Scientific Company, Ottawa, ON) to mix manually by slight shaking, then transferred to Petri plates and kept in the biosafety for air drying.

Similarly, for the whole black peppercorn, 12 g of black peppercorn was taken in 50 ml falcon tube. From the homogenous suspension of cocktail inoculum of *Salmonella*, one ml of inoculum was dispensed onto the sample. Mixing of the inoculated sample was performed by vortex and left for 20 min to absorb inoculum. Then, the next 1 ml and 0.5 ml of bacterial inoculum were added subsequently, mixed well and left for 20 min each time to absorb the inoculum. Inoculated peppercorn was mixed in a vortex to break clumps. The sample was transferred to Petri plates and kept in the biosafety for air drying. After 24 h of air drying, the 24 g of the whole peppercorn was ground in a coffee grinder for 45 min. The black pepper powder obtained was transferred into Petri plates and left in biosafety for air drying at room temperature.

After 18 to 24 h of air drying, samples were transferred to a chamber with silica gel (ACROS Organics™, Fisher Scientific Company, Ottawa, ON) for pre-exposure of *Salmonella* to the desiccation stress. The silica gel used for drying purpose was dried in an oven (Thermo Scientific™ Heratherm™ General Protocol Oven, Fisher Scientific Company, Ottawa, ON) at 105ºC for 5 h and cooled down in a chamber before transferring the sample. The bacteria exposed to desiccation stress were reported to become more resistant to thermal treatments (Rachon, Penaloza, & Gibbs., 2016).

2.4. Equilibration of samples at different relative humidity conditions

Three different chambers were filled with 300 ml of supersaturated solutions of NaCl (Fisher Chemical, Fisher Scientific Company, Ottawa, ON), $MgCl_2$ and $Mg(NO_3)_2$ (ACROS Organic, Fisher Scientific Company, Ottawa, ON) to maintain the relative humidity (RH) values of 33%, 54% and 75%, respectively. The inoculated and non-inoculated samples were equilibrated in those humidity chambers separately for one week at room temperature (20-25°C). After equilibration, the a_w values of samples were confirmed as 0.33, 0.54 and 0.75, corresponding to the respective RH values during equilibration.

2.5. Thermal treatments of samples inside test cells

In this research, aluminum test cells (Chung et al., 2008) were used for modeling the inactivation of *S. enterica* in pet food pellets and black pepper powder. Inoculated product samples were hermetically sealed in an aluminum test cell to avoid change in a_w and maintain an environment close to ideal isothermal condition during thermal treatment. The inoculated and equilibrated pet food pellets and black pepper powder samples in test cells were treated at different temperatures using a water bath (Fisher Scientific Isotemp GPD10 Water Bath, Fisher Scientific company, Ottawa, ON). The test cell diameter and height were 18 mm and 4 mm, respectively with a sample volume of 1 ml (Chung et al., 2008).

Before thermal treatment, the come-up time (CUT) values, which is the time required to achieve the target temperatures (0.5ºC less to 75, 85 or 95ºC) at the center of the pet food pellet and black pepper powder, were obtained using a Type-T thermocouple probe attached to the digital thermometer (Fisher Scientific, Fisher Scientific Company, Ottawa, ON). To determine the come-up time for a pet food pellet, a small hole was made at the geometrical center of a non-inoculated

pet food pellet and the tip of a thermocouple was placed at the center of the pellet (Villa-Rojas et al., 2013). As the thermocouple sensor probe can push through black pepper powder, no additional preparation was required for this sample. The food sample (approx. 0.3 g) in the test cell was heated using a hot water bath and the temperature at the pellet center was monitored to determine the come-up time. Triplicate samples were selected for come-up time measurements.

Food samples with selected a_w (0.33, 0.54, and 0.75) were treated at specific temperatures ranging between 60 to 85°C. Preliminary experiments were conducted to select the treatment times for the specific treatment temperatures for the different a_w samples. Approximately, 0.3 g of pet food pellet and 0.33 g of black pepper powder were sealed inside the test cells and placed inside a water bath for selected times, at the treatment temperatures. After treatments, the test cells were placed in the ice-water bath for 45 seconds to bring down the temperature to 30°C or below and stop the thermal inactivation. All the experiments were performed taking three independent replicates.

2.6. Recovery and enumeration of *Salmonella*

After thermal treatments, test cells were removed from the ice bath and wiped the surface water out thoroughly with a paper towel before opening. The food samples were removed from the test cells and mixed with 100 ml sterile 0.1% peptone water inside a sterile stomacher bag with filter (Fisherbrand™ Lab Blender Bag, Fisher Scientific Company, Ottawa, ON) and left to soak for 30-45 min. The food samples were massaged manually first and then stomached in a stomacher (Seward™ Stomacher™ Model 400C circulator Lab Blender, Fisher Scientific Company, Ottawa, ON) at 260 rpm for 3 min. The homogenized suspension was dispensed into a 15 ml falcon tube and left for 2 min to settle large particles. The supernatant was collected for the serial dilution in

sterile 0.1% peptone water, plated on TSAYE, and incubated at 37°C for 24 h. Rich media like TSB, LB, or BHI and incubation temperature of 37°C enhances the growth of stressed bacteria than selective media like XLD and incubation temperature of 25°C (Gruzdev et al., 2012). Hence, TSA was used to revive the *Salmonella* from the treated sample. *Salmonella* colonies were enumerated, and log reductions were calculated by subtracting log counts calculated for different time intervals from the log population at time 0 (control sample; here come-up time is considered as time 0) for the respective replicates. Surviving *Salmonella* population in heat treated and control samples were determined from isothermal inactivation kinetics of the *Salmonella* in both food samples (Smith et al., 2016). Initially, XLD agar media was used for enumeration of surviving *Salmonella* in treated pet food pellets but discontinued to use it due to inconsistent readings between replicates for each sample and between averages of corresponding treatment times.

2.7. Thermal inactivation kinetics

The Weibull model (Peleg, 2006) (equation 2) was used to describe the thermal inactivation kinetics of *Salmonella* on pet food pellets and black pepper powder as the data showed a non-linear trend. The 5-log reduction time was predicted by the Weibull model using the equation (3).

2.8. Water adsorption isotherms at different temperature levels

2.8.1. Vapor sorption analyzer

Vapor sorption analyzer (VSA) (METER group, Pullman, WA, USA) was used to generate sorption isotherms of pet food pellets and black pepper powder at 20 and 60°C (in duplicates), following the dynamic vapor sorption (DVS) method. Water sorption isotherm of black pepper powder at 60°C could not be completed due to contamination of mirror of VSA by fine powder

and a volatile component. The sample was exposed to preselected relative humidity levels until constant weight was maintained. At equilibrium, the corresponding a_w and water content values were recorded. By setting VSA at 10% incremental change in relative humidity, sorption isotherm curve was constructed taking a_w values from 0.1 to 0.8 with every a_w interval of 0.1 and corresponding water contents of the equilibrated samples.

2.8.2. The high-temperature water activity system

The VSA cannot be used to generate a sorption isotherm curve above 60°C. Hence, a sealed thermal cell containing a commercial relative humidity sensor (HX15-W, Omega Engineering Inc.) was used to measure a_w of food sample at 81°C to get water adsorption isotherm for pet food pellets and black pepper powder. To generate the water adsorption isotherms, the pet food pellets and black pepper powder samples were stored in a silica gel (dried in an oven at 105ºC for 5 h and cooled) chamber at room temperature for one week. Dry food samples (a_w<0.1) were equilibrated at various relative humidity conditions (11.3%, 23%, 33%, 43%, 54%, 75%) using supersaturated salt solutions of LiCl (99%, ACROS Organics, Fisher Scientific Company, Ottawa, ON), CH_3COOK (>99%, Fisher Bioreagents, Fisher Scientific Company, Ottawa, ON), $MgCl_2$ (pure, ACROS Organics, Fisher Scientific Company, Ottawa, ON), K_2CO_3 (Sigma Aldrich Canada Co., Oakville, ON), $MgNO_3$ (98%+, ACROS Organics, Fisher Scientific Company, Ottawa, ON), NaBr (99+%, , ACROS Organics, Fisher Scientific Company, Ottawa, ON) & NaCl (Fisher Chemical, Fisher Scientific Company, Ottawa, ON) for 2 weeks. The equilibrated samples were sealed in the thermal cell and kept in a hot water bath set at 80°C. The weight of equilibrated samples before treatment and after treatment was recorded. Once the a_w of each sample at elevated temperature was constant, the value was recorded. The thermal cells were then removed and cooled

to 25°C in ice-bath. The water content of each sample was determined by oven drying method by heating at 105°C for 8 to 12 h in an oven until constant weight was obtained.

2.8.3. Modeling of water adsorption isotherms at selected temperatures.

The GAB model is commonly used to explain the water sorption isotherms as it is found to be well fitted for different food products (Maroulis, Tsami, Marnos-Kouris, & Saravacos, 1988; Yogendrarajah, Samapundo, Devlieghere, De Saeger, & De Meulenaer, 2015). In the present study also, the GAB equation (equation 4) was used to describe the water adsorption isotherms of pet food pellets and black pepper powder.

2.9. Quality change analysis

2.9.1. Lipid oxidation in pet food pellets after thermal treatment

Change in the quality of pet food pellets after thermal treatment was determined by monitoring the lipid oxidation. To determine the lipid oxidation, the TBARS values for pet food pellets were quantified after thermal treatments, following the standard methods described by Wenjiao, Yongkui, Yunchuan, Junxiu, & Yuwen (2014) and Gomes et al. (2003) with some modifications. TBARS values were quantified colorimetrically by the method based on the spectrofluorometric determination of the pink complex formed after the reaction of one molecule of malondialdehyde (MDA) with two molecules of 2-thiobarbituric acid (TBA).

The equilibrated pet food pellets with 0.33, 0.54, and 0.75 a_w were treated at three temperatures ranging from 65 to 85ºC, for the treatment times at which the maximum reduction of *Salmonella* was observed. In 0.5 g of the treated and corresponding control (untreated) pet food pellets, 5 ml of 20% trichloroacetic acid (100 g/l) (ACROS Organics, Fisher Scientific Company,

Ottawa, ON) and 1.6% phosphoric acid (ACROS Organics, Fisher Scientific Company, Ottawa, ON) was added, followed by homogenization at 1680 ×g RCF for one min using benchtop digital disperser (IKA, T18 digital ULTRA TURRAX disperser). The homogenate was centrifuged at 5320 ×g RCF for 3 min (Beckman Coulter-Allegra 25R Centrifuge) and one ml supernatant was taken in duplicates in separate falcon tubes. One ml supernatant was mixed with one ml of 0.02 molar 2-thiobarbituric acid (Sigma Aldrich, Sigma Aldrich Canada Co., Oakville, ON) and the mixture was placed in a water bath set at 90°C for 35 min and then removed to cool down in an ice-water bath for approximately 10 min. The absorbance of the resulting solution was determined by fluorescence spectroscopy using a Varioskan Flash spectral scanning multimode reader (Varioskan Flash Multimode Reader, Thermo electron corporation), at an excitation wavelength of 532 nm and an emission wavelength of 553 nm at one cm path length cells.

The standard curve was prepared by acid hydrolysis of 1,1,3,3-tetramethoxypropane (TMP) (ACROS Organics, Fisher Scientific Company, Ottawa, ON), an MDA precursor (Mahmoodani et al., 2018). For the standard solution, a stock solution of 0.02 M TMP was prepared by dissolving 0.328 g of TMP in 100 ml of distilled water and kept in a brown bottle. This can be stored for a month. The working solution of TMP was prepared by dissolving 5 ml of 0.02 M TMP solution to 500 ml of distilled water and stored in dark for not more than a week. The different volume of working TMP solutions was mixed with 50% TCA (50% TCA: 50% distilled water) solution. A standard curve ($R^2 = 0.97$) was developed with selected dilutions of working TMP (malonaldehyde) solution in 50% TCA and the corresponding absorbance values at an excitation wavelength of 532 nm and an emission wavelength of 553 nm at one cm path length cells. After obtaining absorbance values for control and treated pet food pellets, the TBARS values of samples were calculated using the value of the slope obtained from the standard curve and was

expressed as mg MDA/kg of dry pet food pellets. The values were reported as the average of three measurements. The TBARS values of control and each treated sample were compared to determine the lipid oxidation after the thermal treatment.

2.9.2. Piperine content in black pepper powder after thermal treatment

Change in quality of black pepper powder after thermal treatment is analyzed by quantifying the change in piperine content in black pepper powder. The non-inoculated but equilibrated black pepper powder with selected a_w (0.33, 0.54, and 0.75) were heat treated at selected temperatures ranging from 60 to 85ºC, for the treatment times at which the maximum reduction of *Salmonella* was observed. The piperine contents from the treated and untreated sample were extracted by conventional solvent extraction method (Azwanida, 2015; Ban et al., 2018; Gberikon, Adeoti, & Aondoackaa, 2015), with some modifications.

2.9.2.1. Piperine extraction

For extraction, 0.33 g of black pepper powder was taken in a conical flask after thermal treatments and mixed with 30 ml of methanol (Fisher Chemical, Fisher Scientific Company, Ottawa, ON) as the solvent. The solubility of piperine in alcohol is 1g/15ml. The sample in methanol was shaken manually after every 15 min for 2 h at room temperature. The sample was then left for 20 h and the residue was filtered using Whatman No. 1 filter (Quantitative Circles 110 mm ϕ). The residue was washed several times with methanol and volume of the filtrate was made up to 50ml in a volumetric flask. The filtrate was centrifuged at 851×g for 20 min and the clear supernatant was collected. Then, 0.1 ml of the collected extract was diluted with 0.9 ml of methanol and mixed well. 0.1 ml of the diluted piperine was again diluted in 0.9 ml of fresh methanol.

2.9.2.2. Estimation of piperine by spectrophotometry

The absorbance of each extract solution was measured at 343 nm wavelength against a reagent blank (solvent without piperine) using a Varioskan flash spectrophotometer (Gupta & Jain, 2011). The concentration of piperine was calculated using the calibration curve from standard piperine (Analytical standard, Sigma Aldrich Co., Oakville, ON) (Gupta & Jain, 2011; Rathnawathie & Buckle, 1983).

Exactly, 1 mg of standard piperine was dissolved in 10 ml of methanol to make 100 µg/ml concentration. It was then diluted with methanol to make different concentrations (2, 3, 4, 5, 6, 7, and 8 µg/ml) of standard solutions. The absorbance of each standard solution was determined by photo-spectrometry at 343 nm wavelength to make the calibration curve (absorbance vs concentration (µg/ml)).

2.10. Statistical analysis

ANOVA for the response variable was conducted with the SAS software (SAS Institute, 2011). Each of the different combinations of the two factors (a_w and temperature) for multiple time points were considered as separate treatments in the analysis. There were three replicates for each treatment. The level of significance was set at a *P* value of <0.05. Post hoc multiple pairwise comparisons of treatment group means were performed with the Tukey-Kramer adjustment (Tukey's honestly significant difference test) to control for the type I error rate (Appendix III-VI).

CHAPTER III

RESULTS

3.1. Sample characteristics

The average population of bacteria in cocktail inoculum of *Salmonella* for all tests was 9.3 to 10.8 log CFU/ml with a standard deviation of ± 0.19.

Pet food pellets*:* The average initial a_w of pet food pellet was 0.641 ± 0.001 at 25.1 °C. *Salmonella* was not detected in the untreated control pet food pellet.

For each test, a sampling of pet food pellets was conducted in duplicates for inoculation and drying of the samples, before the thermal treatment. The average weight of individual pet food pellet weighed approximately 0.30 ± 0.02 g. The average *Salmonella* count of 9.5 to 10.0 log CFU/g was detected in inoculated, air-dried pet food pellets. Within a single inoculated sample of a pet food pellet, at least two subsamples were tested for the *Salmonella*. The standard deviation of ± 0.17 in randomly picked subsamples within one experimental set of inoculated samples indicated the uniformity of the *Salmonella* population in each pellet. The average population of *Salmonella* in silica gel dried samples was 9.4 to 9.6 log CFU/g with the maximum standard deviation of ± 0.17 which confirmed the uniform distribution of *Salmonella* among the pellets.

Black pepper*:* The average a_w of autoclaved black peppercorn was 0.504 ± 0.001 at 25.0°C. The absence of bacterial contamination was confirmed by random sampling, diluting and plating of 0.33 g of black peppercorn on TSA and XLD agar plates. The average *Salmonella* count of 8.1 to 9.5 log CFU/g was detected in inoculated and air-dried black peppercorn samples. The 0.33 g of black pepper powder was sampled for all thermal treatment experiments. The average *Salmonella* population in air-dried and silica gel dried black pepper powder was between 8.0 to 9.1 log CFU/g.

The standard deviation of <0.1 between the subsamples within the same lot of inoculated black pepper powder confirmed the even distribution of the *Salmonella* population in the black pepper powder sample.

For the untreated but inoculated and dried pet food pellets, the difference in population of *Salmonella* between XLD medium and TSA medium was 0.08-0.2 log CFU/g and for black pepper powder, that difference was 0.6-0.8 log CFU/g. Hence, the number of colonies forming unit in TSAYE and XLD agar media obtained from inoculum and inoculated food samples were comparable.

3.2. Thermal inactivation of *Salmonella* in pet food pellets and black pepper powder

For pet food pellets, the average come-up times determined were 3.04, 3.05 and 3.12 min for 75, 85 and 95°C, respectively (Appendix I). The treatment temperature range for pet food pellets was 65 to 85°C. For convenience, 3 min was considered as the come-up time regardless of a_w and treatment temperature. During experiments, the samples with higher a_w were treated at lower temperatures compared to lower a_w samples to get enough data points for the analysis. The cooling time required after thermal treatment was 45 seconds for the pet food pellets at all temperatures and water activities.

For black pepper powder, the average come-up times were found to be 2.18, 2.39 and 2.44 min for 75, 85, and 95°C (Appendix I). As the range of the treatment temperature was 60 to 85°C, the come-up time was selected as 2 min for all thermal treatments for black pepper powder.

The log reductions in food samples after thermal treatments were calculated based on the *Salmonella* inactivation after come-up times. The population of *Salmonella* was reduced substantially within the come-up times during thermal treatments of pet food pellets and black

pepper powder samples. During come-up times, the log reductions were smaller at lower water activities and temperatures, which increased with increase in a_w and temperature. For pet food pellets, the inactivation levels of *Salmonella* observed were 0.41, 0.48, 0.69 log CFU/g for 0.33 a_w sample at 75, 80, and 85°C, within the come-up time of 3 min with a standard deviation of 0.29, 0.18, and 0.24 respectively. For 0.54 a_w pet food pellets, the *Salmonella* inactivation levels during come-up times were 0.46, 0.82, and 1.32 log CFU/g at 75, 80, and 85°C with a standard deviation of 0.27, 0.24, and 0.05, respectively. At 65, 70, and 75°C, the *Salmonella* inactivation levels in 0.75 a_w pet food pellets were 0.38, 1.46, and 1.88 log CFU/g with a standard deviation of 0.12, 0.12, and 0.28, respectively.

Similarly, for black pepper powder within the come-up time of 2 min, *Salmonella* reductions of 0.29, 0.31, and 1.49 log CFU/g were obtained in 0.33 a_w sample treated at 75, 80, and 85°C, respectively. At 70, 75 and 80°C, the *Salmonella* inactivation in 0.54 a_w black pepper powder sample were 0.19, 0.88, and 1.24 log CFU/g, respectively. For 0.75 a_w black pepper powder sample, the *Salmonella* inactivation within come-up time were 0.24, 0.56, and 1.35 log CFU/g with a standard deviation of 0.25, 0.10, and 0.05 at 60, 65, and 70°C, respectively. The average population of *Salmonella* after come-up times in pet food pellets were between 7.19 to 9.2 log CFU/g and in black pepper powder were between 7.50 to 8.70 log CFU/g.

3.3. Effect of time, temperature, and a_w on *Salmonella* inactivation

For pet food pellets with 0.33 a_w, at 75, 80 and 85°C treatment temperatures from the surviving bacteria after come-up time, maximum reductions of *Salmonella* were 1.85, 3.18, and 3.27 log CFU/g achieved at 23, 19, and 7 min, respectively. Similarly, at 75, 80, and 85°C, maximum inactivation levels of 3.16, 3.53 and 2.60 log CFU/g were observed at 23, 9, and 3 min,

respectively for 0.54 a_w pet food pellets. For 0.75 a_w pet food pellets, 2.85, 2.12, and 1.80 log CFU/g were the maximum reductions of *Salmonella* obtained after 15, 9, and 5 min at treatment temperatures of 65, 70 and 75°C, respectively.

In the case of black pepper powder with 0.33 a_w, the maximum inactivation levels of *Salmonella* observed when treated at 75, 80, and 85°C for 48, 16, and 8 min were 2.59, 2.68, and 2.60 log CFU/g, respectively. The *Salmonella* count reduced by 2.15, 2.74, and 2.57 log CFU/g in 0.54 a_w black pepper powder sample when treated at 70, 75, and 80°C for 40, 20, and 8 min, respectively. The maximum inactivation of *Salmonella* in black pepper powder samples with 0.75 a_w treated for 32, 12, and 4 min at 60, 65, and 70 °C were 1.63, 2.05, and 2.17 log CFU/g, respectively.

These *Salmonella* population after come-up times were considered as initial population or the population corresponding to treatment time of zero. Surviving *Salmonella* populations in heat treated and control samples were determined from isothermal inactivation kinetics of the *Salmonella* on inoculated and equilibrated pet food pellets and black pepper powder at different experimental conditions (specific a_w, temperature, and time). Both primary models (the log-linear model and the Weibull model) were initially selected to fit each of the nine data sets. Fig. 2 and 3 show the typical non-linear upward concave semilogarithmic inactivation curves as influenced by fixed initial a_w. The Weibull model was found to fit the inactivation data points better than the log-linear model based on the comparison of R^2 values for each model. So, the Weibull equation was used to fit the non-linear inactivation trend for all data (equation 2). Fig. 2 and 3 show that the slope increased with the increase in temperature and the tailing effect increased with the decrease in treatment temperature.

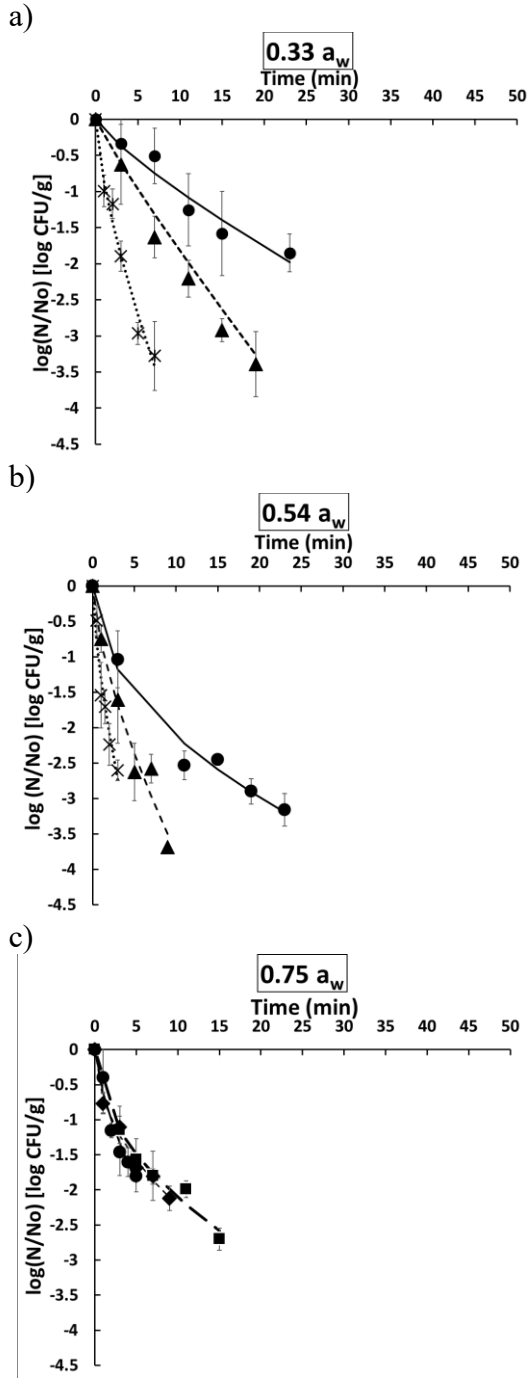


Fig. 2. Thermal inactivation kinetics of *S. enterica* cocktail inoculated on pet food pellets with different water activities (0.33(a); 0.54 (b), and 0.75(c)) and treated at different temperatures (■, 65ºC;◆, 70ºC ;●, 75ºC;▲, 80ºC;✕, 85ºC). Solid, dashed and dotted lines are predicted values by the Weibull model.

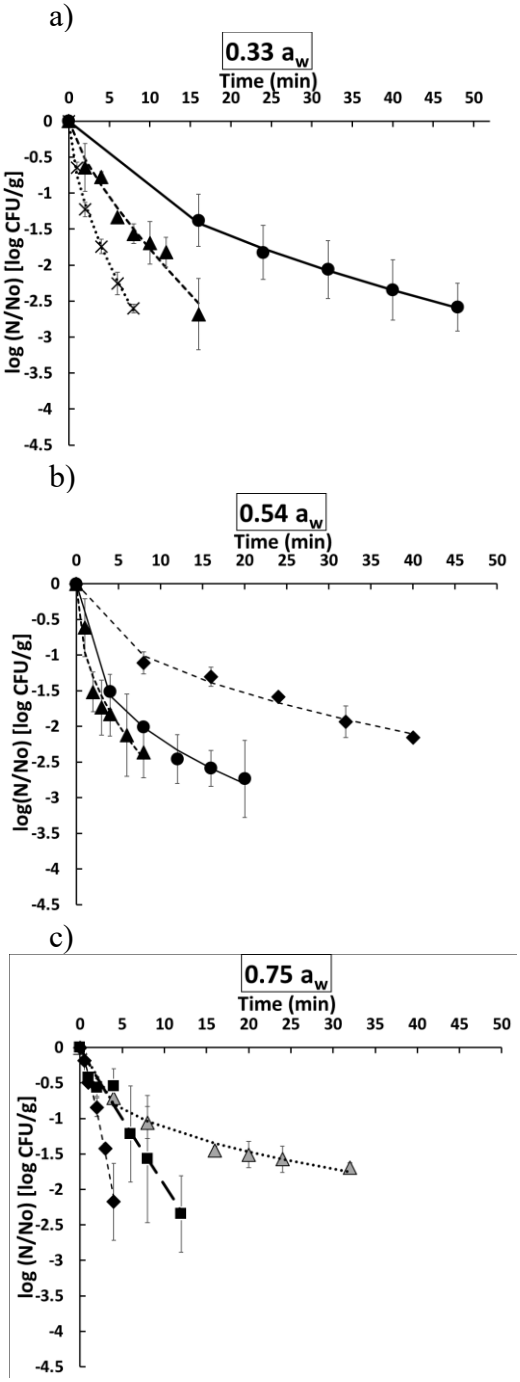


Fig. 3. Thermal inactivation kinetics of *S. enterica* cocktail inoculated in black pepper powder with different water activities (0.33(a), 0.54(b), and 0.75(c)) and treated at different temperatures (△, 60ºC;■, 65ºC;◆, 70ºC; ● , 75ºC;▲ , 80ºC; ✕, 85ºC). Solid, dashed and dotted lines are predicted values by the Weibull model.

The temperature and treatment times used for thermal inactivation experiments were dependent on the a_w of pet food pellets and black pepper powder. It was not possible to compare the effect of a_w on log reductions of *Salmonella* statistically across the data as the treatment time and temperature levels were different for different a_w levels, except for 75ºC in pet food pellets. This means 75ºC and 3 min treatment time were the only common temperature and time levels used to treat the pet food pellets for the selected three a_w levels. So, statistical comparison of the effect of a_w level on thermal inactivation of *Salmonella* on pet food pellets was conducted for only these treatments. Thermal inactivation levels of *Salmonella* were significantly different ($P<0.05$) between pet food pellets with 0.33 and 0.75 a_w, but there was no significant difference in inactivation data between pet food pellets with 0.33 and 0.54 a_w or 0.54 and 0.75 a_w levels. In the case of black pepper powder, a statistical comparison of the effect of a_w between 0.33 and 0.54 a_w levels was conducted, as the only common treatment temperature and time levels were 75ºC and 16 min. The inactivation levels of *Salmonella* in black pepper powder with 0.33 and 0.54 a_w after treatment at 75ºC for 16 min were statistically different ($P<0.05$). The 5-log reduction times at different treatment conditions were used as explained below to describe the effect of a_w and temperature on thermal inactivation levels of *Salmonella* on pet food pellets and black pepper powder.

3.4. Modeling of thermal inactivation kinetics and 5-log reduction time calculation

In both samples, *Salmonella* inactivation kinetics at 0.33, 0.54 and 0.75 a_w exhibited non-linear trend with tailing effect and an upward concavity. At higher water activities and temperatures, the curve showed an increase in slope. With a decrease in temperature, an increase in upward concavity and tailing effect was observed. According to Smith et al. (2016), the log-

linear model was the appropriate model for isothermal inactivation of *Salmonella* in organic wheat flour. However, Villa-Rojas et al. (2013) reported that the inactivation data for the *Salmonella* on almond fitted better with the Weibull model, but the experiment was conducted for samples with a_w above 0.6. According to Santillana Farakos, Hicks, & Frank (2014), the inactivation kinetics of *Salmonella* in low a_w protein powder at different temperatures and a_w levels, and different salinity was described by the Weibull model, but they did not test the fitting of data with the log-linear model. Fig. 2 shows the curve for the inactivation rate of *Salmonella* in pet food pellets at 0.33 a_w and Fig. 3 shows the inactivation data of *Salmonella* in black pepper powder. In Fig. 2 and 3, the upward concavity and tailing effects were observed to be greater at low temperatures, and slope of the curve increased with temperature increase, which is similar to the result reported by Villa-Rojas et al. (2013) for almond.

The upward concavity of the curve in *Salmonella* inactivation versus time graph suggests that there was a rapid decline in the number of *Salmonella* at the initial phase of thermal treatment. The rate of inactivation decreased with time, suggesting that the varying level of thermal resistance of surviving bacteria which take more time for inactivation (Villa-Rojas et al., 2013). At elevated temperatures, heat shock proteins (HSPs) are rapidly induced in bacterial cells and many HSPs aid in refolding heat-damaged proteins (e.g. GroEL and DnaK) or denature proteins for elimination (e.g., proteases such as Lon and ClpAP) (Bigley & Hill, 2015).

The values for α and β calculated from the experimental data for each survival curve along with regression coefficient (R^2) value for corresponding experimental data set are presented in Table 1 for pet food pellets and Table 2 for black pepper powder. The predicted 5-log reduction times for each sample are also presented in the respective tables (Table 1, and 2).

From the inactivation of *S. enterica* in pet food pellets, the R^2 values of > 0.9 were obtained for the Weibull model except for two treatments, (a_w=0.33, T=75°C, and a_w=0.75, T=75°C, with R^2 value of 0.78 and 0.86, respectively) showing the better fitting of experimental data to the Weibull model. In black pepper powder, the R^2 values for all the inactivation data were greater than 0.9, except for two (For a_w=0.54/T=80°C, and a_w=0.75/T=65°C, which had R^2 values of 0.83 and 0.76, respectively).

Table 1. Calculated values of α, β parameters, and 5-log reduction times from the Weibull model for the thermal inactivation of the cocktail strain of *S. enterica* in pet food pellets.

Sample a_w	Temp. (°C)	α-value	β-value	R^2-value	Predicted 5-log reduction time (min)
0.33 ± 0.01	**75**	3.58	0.81	0.78	71.8
	80	2.07	0.91	0.96	30.3
	85	0.37	0.70	0.95	12.1
0.54 ± 0.01	**75**	0.39	0.49	0.96	57.3
	80	0.40	0.67	0.94	15.4
	85	0.20	0.68	0.91	7.3
0.75 ± 0.01	**65**	0.42	0.50	0.95	56.7
	70	0.37	0.49	0.95	52.1
	75	0.60	0.69	0.86	20.5

The values of β were less than one for all treatment conditions in both samples, except for black pepper powder with 0.75 a_w, treated at 75°C, and the inactivation curve was downward concave. The β<1 means the inactivation of most of the heat-sensitive *S. enterica* occurred at the beginning of thermal treatment and remaining population were more heat resistant or developed resistance or they did not get the same lethal dose (Bevilacqua et al., 2015; van Boekel, 2002). In

the case of 0.75 a_w black pepper powder treated at 75°C, the presence of a shoulder in the curve was observed with a value of β greater than one. The downward concavity and presence of shoulder can be due to different reasons such as, i) formation of microbial clump where few exposed microbes are killed initially, ii) cells preparing to combat the effect of lethal treatment and showed slow decline in population at the beginning of treatment, iii) protective effect of the food components such as proteins or fats, or iv) large population escaping the death with heat injury, which on continuous exposure to heat get killed rapidly (Bevilacqua et al., 2015).

Table 2. Calculated values of α, β parameters, and 5-log reduction times from the Weibull model for the thermal inactivation of the cocktail strain of *S. enterica* in black pepper powder.

Sample a_w	**Temp. (°C)**	**α-value**	**β-value**	**R^2-value**	**Predicted 5-log reduction time (min)**
0.33 ± 0.01	**75**	1.88	0.55	0.90	157.8
	80	1.55	0.75	0.91	39.5
	85	0.41	0.61	0.99	22.9
0.54 ± 0.01	**70**	1.25	0.46	0.97	265.2
	75	0.11	0.36	0.93	99.3
	80	0.18	0.45	0.83	38.2
0.75 ± 0.01	**60**	0.80	0.38	0.95	509.0
	65	2.07	0.95	0.76	27.1
	70	1.10	1.23	0.93	8.0

According to the existing guidelines of FDA (2018), the validation of pasteurization process for different types of food products requires the reduction of foodborne pathogens like *S. enterica* by 5-log to ensure the safety of food products. As shown in Table 1 and 2, it required

comparatively less time to achieve the 5-log reduction of *S. enterica* in the sample with high a_w and temperature. The predicted 5-log reduction times increased with increase in a_w of the sample and a decrease in treatment temperature (Fig. 4 and 5).

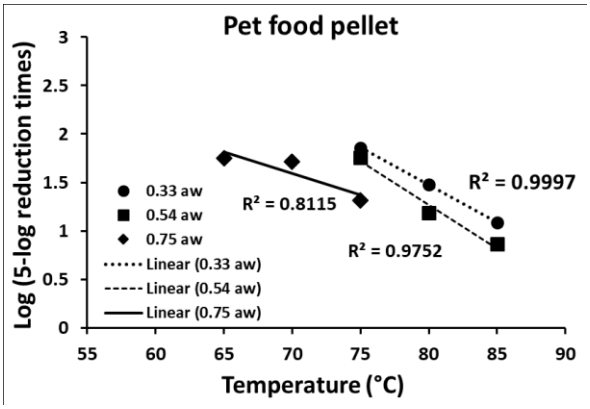


Fig. 4. Effect of temperature on 5-log reduction times of *S. enterica* cocktail on pet food pellets at three different water activities (0.33, 0.54, and 0.75). Solid, dashed, and dotted lines are the log linear trendline.

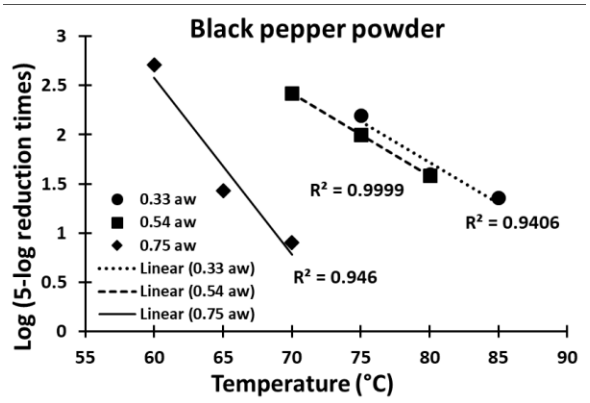


Fig. 5. Effect of temperature on 5-log reduction times of *S. enterica* cocktail in black pepper powder at three different water activities (0.33, 0.54, and 0.75). Solid, dashed, and dotted lines are the log linear trendline.

For pet food pellets, the highest value of the time required to achieve the 5-log reduction in *Salmonella* was 71.8 min in 0.33 a_w at 75°C. The lowest 5-log reduction time obtained was 7.3 min in pet food pellets with 0.54 a_w treated at 85°C. The temperature had a significant effect on the time required for 5-log reduction in *Salmonella*, regardless of a_w. In the case of black pepper powder, the highest 5-log reduction time was 509 min, for the highest a_w tested i.e. for 0.75 a_w sample treated at a temperature of 60°C. For the sample with the same a_w, the 5-log reduction times increased with the decrease in treatment temperature. Likewise, for the sample treated at the same temperature, the 5-log reduction times increased with a decrease in a_w. The relationship between log (5-log reduction times) and treatment temperatures for pet food pellets and black pepper powder with the selected a_w followed a log-linear trend (Fig. 4 and 5).

3.5. Adsorption isotherms of pet food pellets and black pepper powder

Water adsorption isotherms, presenting the relationship between a_w and water content in pet food pellets and black pepper powder at selected temperatures (20, 23.5, 60 and 81ºC) were generated (Figure 6 & 7). For pet food pellets, the isotherms at 20 and 60°C were obtained using VSA and the isotherms at 23.5 and 81°C were generated using a high-temperature water activity meter. Likewise, for black pepper powder, isotherm at only 20°C was generated using VSA, while isotherms at 23.5 and 81ºC were generated using high temperature a_w meter. Measurement of a_w for black pepper powder in VSA and 4TE Aqualab a_w meter was not possible at high temperatures such as 60 and 81°C due to the limitation of the instrument.

An increase in a_w with temperature was observed for pet food pellets with the same water contents. For instance, for pet food pellets with a water content of 0.08 kg water/kg dry pet food pellets, the a_w at 20ºC was around 0.4 while it increased to 0.6 when the temperature was increased to 81ºC. Considerable change in initial a_w values of pet food pellets from 0.33 to 0.50 and 0.54 to 0.62 was observed when the temperature was increased from 20 to 81ºC (red lines in Fig. 6) at constant water content, however, temperature did not change the a_w of pet food pellets with 0.75 a_w (Fig. 6). In the case of black pepper powder, increase in a_w from 0.33, 0.54 and 0.75 at 20ºC to approximately 0.55, 0.70, and 0.83 a_w, respectively, at 81ºC at constant water content (red lines in Fig. 7) was observed. The increase in a_w at a specific water content was higher in black pepper powder compared to pet food pellets. The change in a_w with temperature increase was lower at higher a_w, especially above 0.7 a_w in pet food pellets (Fig. 6). The isotherm curve for 81°C in pet food pellets tapered towards 20°C adsorption isotherm with a rise in the water content of sample over 0.1 kg water/kg dry pet food pellets, and this phenomenon was not observed in the case of black pepper powder. In order to determine the effect of a_w change with temperature increase on

thermal resistance of *Salmonella* on pet food pellets, future experiments should be conducted at controlled a_w conditions with no change in a_w when the temperature is increased.

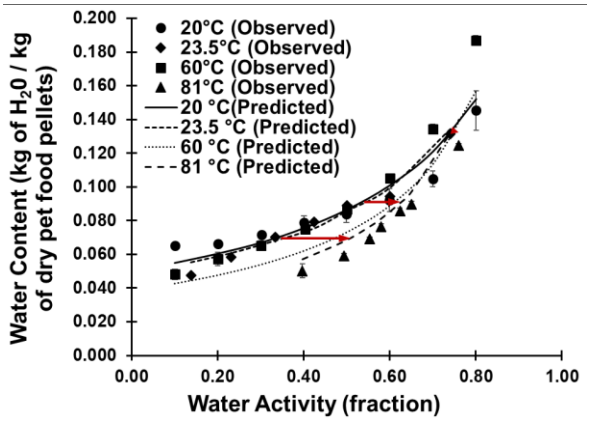


Fig. 6. Adsorption isotherms of pet food pellets at tested temperatures (water content data points are an average of three independent samples). Red lines show the change in a_w of sample when temperature increased from 20 to 81ºC at a specific water content.

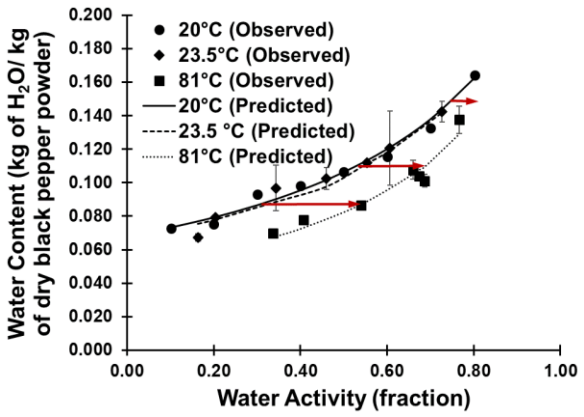


Fig. 7. Adsorption isotherms of black pepper powder at tested temperatures (water content data points are an average of three independent samples). Red lines show the change in a_w of sample when temperature increased from 20 to 81ºC at a specific water content.

The GAB model (equation 4) was used to fit the isotherm data of pet food pellets and black pepper powder.

The GAB equation was used to determine values for different isotherm model parameters (Table 3 & 4.) from the food product's water adsorption behavior. The modeling result for adsorption isotherm of pet food pellets, based on GAB equation showed a decrease in monolayer water content (X_m) (dry basis) from 0.05 to 0.035 when temperature increased from 20 to 81°C (Table 4.). From modeling of the adsorption isotherm data of black pepper powder, the monolayer water content (dry basis) based on GAB equation decreased from 0.068 to 0.049 when temperature increased from 20 to 81°C (Table 4.). The monolayer water contents for both samples were higher at low temperatures, which decreased with increase in temperature.

Table 3. GAB Model parameters for adsorption isotherms of pet food pellets and black pepper powder.

Sample	Isotherm	GAB Model Parameters					
		X_{mo}	ΔH_X	C_0	ΔH_C	K_0	ΔH_K
Pet food pellets	Adsorption	0.006	5288.8	10111.1	1340.8	2.2	-2376.3
Black pepper powder	Adsorption	0.010	4570.4	-1857.8	697.2	1.36	-1512.3

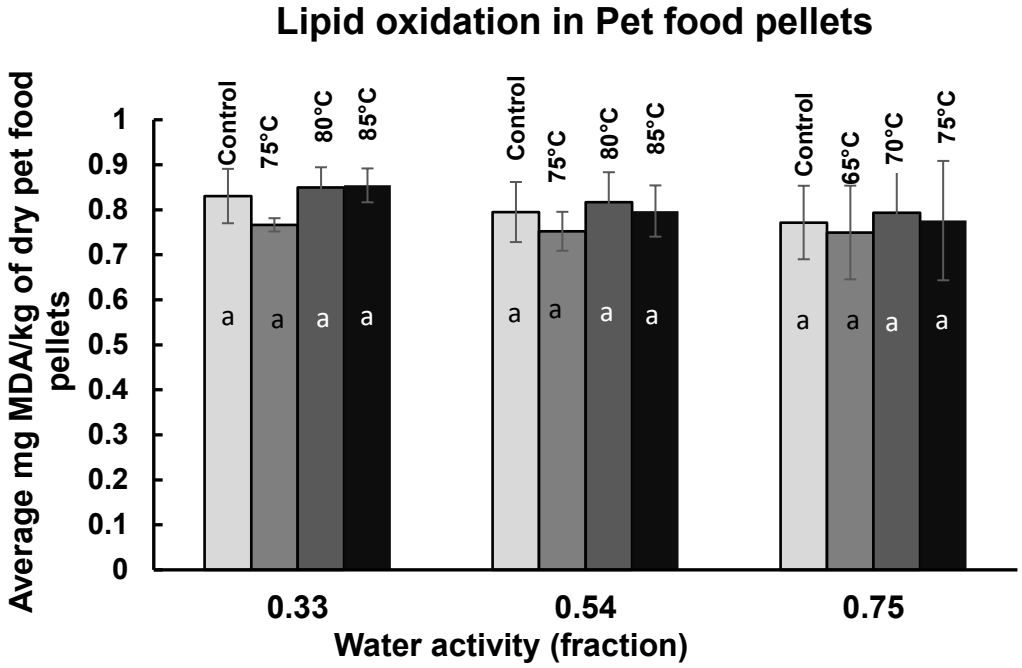
Table 4. Other GAB model parameters for adsorption isotherms of pet food pellets and black pepper powder at 20, 23.5, 60, and 81°C.

Sample	Constant	20°C	23.5°C	60°C	81°C
Pet food pellets	$\mathbf{X_m}$	0.050	0.049	0.039	0.035
	K	0.837	0.847	0.941	0.990
	C	175320.1	174184.7	164103.7	159456.3
	$\mathbf{R^2}$	0.940	0.970	0.997	0.986
Black pepper powder	$\mathbf{X_m}$	0.068	0.066	n/a	0.049
	K	0.728	0.734	n/a	0.811
	C	-2473.5	-2465.2	n/a	-2354.5
	$\mathbf{R^2}$	0.968	0.883	n/a	0.911

Saleh et al. (2018) reported a similar trend of decrease in monolayer water content with an increase in temperature in dried purple flesh sweet potato. Similarly, with an increase in treatment temperature, the values of the GAB parameter, C, decreased in pet food pellets but increased in black pepper powder samples. The parameter C measures the strength of binding water to the

primary binding sites of the food and thus higher C value means the better binding strength of water (Quirijns et al., 2005).

3.6. Lipid oxidation in pet food pellets after thermal treatment



Note: Different letter within the column indicate the average values are significantly different ($P < 0.05$).

Fig. 8. Changes in TBARS content of pet food pellets at different a_w and different temperatures.

The changes in TBARS values representing the lipid oxidation in pet food pellets with 0.33, 0.54 and 0.75 a_w at 65, 70, 75, 80, or 85°C for the times corresponding to maximum log reductions of *Salmonella* were determined (Fig. 8). The MDA values of the treated samples were comparable to those in the control sample. No significant difference ($P \geq 0.05$) was observed in the values of MDA after the treatment of samples at the selected temperatures and a_w of pet food pellets, presenting that the temperatures or a_w did not influence the lipid oxidation during thermal treatments. The higher MDA values corresponding to the lipid oxidation observed in untreated

control pet food pellets must be due to the presence of already oxidized lipids, that might have occurred during manufacturing (e.g., extrusion and drying process at high temperatures) or storage of pet food pellets.

3.7. Piperine content in black pepper powder after thermal treatment

Table 5. Piperine content of treated and untreated black pepper powder equilibrated at three different water activities.

Water activity (fraction)	Treatment temperature (°C)	Treatment time (min)	Piperine content ((%wt of piperine content /wt of black pepper powder dry solids) ± SD)*
Control	0	0	4.65 ± 0.15^{bc}
0.33	0	0	5.76 ± 0.18^{a}
	75	48	5.76 ± 0.18^{a}
	80	16	5.66 ± 0.23^{a}
	85	8	5.72 ± 0.18^{a}
0.54	0	0	5.81 ± 0.30^{a}
	70	40	5.89 ± 0.34^{a}
	75	20	5.08 ± 0.96^{ab}
	80	8	3.70 ± 0.06^{c}
0.75	0	0	4.12 ± 0.10^{bc}
	60	32	3.96 ± 0.12^{c}
	65	12	4.31 ± 0.26^{bc}
	70	4	4.09 ± 0.14^{c}

* Different letter within the column indicates the average values are significantly different ($P < 0.05$)

The piperine contents (dry basis) estimated in black pepper powder after thermal treatment at maximum treatment time corresponding to the sample and temperature are given in Table 5.

The water content in black pepper powder with 0.33, 0.49 (control), 0.54, and 0.75 a_w were 9.3, 9.8, 10.7, and 13.3% respectively. The significant change in piperine content was observed in black pepper powder with 0.33 a_w, for all treated and untreated samples compared to the control (non-equilibrated, untreated black pepper powder) sample. There was no significant change in piperine content between the control black pepper powder and the samples with 0.54 a_w and 0.75 a_w, irrespective of levels of treatment time and temperature except for the untreated and treated (at 70°C for 40 min) black pepper samples with 0.54 a_w. The result shows the significant increase in piperine content after equilibration at 33% and 54% relative humidity values. There was no significant change in the piperine content after heat treatments between the black pepper powder with 0.33 and 0.54 a_w, irrespective of levels of treatment time and temperature except for 0.54 a_w sample treated at 80°C for 8 min. For 0.75 a_w sample, the piperine level was significantly lower than any treated samples with 0.33 and 0.54 a_w, except for 0.54 a_w samples treated at 80ºC. No change in piperine content after superheated steam treatment in black peppercorn and radiofrequency heating in black pepper powder was reported by Ban et al. (2018) and Wei et al. (2018), respectively.

CHAPTER IV

DISCUSSION

In this study, experiments and modeling of thermal inactivation of two serovars of *S. enterica* subspecies *enterica* on pet food pellets and five strains of *Salmonella,* including mentioned two strains of *S. enterica,* were carried out on black pepper powder, as influenced by a_w of the food products.

From the initial experiments, 3 min and 2 min were considered as the come-up times for the pet food pellets and black pepper powder, regardless of the a_w and treatment temperature. These results were comparable to the reported come-up times for other LMFs from previous studies. For example, the come-up time for wheat flour with 0.45 a_w at 80°C treatment temperature was 2.20 min (Villa-Rojas et al.,2017). In case of almond powder, 1.33 to 1.50 min was considered as the come-up time for samples with a_w ranging from 0.601 to 0.970, treated at 56 to 80ºC temperature range (Villa-Rojas et al., 2013). Smith et al. (2016) observed the come-up time of approximately, 1.13 min for wheat flour with a_w in the range of 0.30 to 0.60 and treatment temperatures of 75, 80, and 85°C.

The count of *Salmonella* after come-up time was considered as *Salmonella* population at zero-minute treatment time because there was a considerable reduction in *Salmonella* count in the 3 min come-up time at treatment temperatures between 65 to 85°C in pet food pellets. Similarly, the reduction of *Salmonella* population in black pepper powder during the come-up time of 2 min was also considerable. The reduction was minimal at lower a_w and treatment temperatures.

The type of strain, the growth conditions of pathogens, food composition, a_w, and temperature affect the survival of pathogens in food products (Santillana Farakos & Frank, 2014).

The thermal resistance of the foodborne pathogens like *S. enterica* increases with the decrease in a_w (Beuchat et al., 2011; Lang et al., 2017; Villa-Rojas Sa., 2013). Result in this study showed that low a_w samples require more time than high a_w samples to achieve the same log reduction of *Salmonella* at any treatment temperature. The increase in thermal resistance of *Salmonella* with decrease in a_w of the sample was observed in previous studies conducted in different LMFs including chicken seasoning, pet food formulation, chicken litter, whey protein, peanut butter, all-purpose wheat flour, and almond (Chen et al., 2013; Rachon et al., 2016; Santillana Farakos et al., 2013; Song & Kang, 2016; Syamaladevi et al., 2016a; Villa-Rojas et al., 2013).

In this study, the Weibull model was found to fit the inactivation data of pet food pellets and black pepper powder better than the log-linear model based on the comparison of R^2 values for each model. Microbial inactivation data was found to fit best with the Weibull model for other different LMFs such as whey protein (a_w =0.39-0.58), and almond (Santillana Farakos et al., 2013; Villa-Rojas et al., 2013). The inactivation curves of *S.* Typhimurium and *S.* Enteritidis in black peppercorns, pecan, and almonds obtained after steam sterilization were non-linear with the presence of a shoulder (Ban et al., 2018). Several studies reported that the inactivation of *Salmonella* on LMFs fitted better with a non-linear model like the Weibull model (Ban et al., 2018; Rachon et al., 2016; van Boekel, 2002). In a study by Jeong & Kang (2014), the curve for inactivation of *S.* Typhimurium in black and red pepper powder by radiofrequency heating also followed the non-linear trend, but they did not mention about the modeling of their inactivation data. The genetic or phenotypical heterogenicity of the cells might affect the linearity of the inactivation trend resulting in non-linear inactivation curve (Smelt & Brul, 2014; van Boekel, 2002).

The β-values of the Weibull model were considerably smaller than one, which resulted in an upward concavity of the curve. It shows the presence of tailing effect that may be due to the rapid destruction of the heat-sensitive bacteria at the beginning of the process and gradual decrease in the rate of *Salmonella* inactivation (Bevilacqua et al., 2015). The decrease in the rate of inactivation might be due to the presence of more heat-resistant bacteria, which requires further treatment (Bevilacqua et al., 2015). In this study, less tailing effect with an increase in temperature was observed. Enachie et al. (2015) reported a similar result in their study on *Salmonella* inactivation in peanut butter. Instead of tailing effect, shoulder effect was observed with 0.75 a_w black pepper powder samples treated at 70°C. This means the remaining cells of *Salmonella* got more susceptible to heat or rate of inactivation increased with the increase in treatment time.

Regulatory agencies, such as, Food and Drug Administration (FDA) and Grocery Manufacturers Association (GMA) have suggested development and validation of food treatments that can ensure 4 to 5-log reduction of foodborne pathogens. The 5-log reduction target could not be achieved experimentally due to the reduction of *Salmonella* during the come-up time and higher detection limits which limited the determination of more than 4-log reduction during treatments.

For the same a_w and temperature conditions, the 5-log reduction time was greater for black pepper powder in comparison to pet food pellets, indicating that the *Salmonella* survival during heat treatments may depend on the food components. For instance, at 75°C and 0.33 a_w, the 5-log reduction time for *Salmonella* in pet food pellets was 71.8 min while in black pepper powder, it was 157.8 min. However, a clear comparison was not possible as the two-strain cocktail of *Salmonella* was used in the case of pet food pellets while a five-strain cocktail was used in the case of black pepper powder.

The exponential decrease in the values of time required for 5-log reduction was observed when the temperature was increased, showing the high-temperature sensitivity of the *Salmonella* cocktail used for both products at the selected a_w (Fig. 4 & 5). In the case of pet food pellets, the much sharper slope was observed in the relationship between 5-log reduction time and temperature at lower water activities (0.33 and 0.54 $a_{w)}$ compared to flatter slope for 0.75 a_w, indicating the greater temperature sensitivity of *Salmonella* at 0.33 and 0.54 a_w conditions. For both products, the relationships between log (5-log reduction time) and temperature were similar and with comparable slopes for 0.33 and 0.54 a_w, but for 0.75 a_w, they were different (Fig. 4 & 5). In the case of black pepper powder, the relationship between 5-log reduction time and temperature at lower water activities (0.33 and 0.54 a_w) showed flatter slope compared to that for 0.75 a_w, presenting less temperature sensitivity of *Salmonella* at lower a_w (0.33 and 0.54). A similar result was observed for the relationship between the log D values and inactivation temperature for *Salmonella* in wheat flour with 0.30, 0.45, and 0.60 a_w treated at 70, 75, and 80ºC (Taylor, Tsai, Rasco, Tang, & Zhu, 2018). With an increase in treatment temperature from 60 to 70ºC, the slope for inactivation of *Salmonella* in 0.75 a_w black pepper powder increased from upward concavity at 60ºC to almost linear at 65ºC or even with slight shouldering effect at 70ºC. An increase in the value of the Weibull parameter, β, in 0.75 a_w black pepper powder from 0.38 at 60 ºC to 1.23 at 70ºC also shows the higher temperature sensitivity of *Salmonella* in black pepper powder compared to that in pet food pellets where values of β in 0.75 a_w samples were below 0.69 for 65, 70, and 75ºC.

In experiments using *S.* Tennessee inoculated talc in peanut paste (a_w=0.6), the 5-log reduction time at 85°C calculated from the Weibull model was 5.36 min (Enache et al., 2015). This result is comparable to the result in the current study where the Weibull model calculation

showed that the *Salmonella* in pet food pellets at 0.54 a_w required 7.3 min for 5-log reduction. For black peppercorn with 0.6 a_w, the radiofrequency heating for 2.5 min (minimum temperature 74.8°C, the surface temperature of 96.5°C) resulted in a 5-log reduction of *Salmonella* (Wei et al., 2018). In black pepper powder, 8 min was 5-log reduction time predicted from the Weibull model for 0.75 a_w sample, treated at a uniform temperature of 70°C. Comparison of these result with previous studies is difficult because of the difference in the sample form, a_w, treatment temperature, and the strain of *Salmonella* used. When *S.* Enteritidis inoculated organic wheat flour with a_w 0.45 was treated in a test cell at 80°C, it took 15 min for 4-log reduction. A comparable result was observed for pet food pellets, where the 5-log reduction time was predicted as 15.4 min from the Weibull model for slightly higher a_w (0.54) sample treated at 80°C. Rachon et al. (2016) observed that the 5-log reduction times calculated from D-value at higher temperatures were significantly lower than the times calculated using the Weibull model. Rachon et al. (2016) calculated 39.5 min as a 5-log reduction time from the Weibull model for pet food at 0.65 a_w treated at 70°C. In the current experiment, 52.1 min was required to achieve a 5-log reduction of *Salmonella* in pet food pellets with 0.75 a_w treated at 70°C. Despite the higher a_w of pet food pellets, the 5-log reduction time was also higher in the current study. This difference could be due to the difference in form of pet foods used in the study by Rachon et al. (2016), which was in powder form and the samples in the current study were in pellet form.

The adsorption isotherms at selected temperatures for pet food pellets and black pepper powder developed in this project may help to understand the change in a_w during thermal treatments at those temperatures (Fig. 6 & 7). This is important data and information because of the following reasons. During thermal treatments, in a closed environment, the water content of the sample remains same, but a_w of food samples may change during thermal treatments (Liu,

Tang, Tadapaneni, Yang, & Zhu, 2018). Hence, the a_w of the pet food pellets and black pepper powder will change due to the increase in temperature during thermal treatments. The degree of change in a_w of food sample is influenced by various factors, including water content, temperature and the interaction of the food constituents with water molecules (Labuza & Altunakar, 2007; Liu et al., 2018). Pet foods contain mainly proteins and fat, and their interactions with water molecules may differ. The water molecules may interact with a hydrophilic end of the protein structure but there may be less interaction between hydrophobic lipid molecules and water. So, it is difficult to predict the changes in a_w in pet food pellets with a change in temperature, which makes it necessary to generate their isotherms.

The GAB equation was found to represent adequately the experimental sorption data in different food products, such as, all-purpose flour (Syamaladevi et al., 2016a), spray dried tamarind pulp powder (Muzaffar, & Kumar, 2016), whole black peppercorn (Yogendrarajah, et al., 2015), spray dried orange juice (Sormoli & Langrish, 2015), and dried fruits (Maroulis et al., 1988). The results obtained from the modeling of the adsorption isotherm data of pet food pellets and black pepper powder based on the GAB equation showed the decrease in monolayer water content (X_m) (dry basis) when the temperature increased. The monolayer water contents for adsorption isotherms in other LMF products such as spray dried tamarind pulp powder, purple flesh sweet potato, spray dried orange juice, and whole black peppercorn also decreased with an increase in temperature (Muzaffar, & Kumar, 2016; Saleh, et al., 2018; Sormoli & Langrish, 2015; Yogendrarajah et al., 2015). This might be due to a decrease in the number of water binding sites with an increase in temperature as a result of physical, chemical and structural changes in food products (Quirijns, et al., 2005). This monolayer water content could be a measure of bound water in food materials which may not be available for chemical reactions or microbial growth, that can

cause spoilage in the food (Saleh et al., 2018). The monolayer water content is considered as the value of water content for maximum stability of dry food products (Labuza, 1975; Yogendrarajah, et al., 2015). Similarly, with an increase in treatment temperature, the values of the GAB parameter, C, for adsorption isotherms were found to decrease in the case of pet food pellets. A similar result has been reported for various LMFs which includes Sultana raisins (Maroulis et al., 1988), and purple flesh sweet potato (Saleh, et al., 2018). But the value of C increased with increase in temperature for black pepper powder samples. Yogendrarajah et al. (2015), also reported an increase in C value (estimated from GAB equation) for adsorption isotherms in whole black peppercorns with an increase in temperature. The increase in C value with increase in temperature indicates the increase in strength of binding of water to primary binding sites of food (Quirijns et al., 2005).

For pet food pellets, the influence of temperature on thermal inactivation kinetics was noticeable at initial a_w values of 0.54 and 0.33, however, the inactivation kinetics of pet food pellets with 0.75 a_w was similar at all the treatment temperatures used in this study (Fig 2). This peculiar observation may be because of the a_w change during thermal treatments. It was previously reported that a_w change during thermal treatment could influence the thermal inactivation kinetics of microorganisms in foods and this change in a_w may depend on the type of food products and their constituents (Syamaladevi et al. 2016a, Taylor et al., 2018; Xu et al., 2019). In the current study, the a_w of pet food pellets increased significantly from 0.33 to ~0.5 and from 0.54 to ~0.62 when the temperature was changed from 20 to 81ºC (Fig 6). However, little or no change in a_w was observed with temperature increase at high a_w (>0.70). The a_w of pet food pellets did not change from 0.75 when the temperature was increased from 20 to 81ºC (Fig 6). Very less or no difference in a_w of 0.75 a_w pet food pellets with an increase in treatment temperature from 20 to 81ºC might

be the reason for almost similar inactivation kinetics for *Salmonella* in 0.75 a_w pet food pellets treated at 65, 70 and 75ºC (Fig. 2). This also shows the less heat sensitivity of *Salmonella* in 0.75 a_w than 0.33, and 0.54 a_w pet food pellets as observed in Fig. 4. A decrease in a_w with temperature increase was reported in the case of peanut butter and the greater thermal resistance of *Salmonella* in peanut butter compared to all-purpose flour was attributed to this decrease in a_w in peanut butter in comparison to the increase in a_w of all-purpose flour (Syamaladevi et al., 2016a). The adsorption isotherms observed for pet food pellets in the present study was comparable to the adsorption isotherms of almond flour obtained by Xu et al., (2019). The study by Xu et al. (2019), showed that the degree of change in a_w with increasing temperature in wheat flour, whey protein, and almond flour was inversely related to the fat content of the samples. The higher D_{80^oC}-value for *Salmonella* in almond flour compared to the wheat flour at same a_w is due to little or no change in a_w of almond flour compared to wheat flour which observed an increase in a_w with a rise in temperature.

However, in the case of black pepper powder with all the tested a_w, treatment temperature significantly influenced the thermal inactivation kinetics. Also, the adsorption isotherms of black pepper powder at different temperatures indicated that the influence of temperature on a_w change was considerable at all the tested a_w in this study. For instance, the a_w of black pepper powder changed from 0.33 to ~0.55 and from 0.54 to ~0.70, and from 0.75 to ~0.83 when the temperature was increased from 20 to 81ºC (Fig 7). This increase in a_w of the food sample inside the test cell during heat treatment might help to reduce the thermal inactivation time at the treatment temperature. The influence of temperature on 5-log reduction time of *Salmonella* at 0.75 a_w was more noticeable in the case of black pepper powder compared to pet food pellets. For instance, the 5-log reduction time decreased from 509 min to 8 min when the temperature was increased from

65 to 75ºC for black pepper powder at 0.75 a_w while it decreased from 56.7 to 20.5 min when the temperature increased from 65 to 75ºC for 0.75a_w pet food pellets (Table 1 & 2). The change in a_w with temperature increase could be also contributed to the huge difference in 5-log reduction times at the tested temperatures in the case of 0.75 a_w black pepper powder in comparison to the unchanged a_w of pet food pellets (Table 1 & 2). Further, the slope of the relationship between log (5-log reduction times) and the temperature was much sharper for black pepper powder at 0.75 a_w compared to that of pet food pellets at the same a_w (Fig 4 & 5). Syamaladevi et al. (2016a) and Xu et al. (2019) observed an increase in a_w and decrease in thermal resistance in all-purpose flour and wheat flour, respectively, like in black pepper powder with an increase in temperature at same water content (dry basis). Black pepper powder is rich in carbohydrates, starch being the major component, along with alkaloids (Ravindran & Kallupurackal, 2012). In a carbohydrate-rich compound, carbohydrate dissolves in water as hydrogen bond is formed with water which breaks at an elevated temperature resulting in free water that escapes as water vapor, thereby increasing the a_w of the food (Tadapaneni et al., 2017).

Thermal treatment not only destroys the foodborne pathogens but also can change the quality of treated food products depending upon the intensity of heat, time of exposure, and product characteristics (Berk, 2013). In this study, for quality parameter analysis, pet food pellets equilibrated at 0.33, 0.54 and 0.75 were treated at three different temperatures ranging from 65 to 85°C for times at which maximum *Salmonella* inactivation was observed. Any distinct changes in TBARS values between heat treated and untreated pet food pellets were not observed in this study. A similar observation was reported for lipid oxidation for whole milk powder by Mahmoodani et al. (2018). Mahmoodani et al. (2018) reported higher lipid oxidation rate in fat-rich whole milk powder at higher temperatures. But, TBARS values of all samples at room temperature and 40°C

were not significantly different initially but difference increased with the storage time. TBARS-value for whole milk powder at 40°C was high at 7 months storage time after which the values declined. Similarly, Rao & Artz (1989) reported an increase in lipid oxidation with the increase in extrusion temperature (Hsieh & Huff, 1998). TBARS value in pork treated at 0.1 MPa was higher for samples treated at 55 and 60 °C before cold-storage compared to untreated samples (Huang, Wang, Wu, & Li, 2016). Most of these studies have presented the TBARS value of treated and untreated samples after storage time. The TBARS value immediately after treatment was analyzed and none of the samples was stored to study effects of storage in the current study.

Thermal treatment can alter the color, flavor and volatile components of heat sensitive spices like black pepper (Hertwig et al., 2015; Kim et al., 2012). The quality of black pepper is determined based on the piperine content which contributes to the major pungency of black pepper (Gorgani et al., 2017; Rathnawathie, & Buckle, 1983). The change in piperine content was analyzed after all the treatments to observe the influence of a_w and treatment temperature in the quality of black pepper powder in the current study. Previous studies reported no significant change in piperine content after the thermal treatment of black pepper powder. The piperine content of black pepper powder didn't change much after 180°C super-heated steam treatment for up to 20 seconds (Ban et al., 2018). Treatment of black pepper powder by radio-frequency heating for 2.5 min, that generated the maximum average temperature of 107.3°C, didn't affect the piperine content in black pepper powder (Wei et al., 2018). The piperine content in black pepper was affected by the a_w. Increase in piperine content was observed in the equilibrated black pepper powder samples with 0.33 and 0.54 a_w. The quality changes were not significant in heat treated black pepper powder with 0.33 a_w and 0.54 a_w except for 0.54 a_w sample treated at 80°C for 8 min and all heat treated 0.75 a_w black pepper powder samples in which piperine content was

significantly less (Table 5.). This shows the effect of the thermal treatment in the quality of black pepper powder depending upon the water content.

The thermal inactivation levels of *Salmonella* were influenced by treatment temperature and a_w as explained before in terms of statistical analysis and 5-log reduction time data. The lipid oxidation in pet food pellets was not affected by the treatment temperature and time used in this study. This information obtained in this study can be used to select the appropriate initial a_w, treatment temperature and time required to achieve an adequate reduction in *Salmonella* in pet food pellets and black pepper powder.

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

5.1. Conclusions

The main objective of this study was to determine the effect of a_w on thermal resistance of *Salmonella* and quality parameter in LMFs. This thesis was able to generate the data describing the effect of a_w, temperature, and time during thermal treatments of pet food pellets and black pepper powder.

There are many data available on modeling for thermal inactivation of *Salmonella* influenced by a change in a_w in various types of food products with different physical characteristics and chemical composition. But there are only a few studies that used the methodology most relevant to real food systems. Two different types of LMFs were equilibrated at three low-moisture conditions and treated at three different temperatures. For a more realistic approach, the cocktail strain of resistant *Salmonella* in real food products was selected and treated them in a conventional way by sealing them in aluminum test cells. The treatment method followed was widely accepted and the thermal treatment method used a water bath, set at different temperatures ranging from 60 to 85°C. Thermal treatments below 100°C may take longer times to achieve recommended log reductions in low-moisture food products. There is a need to develop a proper protocol to get the recommended inactivation of foodborne pathogens by thermal treatments at minimum temperatures and comparatively shorter times, at specific a_w values.

The study shows, prolonged heating or higher temperature is required to achieve recommended log reductions in *S. enterica* in the tested food products at low a_w conditions. Thermal inactivation of *S. enterica* in pet food pellets and black pepper powder was found to be

non-linear and could not be defined by the first order kinetics model. The Weibull model was used to fit the inactivation data better for both pet food pellets and black pepper powder. The study showed that a decrease in a_w required an increase in the inactivation time and treatment temperature to achieve the required reduction levels. The upward concavity with tailing effect (Fig 2. and 3.) showed the rapid rate of inactivation of less resistant *Salmonella* in the initial phase of treatment which slowed down afterward, that can be due to the heat resistant bacteria, which require more heat or time for the inactivation.

The validation of the pasteurization process for different types of food product requires the reduction of foodborne pathogens like *Salmonella* by 2 to 5-log, to ensure the safety of food products. With modeling of the thermal inactivation data with the Weibull model, the treatment times for 5-log reductions in *Salmonella* population in both products at different a_w and temperature conditions were determined. The 5-log reduction times increased with a decrease in a_w at a specific time for pet food pellets and black pepper powder. This result concludes that along with treatment temperature and time, a_w of food products also plays determining role in thermal destruction of foodborne pathogens. The 5-log reduction time data shows that heat treatment times can be quite long, especially at low a_w and temperature conditions. For example, the 5-log reduction times were ~72 and 157 min for pet food pellets and black pepper powder, respectively at 0.33 a_w and 75ºC. If the treatment temperature was 60ºC, the treatment time required to achieve 5-log reductions in *Salmonella* in black pepper powder with 0.75 a_w would be 509 min. This information may be used by food companies handling these products or similar products to develop and validate thermal treatments or reduce the number of experimental trials for process development and validation to reduce the occurrence of *Salmonella*. The high standard deviation

was observed for inactivation of *Salmonella* for most of the treatments. This can be attributed to the equilibration time effect, as treatments were done in triplicates but on different days.

Adsorption isotherms were developed at elevated treatment temperature (81ºC) for the tested products for the first time to understand the changes in a_w during thermal treatments. This information also helps to relate the a_w change with the thermal resistance of *Salmonella* and further improve processes. Water activity values of pet food pellets and black pepper powder were determined at 20°C and 81°C for the same water contents using the adsorption isotherms. When the temperature was increased from 20 to 81°C, a_w increased in both samples.

The change in quality parameters during thermal treatments may depend upon the type of product and quality parameter itself. Thermal treatments below 100°C caused little to no lipid oxidation in pet food pellets. The values for TBARS for treated samples didn't change considerably. However, piperine content in black pepper powder was found to be significantly different in some thermal treatment conditions. The data obtained for the change in lipid oxidation in pet food pellets and piperine content in black pepper powder from this thesis can help optimize the heat treatment conditions since that will give information on the changes in the crucial quality parameters in the tested products at different a_w and treatment temperature conditions. Also, further studies on quality changes corresponding to the thermal treatment conditions to achieve the required log reductions in target pathogens can help to develop standard process protocols for the LMFs, selected in this study. It may be also possible to develop the effective thermal treatment protocols by modifying the a_w to achieve the required level of pathogen inactivation at relatively short time at low treatment temperature with minimal change in quality parameters of the LMFs. Such protocols will help manufacturing companies to prepare safe LMFs at a reasonable cost and minimize recalls of LMFs and outbreaks of foodborne illnesses.

5.2. Recommendations

This study shed some more light upon the significance of a_w in thermal inactivation of foodborne pathogens. Further research should be carried out on inactivation modeling for other LMFs. The a_w of food product changes during thermal treatments with a change in treatment temperature. As the change in a_w of food products depends upon the composition and matrix of foods, further studies should be conducted for food products with different compositions. Future studies can focus on considering this change in a_w during thermal treatments to relate to the thermal resistance of pathogenic microorganisms in LMFs. More research work may be worthwhile, following this study to ensure the development of effective process controls in eliminating *Salmonella* contamination in LMFs.

1. The high standard deviation between replicates shows that the inoculation process and incubation time influences the thermal resistance of *Salmonella*. Hence future work on the effect of inoculation methods and incubation time would be helpful in getting results that would provide further insight into the practical approach.
2. There are conflicting opinions on the inactivation trend of *Salmonella* by thermal treatments. Studies on heat inactivation of foodborne pathogens in different types of LMFs and comparative analysis of the results would be helpful in determining the inactivation trends in such products.
3. The change in a_w with an increase in treatment temperature is worthwhile for further study as still there are not enough data available on a_w change at different temperature levels influenced by the food matrix. This data should be related to the thermal resistance of the target pathogens in LMFs to accurately design thermal processes.

4. In general, quality parameters of LMFs are less temperature sensitive compared to microorganisms. More studies on the optimization of thermal treatment conditions for reducing quality changes in different LMFs, while achieving the required reduction in target pathogens are needed.
5. Though many studies have been conducted on the effect of a_w on thermal inactivation of different pathogenic bacteria, the mechanisms behind the thermo-tolerance of bacteria in response to a decrease in a_w have not been understood well. More research and a better understanding of the fundamental mechanisms behind the increased thermal resistance of bacteria at low a_w conditions would advance science and help in designing better thermal process protocols for LMFs.

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APPENDICES

APPENDIX - I

Table 1. The come-up time for pet food pellets to reach different temperatures.

Temperature of the water bath (°C)	Average come-up time (min)
75	3.04
85	3.05
95	3.12

Table 2. The come-up time for black pepper powder to reach different temperatures.

Temperature of the water bath (°C)	Average come-up time (min)
75	2.18
85	2.39
95	2.42

APPENDIX - II

Table 3: The change in the population of bacteria in 3 min come-up time for different pet food pellets.

Sample	log(N/No)	log(N/No)'	log(N/No)''	AVG	STDEV
a_w=0.33 / Temp.=75°C	-0.42	-0.70	-0.11	-0.41	0.29
a_w=0.33 / Temp=80°C	-0.30	-0.50	-0.65	-0.48	0.18
a_w=0.33 / Temp.=85°C	-0.42	-0.80	-0.86	-0.69	0.24
a_w=0.54 / Temp=75°C	-0.75	-0.43	-0.21	-0.46	0.27
a_w=0.54 / Temp=80°C	-0.56	-0.90	-1.02	-0.82	0.24
a_w=0.54 / Temp=85°C	-1.28	-1.32	-1.38	-1.32	0.05
a_w=0.75/ Temp=65°C	-0.48	-0.25	-0.41	-0.38	0.12
a_w=0.75 / Temp=70°C	-1.44	-1.59	-1.35	-1.46	0.12
a_w=0.75 / Temp=75°C	-1.60	-1.88	-2.16	-1.88	0.28

Table 4: The change in the population of bacteria in 2 min come-up time for different black pepper powder samples.

Sample	log(N/No)	log(N/No)'	log(N/No)''	AVG	STDEV
a_w=0.33 / Temp.=75°C	-0.54	-0.15	-0.18	-0.29	0.22
a_w=0.33 / Temp=80°C	-0.22	-0.58	-0.14	-0.31	0.23
a_w=0.33 / Temp.=85°C	-1.48	-1.50	-1.50	-1.49	0.01
a_w=0.54 / Temp=70°C	-0.20	-0.20	-0.18	-0.19	0.01
a_w=0.54 / Temp=75°C	-0.83	-0.93	-0.89	-0.88	0.05
a_w=0.54 / Temp=80°C	-0.60	-1.78	-1.33	-1.24	0.60
a_w=0.75 / Temp=60°C	-0.41	-0.36	0.05	-0.24	0.25
a_w=0.75 / Temp=65°C	-0.55	-0.67	-0.47	-0.56	0.10
a_w=0.75 / Temp=70°C	-1.41	-1.32	-1.31	-1.35	0.05

APPENDIX - III

SAS Code for *Salmonella* inactivation in Pet food pellets with 0.33, 0.54 and 0.75 a_w treated at three temperatures ranging from 65 to 85 °C.

```
options nodate linesize=140;
data petfooddat;
length trtmtID $3. trtmt $18. rep$3. ;
input trtmtID trtmt      aw temp time rep logreduction;
run;

/*proc print data = petfooddat;
run;*/
/* Also see CuttingBoard_CheckDistribnFit.R ; Data is almost normally distrubuted */

title'Analysis on count of bacteria';

/*ods graphics on;
title “Summary statistics for pet food data”;
proc means data=petfooddat maxdec=2;
class trtmtID trtmt      aw temp time ;
var logreduction;
run;
ods csv close;
ods graphics off;*/

/*************************************************************************
**********************************************************/
```

```
/***Below Proc Mixed code with call to pdmix800 macro was run to generate Stats1c file with
letter groupings for significant differences***/

/*************************************************************************************
*************************************************************/

/********************************************** First ran the macro for the pdmix800
*************************************************/

ods graphics on;

proc mixed data = petfooddat plots=all nobound;

title2 ’Analysis of pet food data’;

class trtmt       ;

model logreduction = trtmt;

lsmeans trtmt  / cl adjust = tukey;

ods output diffs = ppp lsmeans = mmm ;

ods listing exclude diffs lsmeans;

run;

/* %include 'pdmix800.sas';   commented because could not load this file ; so ran the macro for
pdmix800 first and then ran the proc mixed code */

%pdmix800(ppp, mmm, alpha = 0.05, sort = yes);

title3 ' ';

title4 'PROC MIXED ANOVA';

run;

ods csv close;

ods graphics off;

/*************************************************************************************
*************************************/

/*************************************************************************************
*************************************/

/*************************************************************************************
*************************************/

/*************************************************************************************
*************************************/
```

APPENDIX - IV

SAS Code for *Salmonella* inactivation in black pepper powder with 0.33, 0.54 and 0.75 a_w treated at three temperatures ranging from 60 to 85 °C.

```
options nodate linesize=140;
data bppdat;
length trtmtID $3. trtmt $18. rep$3. ;
input trtmtID trtmt      aw temp time rep logreduction;
run;
/*proc print data = bppdat;
run;*/
/* Also see CuttingBoard_CheckDistribnFit.R ; Data is almost normally distrubuted */

title'Analysis on bpp data';

/*ods graphics on;
title “Summary statistics for bpp data”;
proc means data=bppdat maxdec=2;
class trtmtID trtmt      aw temp time ;
var logreduction;
run;
ods csv close;
ods graphics off;*/

/*************************************************************************
*************************************************************/
/***Below Proc Mixed code with call to pdmix800 macro was run to generate Stats1c file with
letter groupings for significant differences***/
```

```
/*************************************************************************
*************************************************************/

/********************************************** First ran the macro for the pdmix800
*************************************************/

ods graphics on;


proc mixed data = bppdat plots=all nobound;

title2 ’Analysis of bpp data’;

class trtmt       ;

model logreduction = trtmt / ddfm = kr ;

lsmeans trtmt  / cl adjust = tukey;

ods output diffs = ppp lsmeans = mmm ;

ods listing exclude diffs lsmeans;

run;

/* %include 'pdmix800.sas';   commented because could not load this file ; so ran the macro for
pdmix800 first and then ran the proc mixed code */

%pdmix800(ppp, mmm, alpha = 0.05, sort = yes);

title3 ' ';

title4 'PROC MIXED ANOVA';

run;

ods csv close;

ods graphics off;
/*************************************************************************
*************************************/

/*************************************************************************
*************************************/

/*************************************************************************
*************************************/

/*************************************************************************
*************************************/
```

APPENDIX - V

SAS code for lipid oxidation of heat-treated pet food pellets.

```
options nodate linesize=140;
data PetFoodLipiddat;
length trtmtID $3. trtmt $18. rep$3. ;
input trtmtID trtmt      aw temp time rep lol;
run;
/*proc print data = PetFoodLipiddat;
run;*/
/*ods graphics on;
title “Summary statistics for PetFoodLipidOxidn data”;
proc means data= PetFoodLipiddat maxdec=2;
class trtmtID trtmt      aw temp time ;
var lol;
run;
ods csv close;
ods graphics off;*/
/*************************************************************************
**********************************************************/
/***Below Proc Mixed code with call to pdmix800 macro was run to generate Stats1c file with
letter groupings for significant differences***/
/*************************************************************************
**********************************************************/
/********************************************* First ran the macro for the pdmix800
*****************************************************/
ods graphics on;
proc mixed data = PetFoodLipiddat plots=all nobound;
title2 ’Analysis of PetFoodLipidOxidn data’;
class trtmt       ;
model lol = trtmt / ddfm = kr ;
```

```
lsmeans trtmt  / cl adjust = tukey;

ods output diffs = ppp lsmeans = mmm ;

ods listing exclude diffs lsmeans;

run;

/* %include 'pdmix800.sas';   commented because could not load this file ; so ran the macro for
pdmix800 first and then ran the proc mixed code */

%pdmix800(ppp, mmm, alpha = 0.05, sort = yes);

title3 ' ';

title4 'PROC MIXED ANOVA';

run;

ods csv close;

ods graphics off;
/*************************************************************************************
*****************************************/

/*************************************************************************************
*****************************************/

/*************************************************************************************
*****************************************/

/*************************************************************************************
*****************************************/
```

APPENDIX - VI

SAS code for piperine content in thermally treated black pepper powder.

```
options nodate linesize=140;
data BPPPiperinedat;
length trtmtID $3. trtmt $18. rep$3. ;
input trtmtID trtmt      aw temp time rep piperine;
run;

/*proc print data = BPPPiperinedat;
run;*/
/* Also see BPPPiperine_CheckDistribnFit.R ; Data is almost normally distrubuted */

title'Analysis on BPPPiperine data';

/*ods graphics on;
title “Summary statistics for BPPPiperine data”;
proc means data= BPPPiperinedat maxdec=2;
class trtmtID trtmt      aw temp time ;
var piperine;
run;
ods csv close;
ods graphics off;*/
/**********************************************************************************
*****************************************************/
/***Below Proc Mixed code with call to pdmix800 macro was run to generate Stats1c file with
letter groupings for significant differences***/
```

```
/*************************************************************************
*************************************************************/

/********************************************** First ran the macro for the pdmix800
*************************************************/

ods graphics on;

proc mixed data = BPPPiperinedat plots=all nobound;

title2 ’Analysis of BPPPiperine data’;

class trtmt       ;

model piperine = trtmt / ddfm = kr ;

lsmeans trtmt  / cl adjust = tukey;

ods output diffs = ppp lsmeans = mmm ;

ods listing exclude diffs lsmeans;

run;

/* %include 'pdmix800.sas';   commented because could not load this file ; so ran the macro for
pdmix800 first and then ran the proc mixed code */

%pdmix800(ppp, mmm, alpha = 0.05, sort = yes);

title3 ' ';

title4 'PROC MIXED ANOVA';

run;

ods csv close;

ods graphics off;
/*************************************************************************
*************************************/


/*************************************************************************
*************************************/

/*************************************************************************
*************************************/

/*************************************************************************
*************************************/
```