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Thermal Inactivation Kinetics of Salmonella in Milk Powder as Impacted by Water Activity and Powder Type

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Thermal Inactivation Kinetics of *Salmonella* in Milk Powder as Impacted by Water Activity and Powder Type

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Abstract

Historically, low-water activity (a_w) foods (a_w <0.65) were considered to be microbiologically safe for consumption. However, these foods have been implicated in outbreaks of pathogens such as *Salmonella enterica*, at a frequency that surely challenges this assumption of safety. Although usually implicated in outbreaks involving poultry and egg products, *Salmonella* spp. have frequently been the culprit in several outbreaks and recalls associated with low-water activity foods (LWAF) due to contamination resulting from the environment, animals, or even the employees during pre- or post-processing. One such LWAF that has been associated with *Salmonella* spp. outbreaks is milk powder. Milk powders are used in a variety of products ranging from infant formula to confectionary goods. With their widespread use, improving their safety is imperative. A key step in improving their safety is to thoroughly investigate preventive controls related to milk powder processing. The Food Safety Modernization Act (FSMA) established the Preventive Controls for Human Foods Rule, which mandates that processors design and establish a food safety plan outlining any potential hazards and the steps they will take to ensure those hazards do not compromise the safety of the product. Presently, the dairy industry does not have an established kill-step for milk powders post-spray drying. Spray drying may reduce some microbial populations, however, desiccation (e.g., spray drying) is not considered a kill-step. The work outlined in this thesis aims to assist our dairy industry partners in establishing their own thermal process for milk powders post-spray drying. Moisture sorption isotherms of two milk powders (nonfat dry milk: NFDM and a milk protein concentrate with 85% protein content: MPC-85) were determined and showcased that the temperatures at which the isotherms were determined (23, 40, or 60°C) significantly affected both the adsorption and desorption isotherms ($p < 0.0001$) for each powder. Secondly, the thermal inactivation kinetics of

Salmonella spp. in both powders were evaluated at two different water activities (0.20 and 0.30) and three temperatures (75, 80, and 85°C) to determine the effect of water activity on the thermal resistance of a cocktail of *Salmonella* spp. The *D*-values of *Salmonella* were heavily influenced by the thermal treatment temperature ($p < 0.0001$), but not the water activity ($p > 0.05$). The overall findings contained within this thesis provide valuable information about the thermal inactivation kinetics of *Salmonella* spp. to our partners in the dairy industry so that they may implement an appropriate thermal process for their milk powders.

Acknowledgements

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Dedication

This thesis is dedicated to my mom and dad, Tina and David Kadas.

To Mom and Dad,

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List of Acronyms

A_w- water activity

CUT- come-up-time

DDI- dynamic dewpoint isotherm

EMC- equilibrium moisture content

ERH- equilibrium relative humidity

LWAF- low-water activity food(s)

MPC-85- milk protein concentrate with 85% protein content

NFDM- nonfat dry milk

RH- relative humidity

TDT- thermal-death-time

VSA- vapor sorption analyzer

Chapter 1: Literature Review

1.1. Burden of Foodborne Illness Related to LWAF

Low-a_w foods (LWAF) have a_w <0.65 (FAO, 2003) and were previously regarded as microbiologically safe due to the lack of available water and inhibition of microorganism growth (Beuchat et al., 2013; Gurtler et al., 2014). Foods with a_w <0.65 include chocolate, cereal, cocoa powder, some dried fruits and vegetables, powdered infant formula (PIF), nut butters, spices, and milk powders (Beuchat et al., 2013). Despite the reduced water activity, notable outbreaks in LWAF include peanut butter (CDC, 2007; CDC, 2009), chocolate (Werber et al., 2005), PIF (Jourdan-Da Silva et al., 2018; FDA, 2022), and cereal (CDC, 2008). Various serotypes (including but not limited to, *S.* Typhimurium, *S.* Tennessee, *S.* Newport, and *S.* Montevideo) of *Salmonella* were implicated in LWAF outbreaks between 2000 and 2019, which caused 3880 illnesses, 659 required hospitalization, and 15 deaths (Dhowlaghar & Zhu, 2021). From 2012 to 2016, *Salmonella* was implicated in ~30% of outbreaks associated with LWAF (Smith et al., 2016; CDC, 2016). The frequency of *Salmonella* associated with LWAF outbreaks is attributed to its ability to survive for prolonged periods of time in LWAF and presumed increased thermal resistance in LWAF (Beuchat et al., 2013). The long-term survival of *Salmonella* in LWAF and the fact that many LWAF are ready-to-eat (RTE) (e.g., peanut butter and chocolate) creates a particularly unique food safety risk for LWAF processors and consumers.

In the case of dairy powders, the two pathogens of the greatest concern are *Cronobacter sakazaii* (formerly *Enterobacter sakazaii*) and *Salmonella enterica* subsp. *enterica*. *Cronobacter* spp. can cause septicemia, meningitis, and necrotizing enterocolitis in infants (Hayman et al., 2020). *Salmonella* infections are characterized by (sometimes bloody) diarrhea, fever, and

abdominal cramps. In some cases, *Salmonella* can cause severe disease, such as septicemia, if infection occurs in the blood (CDC, 2019). *Salmonella* is a leading cause of foodborne illness in the U.S., and children under the age of 5 are at an increased risk of infection due to underdeveloped immune systems (CDC, 2019). Contamination of PIF or other milk powders with these two pathogens could have devastating consequences.

1.2. Economic Impact of Milk Powders and Recalls

The milk powder industry has the potential to grow by $25.38 billion (USD) from 2020-2024 (Technavio, 2020). In 2013, milk production around the globe was estimated to be around 466 million metric tons (mt), with the largest producers being the United States, the European Union (EU), and India (Lagrange et al., 2015). Furthermore, from 2009 to 2013, global production of powdered milk increased from 3.7 to 4.5 million tons (Lagrange et al., 2015; Steinbrunner et al., 2021). With both trade and the human population growing dramatically each year, these increases are not surprising.

About 5% of the produced cow milk is traded globally, with the products traded being primarily manufactured dairy products such as dry milk powders, butter, and cheese (U.S. Dairy Export Council, 2014; Lagrange et al., 2015). Around the world, milk powders are primarily used for nutrition products for children, bakery and confectionary products, and fortification of dairy products (Lagrange et al., 2015). In the U.S., for example, the American Dairy Products Institute (ADPI) reports the country's top uses being dairy (64%), bakery and confectionary (7% and 19%, respectively), prepared mixes (3%), and nutritional products (3%) (ADPI, 2012). With the variety of uses of dairy powders, ensuring their safety is vital to prevent costly recalls and outbreaks.

From the period of 2009-2015, the Foodborne Disease Outbreak Surveillance System (FDOSS) reported that dairy foods were linked to 136 foodborne outbreaks in the U.S. (Dewey-Mattia et al., 2018). Most recently, the recall of infant formula containing *C. sakazakii* rattled consumers as the outbreak resulted in the death of two infants (FDA, 2022). The Abbott Nutrition plant in Sturgis, Michigan was a main producer of infant formula in the U.S., and the consequences of the plant being shut down while the FDA's investigation was ongoing were felt all over the country as parents panicked due to being unable to find formula to feed their infants.

1.3. FSMA and the Dairy Industry

The Food Safety Modernization Act (FSMA) was signed into law in 2011, aiming to reprioritize the United States' approach to foodborne illness from reactive to preventive. To achieve this goal, the United States Food and Drug Administration (FDA) published the Preventive Controls for Human Foods (PCHF) Rule (Gombas, 2019), requiring processors to have a food safety plan outlining hazards that could compromise product safety and to develop preventive measures (FDA, 2020).

Presently, the dairy industry does not have a standard thermal or nonthermal pasteurization process for dairy powders. In the production plant, the milk is spray dried between 180-220°C (Kim et al., 2009). Spray drying at these temperatures may result in some reduction of microorganisms, but spray drying alone is not a kill-step, and post-pasteurization contamination could occur at various points of the processing. Furthermore, spray dryers have the potential to be reservoirs for pathogens that can contaminate the product (Steinbrunner et al., 2021). For example, in 1985, an outbreak of *S.* Ealing in infant formula in the U.K. was traced back to cracks in the walls of the spray dryer (Rowe, 1987). If the powder is contaminated following spray drying and prior to packaging, there is no final step to remove any microbial

contamination. Previous studies have shown that both *Salmonella* spp. and *C. sakazakii* can survive spray drying temperatures if there has been post-pasteurization contamination of the milk (Licari & Potter, 1970; Olsen et al., 2004; Arku et al., 2008). Arku et al. (2008) demonstrated this scenario by inoculating *C. sakazakii* into reconstituted skim milk, followed by spray drying the milk (inlet 160°C and outlet 90°C), and enumerating the surviving *C. sakazakii*. Regardless of inoculation level (ca. 2 and 7 log CFU/g) *C. sakazakii* survived the spray drying process. Both *C. sakazakii* and *Salmonella* spp. may employ cellular mechanisms to resist the desiccation conditions and high temperatures used during spray drying (Arku et al., 2008; Licari & Potter, 1970; Miller et al., 1972).

In 2014, the FDA conducted a sampling assessment to evaluate the prevalence of environmental *Cronobacter* spp. and *Salmonella* spp. in 55 U.S. processing facilities manufacturing milk powders (Hayman et al., 2020). Of these 55 facilities, they detected *Cronobacter* spp. in 38 (69%) of these facilities and *Salmonella* spp. in 3 (5.5%) of these facilities (Hayman et al., 2020). Moreover, many studies have observed prolonged survival of salmonellae in dairy and other confectionary powders (Santillana Farakos et al., 2013; Tsai et al., 2019; Sekhon et al., 2021). The prevalence of foodborne pathogens in LWAF processing facilities and their abilities to survive for extended periods create challenges for manufacturers of products that consumers expect to be RTE. These studies highlight the pervasiveness of both *Cronobacter* spp. and *Salmonella* spp. in food processing environments and that measures must be taken to reduce their prevalence in processing facilities and ultimately in the final powders.

1.4. Thermal Inactivation of Pathogens in Milk Powders

Recognizing the limitations of spray drying as a kill-step and the expectations of the PCHF rule, thermal inactivation studies are conducted for pathogens in dairy powders to provide

information to the dairy industry that will improve the safety of their products and facilitate compliance. Providing information about thermal inactivation kinetics of pathogens can inform processor preventive controls for milk powders (e.g., time and temperature combinations). Thermal inactivation studies of *Salmonella* spp. to determine the *D*- and z-values in LWAF have been done using thermal cells (Rachon et al., 2016; Smith et al., 2016), a novel thermal-water activity cell (Tadapaneni et al., 2017), and more recently, using custom-designed thermal death time (TDT) sandwiches (Lau & Subbiah, 2020; Wei et al., 2020; Wei et al., 2021). A study comparing three methods (thermal-death-time disks [TDT disks] in a hot water bath, aluminum pouches in a hot water bath, and TDT Sandwiches) provided insight into the advantages and limitations of each of these methods (Lau et al., 2021). This study found that even though the thermal inactivation results were not significantly different across the three methods (i.e., the methods were interchangeable), they found that the TDT Sandwich method produced thermal inactivation model parameters with less variance than the other two methods (Lau et al., 2021). The most notable difference between the methods was in the determination of come-up-time (CUT). The study found that the TDT Sandwiches had much smaller variability in CUT values compared to the TDT disks and pouches. This is perhaps attributed to the fact that the pouches and TDT disks are heated in a water bath, so the distribution of temperature may not be as even as the distribution of temperature in the TDT Sandwiches, which simply has the sample in an aluminum pouch sandwiched between two aluminum plates (Lau et al., 2021). These studies push the dairy industry closer to the goal of having recognized and validated thermal processing steps following spray drying.

1.4.1. Effects of Experimental Methods on Thermal Reduction of Pathogens in LWAF

Additionally, thermal inactivation kinetics and survival of *Salmonella* spp. in LWAF can be affected by a variety of factors such as inoculation method (Hildebrandt et al., 2016; Liu et al., 2019; Wiertzema et al., 2019), reduction of a_w (Laroche et al., 2005; Smith et al., 2016; Syamaladevi et al., 2016; Tsai et al., 2019), food composition/matrix (C. Li et al., 2014; H. Li et al., 2014), or a combination of these factors (Santillana Farakos et al., 2014; Jin et al., 2018). Finn et al. (2013) emphasizes that all of these factors must be taken into account when examining the thermal resistance and survival of *Salmonella* spp. in LWAF, and that just accounting for the food's a_w is not sufficient.

Researchers have noted differences in stability and thermal inactivation of *Salmonella* and other pathogens which could be attributed to different inoculation methods for LWAF. Inocula using lawn-based cultures have been reported to produce the most repeatable *D*-values and most stable inoculum compared to broth-based cultures of *Salmonella* when evaluating wheat flour (Hildebrandt et al., 2016; Wiertzema et al., 2019). Dry inoculation methods have also been studied to determine if differences exist when foods are inoculated with a liquid inoculum or a dried carrier. Liu et al. (2019) examined dry methods of inoculation and demonstrated that using silicon dioxide as a dry vehicle for inoculation resulted in equal or higher *D*-values, but equal z-values of *Salmonella* when compared to using a small portion of the powder to inoculate the rest of the nonfat dry milk (NFDM). However, dry inoculation methods have shown variability in their effectiveness, and the protocols are more laborious (Hildebrandt et al., 2017). These studies demonstrate that in addition to the low-a_w conditions of these foods, inoculation protocols can affect the thermal inactivation kinetics of pathogens and can also significantly affect the results of inoculum shelf-stability.

The amount of available water in a food greatly impacts the growth and survival of bacteria. Studies have indicated that as a_w decreases, thermal resistance and survivability of *Salmonella* increases in LWAF (Lian et al., 2015). Laroche et al. (2005) inoculated wheat flour and milk powder with *Saccharomyces cerevisiae* and *Lactobacillus plantarum* and treated the powders at 150 or 200°C. In wheat flour, *S. cerevisiae* was most heat resistant at a_w 0.40, while *L. plantarum* was most heat resistant at a_w 0.35. In milk powder, *S. cerevisiae* was most heat resistant at a_w 0.40, while *L. plantarum* was most heat resistant at a_w 0.30 (Laroche et al., 2005). The general trend is that with decreasing water activity, there is increased thermal resistance of *Salmonella*. However, one study showed that the type of solute used to lower water activity affected the thermal resistance of *Salmonella* (Mattick et al., 2001). The authors used a glucose-fructose solution, sucrose, and NaCl to lower the water activity in tryptic soy broth (a_w 0.65-0.90) and found that for each solute evaluated, at temperatures ≥ 70°C, the *Salmonella* cells at a low a_w were more heat resistant than those at a higher a_w. However, Mattick and authors found that at temperatures below 65°C, the reverse was true. It was concluded that this temperature dependence was independent of solute type used, but the extent of the protection afforded by the solute type did vary with sucrose demonstrating the highest protective effect (Mattick et al., 2001). These studies highlight the varying response of different microorganisms to heat when a_w is adjusted.

Interactions between a_w, temperature, and food composition may impact thermal inactivation. Moreover, the food composition can affect the degree to which a_w impacts thermal resistance of pathogens. For example, *Salmonella* exhibited significantly higher $D_{80°C}$-values in peanut butter (a_w = 0.45) versus all-purpose wheat flour (a_w = 0.45) (Syamaladevi et al., 2016). This difference could potentially be attributed to the fat content of peanut butter providing a

protective effect to the *Salmonella* as previously described (Werber et al., 2005). *S.* Agona exhibited a higher $D_{79°C}$-value at a_w 0.63 in a high-protein matrix compared to a high-fat matrix (Jin et al., 2018). Quinn et al. (2021) found higher $D_{60\text{-}90°C}$-values for *Salmonella* than *L. monocytogenes* from treatments of peanut butter (45% fat) and most infant formula (27% fat) samples but found insignificantly different *D*-values for pathogens on wheat flour (<2% fat). These results were consistent with other similar studies of foodborne pathogens on LWAF (Syamaladevi et al., 2016; Rachon et al., 2016; Liu et al., 2019).

Pathogen resistance specifically in dairy powders has been the subject of much study because of past outbreaks and recalls, as both a_w and composition of the powder likely play significant roles in the thermal inactivation rates. A study performed by Lang et al. (2017) found that at 85°C, the *D*-values of all four microorganisms tested (*Escherichia coli*, *S.* Typhimurium, *S.* Senftenberg, and *C. sakazakii*) increased by 2.3-3.0-fold as a result of lowering the a_w of WMP from 0.58 to 0.11. Wei et al. (2020) treated *Salmonella*-inoculated NFDM and calculated a $D_{80°C}$-value of 9.82 min and a $D_{80°C}$ of 10.46 min at a_w 0.30 and 0.20, respectively. These two studies highlight that as water activity decreased, the *D*-values increased for the same temperature, thereby demonstrating the increased thermal resistance of *Salmonella* in LWAF. Sekhon et al. (2021) reported the *D*- and z-values of *Salmonella* for both dry and hydrated NFDM and whole milk powder (WMP) during extended storage periods (180 days). The *D*-values (80, 85, and 90°C) of *Salmonella* for the WMP (a_w 0.205±0.001 on Day 1 and a_w 0.243±0.005 on Day 180) were found to increase from 18.9, 9.9, and 4.4 min on Day 1 to 29.4, 13.6, and 6.5 min on Day 180 of storage (~20°C), while those from NFDM remained similar throughout storage (Sekhon et al., 2021). No significant differences were found in the *D*-values once the powders were hydrated, but the authors did find a difference in the z-value of *Salmonella* in the NFDM and

WMP when they were hydrated (16.3°C vs. 6.4°C, respectively), highlighting the impact of food composition and moisture on *Salmonella* thermal resistance (Sekhon et al., 2021).

1.5. Use of a Surrogate

Surrogates are used for process validations in laboratories and production facilities where introduction of a pathogen is not recommended. Surrogates to be used in thermal process validations are selected based on key criteria: must be nonpathogenic to humans, must be easily distinguishable from the native microflora of a food, and must exhibit higher resistance to the treatment, but still exhibit a similar death trend to the pathogen for the desired process (Hu & Gurtler, 2017). In the case of LWAF, there are two bacteria commonly used or investigated as *Salmonella* surrogates in thermal processes: *E. faecium* NRRL B-2354 and *Pediococcus acidilactici* ATCC 8042 (Acuff et al., 2020). The use of either one depends largely on the specific food to be processed and the target pathogen(s).

E. faecium NRRL B-2354 is a very common surrogate that has been shown to be appropriate for *Salmonella* in a number of thermally treated LWAF such as milk powders, confectionary, and dried basil leaves (Rachon et al., 2016; Verma et al., 2018; Tsai et al., 2019; Dhowlaghar & Zhu, 2021; Steinbrunner et al., 2021; Verma et al., 2021; Wei et al., 2021). Rachon et al. (2016) found that the $D_{80°C}$-values of *E. faecium* NRRL B-2354 in chicken meat powder, culinary seasoning, and pet food were around 3 to 4 times higher than the $D_{80°C}$-values of *Salmonella* in the same products. A study on evaluating *E. faecium* NRRL B-2354 as a surrogate for *Salmonella* during the extrusion of oat flour performed by Verma et al. (2018) found *E. faecium* to exhibit higher heat resistance compared to *Salmonella* for all conditions evaluated (different fat contents, moisture contents, and screw speeds). Another study performed

by Verma et al. (2021) found that the *D*-values for *E. faecium* NRRL B-2354 were about 1.4 to 2.8 times greater than those of *Salmonella* for all conditions tested in thermally treated dried basil leaves. Although *E. faecium* NRRL B-2354 demonstrated its suitability as a surrogate in these studies, its suitability as a surrogate for *Salmonella* must still be evaluated for similar products and processes.

However, *E. faecium* has not always shown itself to be an appropriate surrogate for *Salmonella* in all LWAF. Rachon et al. (2016) found *E. faecium* was not an appropriate surrogate for *Listeria monocytogenes* and *Salmonella* in sugar-containing confectionary, perhaps due to the sugar offering a protective effect on the pathogen, thereby increasing its thermal resistance. *P. acidilactici* was noted to be more suitable than *E. faecium* on low-moisture pet food products treated between 60-90°C, as its *D*-values were still higher than those of *Salmonella* but lower than those of *E. faecium* (Ceylan & Bautista, 2015). Tsai et al. (2019) found *E. faecium* was not always an appropriate surrogate for *Salmonella* in cocoa powder depending on the a_w of the powder. When the a_w of the cocoa powder was higher ($a_w = 0.45$), *E. faecium* was less resistant to the storage conditions than *Salmonella*. However, at a lower a_w (0.30) *E. faecium* was more resistant to the storage conditions than *Salmonella* (Tsai et al., 2019). These studies emphasize the importance of intrinsic factors (such as a_w) of foods when it comes to process validation for surrogates. Therefore, a potential surrogate must be validated for each process, target pathogen, and food product to ensure appropriate processing and confidence.

1.6. Kill-Ratio from Thermal Inactivation Kinetics

To provide meaningful comparisons of thermal inactivation kinetics of pathogen to surrogate, the development of a kill-ratio of pathogen to surrogate thermal inactivation kinetics

may be used. It is thought that a kill-ratio of *Salmonella* to *E. faecium* can be used to aid industry partners in developing thermal treatments for dairy powders which prioritize safety with minimal loss of quality. Verma et al. (2021) described the kill-ratio as the ratio of the *D*-values of *Salmonella* and *E. faecium*. As stated previously, the surrogate must exhibit a similar death trend to the pathogen. If the surrogate is excessively resistant to the treatment, the dairy powder may be thermally treated beyond what is necessary to inactivate *Salmonella* and negatively affect the quality of the powder. Furthermore, a robust kill-ratio can still be accurate to predict nonlinear inactivation trends. Wei et al. (2020) found that the inactivation curves of *Salmonella* for all temperatures and a_w levels tested (75, 80, and 85°C, and a_w 0.10, 0.20, and 0.30, respectively) for both NFDM and WMP follow a log-linear trend with high R^2 values. However, studies have shown that nonlinear models, such as the Weibull model, can at times be more appropriate for accurately characterizing *Salmonella*'s thermal resistance in LWAF (Podolak et al., 2010; Santillana Farakos et al., 2013). These other models can account for curvature in the inactivation trend and allows for other variables to be considered in the inactivation, such as temperature and a_w and the role those factors play in the thermal inactivation kinetics of *Salmonella*.

1.7. Conclusion

Low-water activity foods are a significant component of many diets worldwide, and proper thermal processing will improve the safety of these products. Milk powders remain vulnerable to pre- and post-processing contamination and subsequent recalls resulting from foodborne illness outbreaks due to *Salmonella* and other pathogenic bacteria. Thermal inactivation research will aid the dairy industry in making informed decisions about implementation of thermal processes in their facilities for milk powders.

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Chapter 2: Moisture Sorption Isotherms of Nonfat Dry Milk and Milk Protein Concentrate with 85% Protein Content

2.1. Introduction

The availability of water in the food system affects both the product stability and microbiological safety of the food, of which the latter is of the utmost importance. To address how water influences the microbiological safety of a food, water activity (a_w) must be defined. Water activity, as it pertains to food, is the ratio of the vapor pressure of a food when in complete equilibrium with the surrounding environment, to the vapor pressure of pure water under identical conditions (i.e., temperature and pressure) (Schmidt & Lee, 2012; FDA, 2014). Water activity is used as a metric of the availability of water in a food. In terms of microbiological safety, a_w is used to describe the amount of water in a food that is available for microorganisms to utilize for their growth (Beuchat et al., 2013). Related to, but not interchangeable with the measurement of a_w, is the moisture content of a food. Moisture content is the amount of water in a food. Technically, moisture content is described as the mass of water in a food per mass of dry food (Schmidt & Lee, 2012). The relationship between moisture content and a_w in a food can be depicted through a moisture sorption isotherm of a sample of the food. The moisture sorption isotherm is the moisture content of a food plotted as a function of the a_w of the food (i.e., food relative humidity) at a constant temperature (Schmidt & Lee, 2012).

Historically, a variety of methods have been used to determine the moisture sorption isotherm of a food. Two well-known methods are the saturated salt slurry method and the dynamic vapor sorption (DVS) isotherm method. These methods, although useful, have their limitations. The saturated salt slurry method involves placing the food over salt slurries that are at a temperature-dependent a_w contained in a closed container with desiccators and then waiting

for the food to reach the same a_w as the salt slurry via a minimal weight change. Although advantageous because this method generates precise a_w values, this method requires extensive time and labor due to having to wait for the sample to equilibrate, resulting in potential mold growth during long equilibration periods at $a_w \geq 0.70$ (Yu et al., 2008; Schmidt & Lee, 2012). The DVS method, on the other hand, is a modern and automated version of the saturated salt slurry method. Many modern moisture sorption instruments, such as the vapor sorption analyzer (VSA) used in this study, are built to automatically change the ratio of dry to wet gas to achieve different relative humidity levels and thereby change the water activity in a dynamic stepwise manner (Carter & Fontana, 2016). Although more modernized than the saturated salt slurry method, the DVS method also has its own limitations in that it can also take up to weeks and months to generate isotherms due to the sample having to reach gravimetric equilibrium before proceeding to the next relative humidity level (Mettler and Toledo, 2018).

For the present study, the comprehensive dynamic dewpoint isotherm (DDI) method was chosen due to the faster generation of isotherm curves (e.g., 24-48 hours vs. weeks or months) as well as higher resolution isotherms with hundreds of data points rather than only five or six by using a Vapor Sorption Analyzer (Aqualab VSA, Decagon Devices, Inc., Pullman, WA). The DDI method involves directly measuring a_w with a chilled mirror dewpoint sensor while weight changes in the sample are tracked using a balance. For adsorption, the air is saturated with water before entering the sample chamber and for desorption the air is passed through a desiccator before being able to enter the sample chamber. Once a small change in a_w occurs (usually around 0.015), the airflow is immediately halted, and a snapshot of the sorption is captured via a direct measurement of sample a_w and weight, which is then converted to moisture content (Schmidt & Lee, 2012). This process occurs repeatedly and can produce over 100 data points over a wide

range of a_w and temperature values (0.030-0.950 a_w; 15-60°C) (Schmidt & Lee, 2012; METER Group, 2022). The VSA creates equilibrium relative humidity conditions between 10% and 95%, which corresponds with a_w 0.10 to 0.95 at increments of 1%.

The moisture sorption isotherm varies greatly between products. Even though products can be very similar in physical structure (e.g., different types of milk powders), their isotherms can be very different. This has to do with a variety of factors such as protein content, amorphous lactose crystallization, and the temperature(s) at which the isotherm experiments are conducted (Bronlond & Paterson, 2004; Foster et al., 2005; Shrestha et al., 2007). Therefore, a one-size-fits-all approach is not appropriate when making decisions about a product's shelf life and stability.

The objective of the present study was to determine the moisture sorption isotherms of two milk powders: nonfat dry milk (NFDM) and a milk protein concentrate with 85% protein (MPC-85). Moisture content is a universal metric used in food production, therefore using the moisture sorption isotherms to indirectly describe the a_w could aid processors in making judgements about the properties of their food. Dairy powder producers or retailers may be more likely or able to determine the moisture content of a product or lot. The moisture sorption isotherms produced in this study will assist the dairy industry with correlating the moisture content with the a_w, and thus make better judgements about the product in terms of quality and microbiological safety.

2.2. Materials and Methods

2.2.1. Proximate Analyses of Milk Powders

Proximate analyses of nonfat dry milk (NFDM) (Mars Inc., McLean, VA) and a milk protein concentrate with 85% protein (MPC-85) (Dairy Farmers of America, Kansas City, MO)

were conducted prior to isotherm experiments according to official Association of Official Agricultural Chemists (AOAC) methods to determine initial moisture content, ash, protein, fat, and carbohydrate levels (AOAC 927.05, 930.30, 932.06, 930.29). The proximate compositions of both powders are shown in Table 2.1.

2.2.2. Determination of Moisture Sorption Isotherms of Milk Powders

Moisture sorption isotherms of NFDM and MPC-85 were determined using the dynamic dewpoint isotherm method (DDI). A vapor sorption analyzer was used (VSA) (VSA1075, Meter Group, Inc., Pullman, WA) to determine the isotherms at three temperatures (23, 40, and 60°C). Prior to the experiment, appropriate desiccant and water levels in the VSA were confirmed and the moisture analysis toolkit software was configured for the experimental parameters (DDI method, 10-95% equilibrium relative humidity [ERH] range, temperature, 1% resolution, and the airflow rate of 100 mL/min). The milk powder samples were sealed and held at refrigeration temperatures (ca. 4°C) until use, at which point, they were equilibrated to room temperature (ca. 23°C) for the experiment. Three replications at each temperature were performed for each powder. For the experiment, approximately 1.0 g of powder was placed in a stainless-steel sample cup and loaded into the VSA. The duration of the experiments varied from 2 to 4 days depending on the set temperature, with higher temperatures concluding faster than lower. Upon completion, the data from the experiments were saved in an Excel file (Microsoft, Redmond, WA).

2.2.3. Data Analysis of the Moisture Sorption Isotherms of Milk Powders

Experiments were designed as a randomized complete block, and isotherm data were analyzed with analysis of variance (ANOVA) using JMP Pro 15 (SAS Institute Inc., Cary, NC)

to evaluate the relationship between changing temperatures and milk powder type on the moisture sorption isotherms. For the ANOVA, adsorption and desorption equilibrium moisture content (EMC) served as the response variables, which were analyzed individually with temperature, ERH, and milk powder type as experimental factors. For this test, statistically significant differences were defined by $p < 0.05$. To check for any hysteresis in the adsorption and desorption isotherms, a matched pairs tests were conducted.

2.2.4. Milk Powder Moisture Sorption Isotherm Model Fitting

The data for NFDM and MPC-85 were fit to several mathematical models for predicting adsorption and desorption EMCs, all of which are approved by the American Society of Agricultural Engineers (modified Halsey, modified Henderson, modified Oswin, and modified Chung-Pfost models, and Guggenheim, Anderson, de Boer [GAB] equation). These equations serve as standard equations used for defining sorption isotherms (ASAE, 2002). A list of these model equations can be found in Table 2.2. These models were selected for the present study due to their use in other studies examining the isotherms of similar products (Kumar et al., 2012).

To find the model coefficients, a nonlinear regression was used (JMP Pro 15). To evaluate the goodness of fit of the models to the data, root mean square error (RMSE), and chi-squared (χ^2) were calculated to determine the goodness of fit for each model. Lower values of RMSE and χ^2 (Chapra & Canale, 2010) indicated better fit.

2.3. Results

2.3.1. Milk Powder Isotherm Trends

The moisture sorption isotherms for NFDM and MPC-85 are shown in Figures 2.1-2.4. The NFDM adsorption isotherm exhibited the characteristic sigmoid isotherm shape (Type II; Figure 2.1) due to there being a concurrent increase in the moisture content with an increase in the equilibrium relative humidity as outlined in Brunauer et al. (1940). However, NFDM desorption and MPC-85 adsorption and desorption all displayed J-shaped (Type III) isotherms (Figures 2.2-2.4), which is characteristic of materials with crystalline components (Labuza & Altunakar, 2007). Materials which demonstrate Type III isotherm behavior exhibit small gains in moisture up to a certain point (usually a_w 0.70-0.80), which is when crystalline components begin to dissolve in the adsorbed water (Labuza & Altunakar, 2007). This trend was shown in the NFDM desorption isotherm and the MPC-85 adsorption and desorption isotherms. Overall, the moisture contents increased with increasing a_w. However, the moisture contents for both powders increased more rapidly at 60°C compared to 23 and 40°C, which showed more gradual increases. At 40°C, the moisture content of both powders began to increase rapidly and sharply after a_w 0.30 until reaching a_w 0.50. Furthermore, at 60°C for both powders and after a_w 0.20, the moisture contents increased rapidly until a_w 0.50. The observed trends may be a result of capillary condensation, in which adsorption from the vapor phase into a porous medium continues until the pores are filled with liquid (Tham et al., 2016). As the NFDM and MPC-85 began to adsorb moisture, the powder shifted to both a crystalline and an amorphous state. For powders that contain a mixture of both crystalline and amorphous components, a greater water sorption capacity has been observed compared to pure powder (Hartmann & Palzer, 2011). As a result of this compositional change in the NFDM and MPC-85, higher and more rapid moisture adsorption was observed after the initial moisture uptakes at a_w 0.45, 0.30, and 0.20 for 23, 40, and 60°C, respectively.

Using ANOVA to evaluate the effect of temperature and sample type on moisture sorption isotherms, specific comparisons were made between the milk powders. Temperature had a significant effect on both the adsorption and desorption isotherms ($p < 0.0001$) for both NFDM and MPC-85. The results of an ANOVA conducted for all adsorption across both powders showed that the different replicates (1-3) for each powder were significantly different from one another ($p = 0.0009$), but this difference between replicates was not found for all desorption ($p = 0.9931$). A matched pair analysis revealed hysteresis between the adsorption and desorption curves for both milk powders for all temperatures tested. In other words, at a given water activity, the desorption EMC was higher than the adsorption EMC (i.e., desorption curves were higher than adsorption curves for all powders, batches, and temperatures). Notably, the MPC-85 exhibited greater hysteresis than the NFDM for all batches and temperatures.

2.3.2. Model-Fitted Milk Powder Isotherms

The adsorption and desorption isotherms for NFDM and MPC-85 were fit to the modified Henderson, modified Oswin, modified Halsey, modified Chung-Pfost, and GAB equation (Table 2.3, 2.4, 2.5, 2.6). These models have been used for a variety of other LWAF such as milk powders, cereal grains, and hemp flour (Stencl, 1999; Chung & Pfost, 1967; Oduola et al., 2022). Of the models tested for NFDM adsorption, the Modified Oswin model had the best fit for two out of the three replicates except for replicate 1, where the Modified Halsey model had the best fit. However, the difference in goodness of fit was very minimal between the Modified Halsey versus Modified Oswin models for replicate 1 (RMSE: 1.66 vs. 1.67 and χ^2: 2.76 vs. 2.77, respectively). For NFDM desorption, the GAB equation was found to be the best fit for all three replicates (RMSE: 1.41, 1.19, and 1.49; χ^2: 2.04, 1.43, and 2.22). Notably, when looking at the

MPC-85 modeled isotherms, for both the adsorption and desorption isotherms the RMSE and χ^2 values were considerably lower compared to the NFDM adsorption and desorption models. For MPC-85 adsorption, the GAB equation was the most suitable model for all three replicates (RMSE: 0.35, 0.40, and 0.30; χ^2: 0.14, 0.16, and 0.12), whereas the Modified Chung-Pfost model was the most suitable model for the MPC-85 desorption isotherms for all three replicates (RMSE: 0.79, 0.90, and 0.86; χ^2: 0.73, 0.83, and 0.84).

2.4. Discussion

The results presented in this section illustrate the unique sorption trends of NFDM and MPC-85. The selection of the DDI method allowed for observing sudden changes in the sorption properties of both powders because of changes to the food matrix occurring during the experiments (METER Group, 2022). Although both powders were of bovine milk origin, their individual compositions varied greatly, which may explain why their sorption isotherms vary in shape. There have been a number of studies examining the moisture sorption isotherms of a variety of milk powders and what factors may be responsible for the sorption behavior observed between different milk powders, such as protein concentration, temperature, and the presence of crystalline lactose (Bronlond & Paterson, 2004; Foster et al., 2005; Shrestha et al., 2007; Kumar et al., 2012).

For the present study, it was important to simulate conditions the powders would normally be exposed to during processing, such as a thermal treatment. Three temperatures were chosen (23, 40, and 60°C) to illustrate the sorption behavior of the powders under differing conditions. The 23°C temperature was chosen to illustrate the sorption behavior of the powders at ambient temperature. Since 23°C is considered to be room temperature, the sorption isotherms

at this temperature provided insight into how moisture entered and exited the powder as if it were sitting on a shelf in a grocery store. Furthermore, it was important to run isotherms at 40°C, since it is around this temperature that some proteins begin to denature. A study conducted by Wagner et al. discussed whey protein properties and reported that β-lactoglobulin (the main protein in whey protein) starts to denature around temperatures >40°C (2020). Since some warehouses may not have controlled climates, these powders may be exposed to such temperatures. Therefore, observing differences in the adsorption and desorption of NFDM and MPC-85, which have pointedly different protein contents (37.59±0.65 vs. 86.38±0.17, respectively), at elevated temperatures highlighted that protein content influences sorption behavior. Studies examining proteins on the moisture sorption isotherms of milk powders found that proteins play a role in increased moisture adsorption in milk powders due to binding more water molecules at the same a_w level (Shrestha et al., 2007). This behavior was generally reflected in the adsorption curves of both powders evaluated in the present study.

Although the thermal treatment temperatures that were evaluated in the next study (75, 80, and 85°C), were greater than 60°C, 60°C was the closest temperature to the thermal treatment temperatures and the highest test parameter for temperature within the capabilities of the VSA. At 60°C some loss of protein content was expected due to the temperature being well above 40°C. This loss of protein content is an important consideration for the dairy industry as they look to implement their own thermal process based on the results presented in this work. For both the NFDM and MPC-85, the powders were yellowed upon completion of the 60°C isotherms, indicating that processors may not want to treat dairy powders at significantly higher temperatures if quality degradation would be problematic. The color change is also an important consideration for the dairy industry when they look to determine acceptability.

Processors tend to target $a_w \leq 0.65$ in dry foods, as water activity at or below this point halts microbial growth. Moisture sorption isotherms are not, in and of themselves, a metric for microbiological safety but they link water activity and moisture content trends. Most processors use moisture content over water activity when testing their products since it is much easier (and less expensive) to measure the moisture content. The isotherms can then be used to determine the water activity (at a set temperature) that corresponds to the moisture content, which can inform decisions regarding packaging, shelf-life, and product stability. Furthermore, determining the correlating water activity can help processors understand the potential efficacy of thermal treatments against contaminating pathogens.

2.5. Conclusions

Determining the moisture sorption isotherms of NFDM and MPC-85 helped elucidate the characteristics of two milk powders (NFDM and MPC-85) and how factors such as temperature and powder components can influence the movement of moisture into and out of powders. These isotherms served to inform how these powders may be affected by different temperatures that they may encounter during their production, storage, and use, which greatly influence microbial survival and inactivation.

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2.7. Tables

Table 2.1. Proximate analyses (%, wet basis) of nonfat dry milk powder (NFDM) and a milk protein concentrate with 85% protein content (MPC-85).

Analysis	NFDM	MPC-85
Moisture Content	3.25±0.30	5.59±0.03
Protein Content	37.59±0.65	86.38±0.17
Fat	0.52±0.09	1.23±0.00
Sugar	51.70±0.69	3.01±0.00
Ash	7.86±0.13	6.53±0.01

For protein content, factor 6.38 is used to convert nitrogen to protein.

Table 2.2. Mathematical models used for predicting equilibrium moisture content (EMC) using temperature and water activity as inputs.

Equation	EMC Model	References
Modified Chung-Pfost	$M_e = \frac{1}{C} ln \left[\left(\frac{\mathrm{B} - T}{A} \right) ln\, a_w \right]$	Chung & Pfost, 1967
Modified Henderson	$M_e = \left[-\frac{ln(1 - a_w)}{A(T + B)} \right]^{1/C}$	Henderson, 1952; Thompson et al., 1968
Modified Halsey	$M_e = \left[-\frac{exp\,(A + B * T)}{\ln a_w} \right]^{1/C}$	Iglesias & Chirife, 1976a; Iglesias & Chirife, 1976b
Modified Oswin	$M_e = (A + BT) \left(\frac{a_w}{1 - a_w} \right)^{1/C}$	Oswin, 1946; Chen & Jayas, 1998
GAB	$M_e = \frac{AB(C/T)a_w}{(1 - Ba_w)(1 - Ba_w + (C/T)Ba_w)}$	Yu et al., 1999; Raji & Ojedrian, 2011

*T is temperature (°C), a_w is water activity in decimal (ERH = a_w * 100), *Me* is the equilibrium moisture content (%, d.b.) and A, B, and, C are equation or model coefficients.

Table 2.3. Estimated model coefficients (A, B, and C) with root mean square error (RMSE) and chi-squared (χ^2) values fitted to nonfat dry milk powder (NFDM) adsorption data measured for three batches at three different temperatures (23, 40, and 60°C).

Model	Replicate	A	B	C	RMSE	Chi-Square
Modified Chung-Pfost	1	334.22	-146.93	-0.11	3.33	11.14
Modified Henderson	1	0.00044	289.65	0.85	2.29	5.32
Modified Halsey	1	2.56	-0.0024	1.45	1.66	2.76
Modified Oswin	1	7.63	-0.017	1.67	1.67	2.77
GAB	1	3.69	0.96	632.54	1.79	3.24
Modified Chung-Pfost	2	506.79	-254.82	-0.11	3.06	9.39
Modified Henderson	2	0.00041	310.46	0.85	2.07	4.27
Modified Halsey	2	2.61	-0.0029	1.47	1.75	3.08
Modified Oswin	2	7.63	-0.017	1.69	1.64	2.68
GAB	2	3.94	0.95	333.44	1.85	3.46
Modified Chung-Pfost	3	469.50	-229.18	-0.11	3.09	9.56
Modified Henderson	3	0.00041	290.50	0.86	2.09	4.41
Modified Halsey	3	2.60	-0.0019	1.47	1.81	3.28
Modified Oswin	3	7.69	-0.014	1.70	1.69	2.84
GAB	3	4.04	0.95	331.29	1.86	3.58

Table 2.4. Estimated model coefficients (A, B, and C) with root mean square error (RMSE) and chi-squared (χ^2) values fitted to nonfat dry milk powder (NFDM) desorption data measured for three batches at three different temperatures (23, 40, and 60°C).

Model	Replicate	A	B	C	RMSE	Chi-Square
Modified Chung-Pfost	1	209.76	-47.68	-0.14	2.01	4.08
Modified Henderson	1	0.00039	131.09	1.12	1.67	2.81
Modified Halsey	1	3.89	-0.0092	1.86	1.78	3.16
Modified Oswin	1	9.98	-0.043	2.17	1.59	2.53
GAB	1	5.93	0.86	158.52	1.41	2.04
Modified Chung-Pfost	2	276.78	-68.93	-0.14	1.89	3.61
Modified Henderson	2	0.00031	158.31	1.16	1.51	2.33
Modified Halsey	2	4.05	-0.0090	1.92	1.52	2.31
Modified Oswin	2	9.95	-0.039	2.24	1.34	1.81
GAB	2	5.30	0.87	300.17	1.19	1.43
Modified Chung-Pfost	3	217.24	-44.96	-0.14	1.99	4.11
Modified Henderson	3	0.00040	99.91	1.19	1.65	2.73
Modified Halsey	3	4.56	-0.015	2.03	2.05	4.21
Modified Oswin	3	10.99	-0.060	2.34	1.79	3.19
GAB	3	6.56	0.83	139.46	1.49	2.22

Table 2.5. Estimated model coefficients (A, B, and C) with root mean square error (RMSE) and chi-squared (χ^2) values fitted to milk protein concentrate with 85% protein content (MPC-85) adsorption data measured for three batches at three different temperatures (23, 40, and 60°C).

Model	Replicate	A	B	C	RMSE	Chi-Square
Modified Chung-Pfost	1	426.69	-123.50	-0.18	0.92	0.85
Modified Henderson	1	0.00025	244.02	1.20	0.63	0.41
Modified Halsey	1	3.31	-0.0042	1.85	0.85	0.72
Modified Oswin	1	7.43	-0.016	2.19	0.48	0.23
GAB	1	4.38	0.86	300.93	0.35	0.14
Modified Chung-Pfost	2	540.04	-167.15	-0.18	0.97	0.94
Modified Henderson	2	0.00017	382.14	1.19	0.71	0.50
Modified Halsey	2	3.21	-0.0023	1.84	0.87	0.76
Modified Oswin	2	7.18	-0.0099	2.18	0.53	0.28
GAB	2	4.25	0.87	358.70	0.40	0.16
Modified Chung-Pfost	3	568.04	-172.05	-0.18	0.90	0.81
Modified Henderson	3	0.00017	354.44	1.21	0.64	0.41
Modified Halsey	3	3.32	-0.0030	1.86	0.84	0.71
Modified Oswin	3	7.40	-0.012	2.21	0.47	0.22

GAB	3	4.36	0.87	362.09	0.30	0.12

Table 2.6. Estimated model coefficients (A, B, and C) with root mean square error (RMSE) and chi-squared (χ^2) values fitted to milk protein concentrate with 85% protein content (MPC-85) desorption data measured for three batches at three different temperatures (23, 40, and 60°C).

Model	Replicate	A	B	C	RMSE	Chi-Square
Modified Chung-Pfost	1	209.98	-15.43	-0.18	0.79	0.73
Modified Henderson	1	0.00021	43.85	1.61	1.07	1.16
Modified Halsey	1	5.77	-0.018	2.42	1.40	1.97
Modified Oswin	1	12.39	-0.070	2.88	1.11	1.24
GAB	1	9.18	0.69	161.72	1.13	1.28
Modified Chung-Pfost	2	236.35	-23.73	-0.17	0.90	0.83
Modified Henderson	2	0.00019	61.27	1.59	1.18	1.46
Modified Halsey	2	5.52	-0.015	2.38	1.43	2.06
Modified Oswin	2	11.93	-0.06	2.83	1.16	1.33
GAB	2	8.03	0.73	206.98	1.14	1.31
Modified Chung-Pfost	3	225.20	-19.20	-0.18	0.86	0.84
Modified Henderson	3	0.00020	50.13	1.61	1.14	1.30
Modified Halsey	3	5.66	-0.017	2.40	1.39	1.93
Modified Oswin	3	12.22	-0.066	2.87	1.10	1.21
GAB	3	8.55	0.71	186.31	1.18	1.38

2.8. Figures

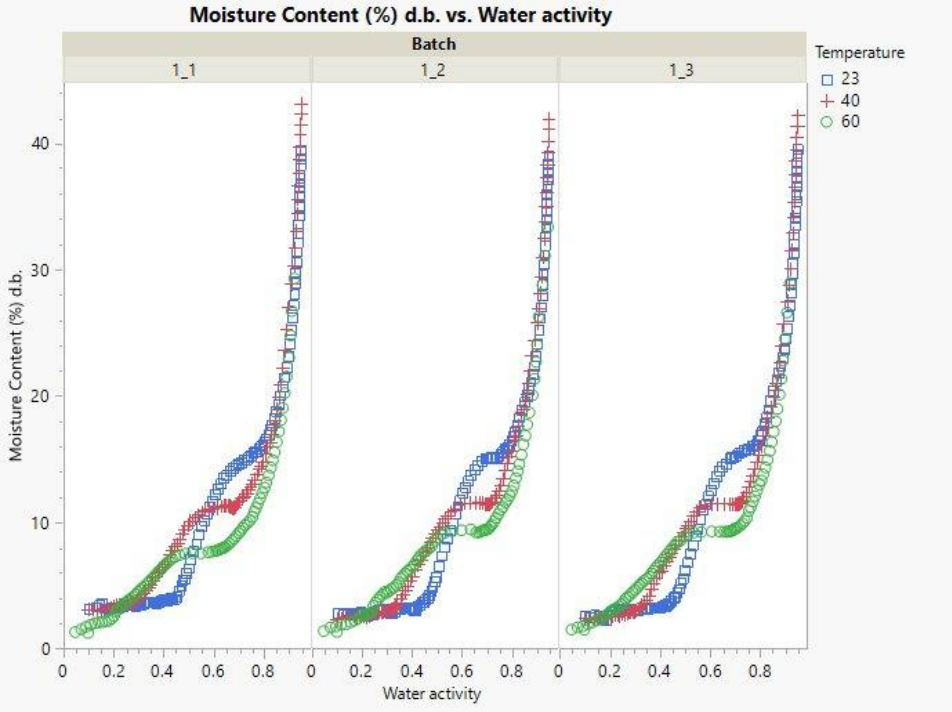

Figure 2.1. Nonfat Dry Milk Powder Adsorption Isotherms at 23, 40, and 60°C.

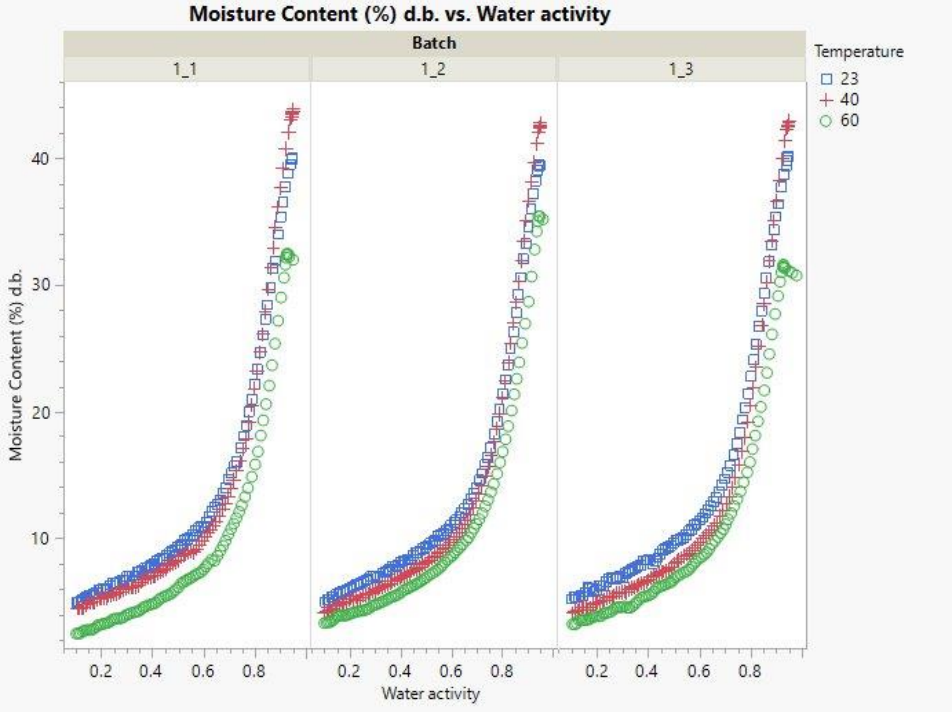

Figure 2.2. Nonfat Dry Milk Powder Desorption Isotherms at 23, 40, and 60°C.

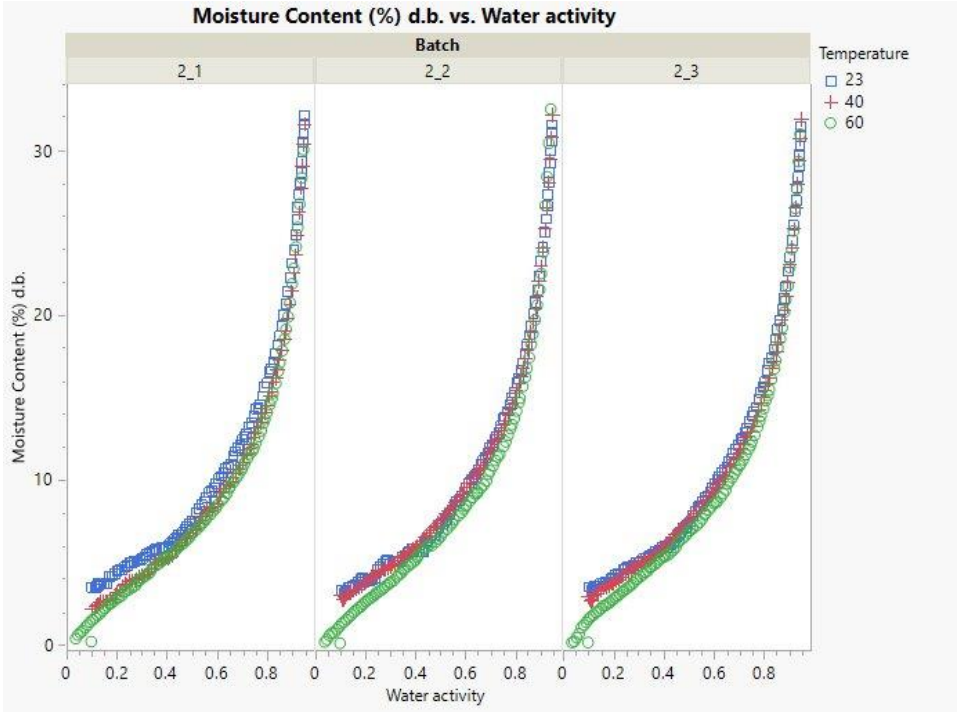

Figure 2.3. Milk Protein Concentrate with 85% Protein Content Adsorption Isotherms at 23, 40, and 60°C.

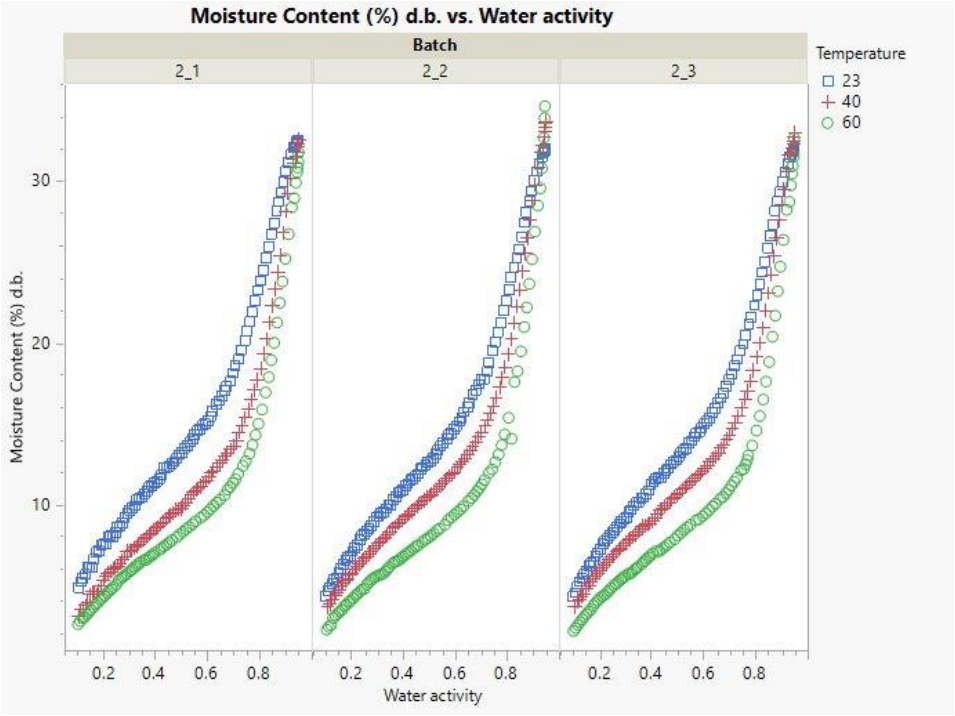

Figure 2.4. Milk Protein Concentrate with 85% Protein Content Desorption Isotherms at 23, 40, and 60°C.

Chapter 3: Thermal Inactivation Kinetics of *Salmonella* in Milk Powder

3.1. Introduction

Salmonella enterica subsp. *enterica* is a leading cause of foodborne illness outbreaks annually in the United States. According to estimates by the Centers for Disease Control and Prevention (CDC), *Salmonella* spp. are responsible for approximately 1.35 million infections, 26,500 hospitalizations, and 420 deaths each year in the U.S. (CDC, 2022). Although outbreaks of *Salmonella* spp. are usually associated with poultry and egg products, *Salmonella* has frequently been associated with recalls and outbreaks of low-water activity foods (LWAF). As an environmental contaminant with the capability of surviving at low water activity (a_w) levels ($a_w < 0.65$) (FAO, 2003), *Salmonella* is a pathogen of concern for processors of LWAF. One such LWAF that has been associated with *Salmonella* contamination is milk powder. Milk powders are used globally in forms such as powdered infant formula (PIF), confectionary goods, and nutritional supplements.

The milk powder industry is projected to grow by 25.38 billion (USD) between the period of 2020-2024 (Technavio, 2020), and thus ensuring the safety of milk powders is imperative. The Food Safety Modernization Act (FSMA) was passed in 2011 and implemented seven final rules for processors. One such rule, Preventive Controls for Human Foods Rule, mandates that processors design a food safety plan that outlines potential hazards and how they will prevent those hazards (through preventive controls) from compromising the safety of the product (FDA, 2020).

Notably, the dairy powder industry does not currently have an established or universally recognized validated kill-step for milk powders post-spray drying. Spray drying does not ensure pathogen lethality, and pathogens such as *Salmonella* spp. and *Cronobacter sakazakii* are capable of surviving the spray drying process (Licari & Potter, 1970; Olsen et al., 2004; Arku et al., 2008). Consequently, milk powders remain vulnerable to pathogen contamination in the final product and have been recently implicated in an outbreak of *C. sakazakii* in infant formula (FDA, 2022).

To assist the dairy industry with FSMA compliance, the thermal inactivation kinetics of *Salmonella* in milk powders were examined to assist in the development of a thermal process post-spray drying. The effect of water activity (a_w 0.20, and 0.30) on the thermal resistance of *Salmonella* will be examined in two milk powders and compared to determine how milk powder type and water activity influence the thermal inactivation kinetics of *Salmonella*.

3.2. Materials and Methods

3.2.1. Milk Powders

The milk powders used in this study were a non-fat dry milk powder (NFDM) (Mars Inc., McLean, VA) and a milk protein concentrate with 85% protein (MPC-85; Dairy Farmers of America, Kansas City, MO). Uninoculated powders were sampled, serially diluted in Buffered Peptone Water (BPW) (Difco, BD, Sparks, MD), and plated onto nonselective media (Tryptic Soy Agar [TSA]; Difco, BD, Sparks, MD or Aerobic Count Plate [APC] Petrifilm™, 3M™, Saint Paul, MN) to determine the background microorganism levels (<0.9 log CFU/g APC [below limit of detection] for NFDM and ca. 3.2 log CFU/g APC for MPC-85). Although there were

approximately 3.0 log CFU/g APC in the uninoculated MPC-85, the background colonies were not found to interfere with the *Salmonella* recovery on the modified selective and differential media and subsequent results of the experiments.

3.2.2. Bacterial Strains, Inoculation, and Equilibration of Milk Powder

Five serovars of *Salmonella enterica* subsp. *enterica* were chosen for the proposed research for their associations with foodborne outbreaks or recalls in LWAF. The strains *S.* Agona 447967 (CDC, 1998), *S.* Mbandaka 698538 (Jackson et al., 2013), *S.* Montevideo 488275 (CDC, 2010; FDA, Office of Regulatory Affairs Regional Laboratory, Jefferson, AR), *S.* Tennessee K4643 (CDC, 2007), and *S.* Reading 180418 (CDC, 2016; University of Georgia; Athens, GA) were provided by the University of Nebraska-Lincoln (UNL; Lincoln, NE). Table 3.1 outlines the outbreaks associated with each strain.

Each bacterial strain was maintained at -80°C on protectant beads in Tryptic Soy Broth with glycerol (Microbank™, Pro-Lab Diagnostics, Ontario, Canada). A single bead for each strain was removed and added to 10 mL of TSB for an overnight culture incubated at 37°C for 24 h. The broth culture was used to streak bacterial lawns of each strain on multiple plates of Tryptic Soy Agar (TSA; Difco, BD, Sparks, MD) using sterile cotton swabs (Dukal® Corporation, Ronkonkoma, NY). After incubation for 24 h at 35°C, the lawns of confluent growth were harvested using a sterile L-spreader and 1 mL of Buffered Peptone Water (BPW; Difco, BD, Sparks, MD) to obtain highly concentrated inocula (ca. 10 log CFU/mL). After the strains were harvested, 2 mL of each *Salmonella* strain (total of 10 mL) that were resuspended in BPW after harvesting were combined to create a cocktail (ca. 11 log CFU/mL) for inoculating the milk powders.

To inoculate the powders (NFDM and MPC-85), 10 mL of the cocktail were pipetted onto 100 g of each powder and homogenized in a spice grinder (SG10 Electric Spice and Nut Grinder, Cuisinart®). This step was repeated twice. Any additional clumps after homogenization were crushed using a sterilized mortar and pestle (18/8 Stainless Steel, Tera). The inoculated powder was transferred to a sanitized aluminum tray (9x13 in) and stored in custom designed relative humidity (RH) chambers (Lau & Subbiah, 2020). The RH chambers were set to the predetermined relative humidity (20 or 30%) needed for the isothermal treatment, and the powders were left to equilibrate for at least 5 days. There were a few instances of the powders being left to equilibrate for a total of 7 days due to inclement weather (i.e., snow). However, the inoculum was stable in the powders after 5 days of equilibration at each RH, and no difference in *Salmonella* survival was observed after equilibration for 7 days. The control for thermal experiments was the equilibrated inoculated powder that received no thermal treatment.

3.2.3. Determination of *Salmonella* Thermal Inactivation Kinetics

After equilibration in the RH chambers, the powders were removed from the chambers and the water activity (a_w) of each powder was checked (TDL; AquaLab; Riverside, CA) to verify they reached the target a_w for the treatment (a_w 0.20 or 0.30 ± 0.02). Samples (6 g of NFDM or 3 g of MPC-85) were aseptically loaded into thermal-death-time disks (Michael et al., 2014), with one disk containing uninoculated powder and a T-type thermocouple to monitor the sample temperature with a datalogger (52-260HZCAL 52-2 Dual Input Digital Thermometer; Fluke; Everett, WA) to determine come-up time (CUT, time required for internal temperature to reach target temperature of water bath) and monitor the powder temperature throughout the treatment.

The disks were placed into a water bath (Precision; Jouan Inc.; Winchester, VA) set to the specified temperature for the treatment (75°C, 80°C, 85°C). The thermal disks were removed from the water bath at predetermined time intervals following CUT and were cooled with ice disks to immediately halt inactivation. The thermal disks were carefully opened, and the powder samples were aseptically transferred to sample bags (Whirl-Pak®, Nasco, Fort Atkinson, WI) with sterile BPW. The samples were serially diluted with BPW and spread plated onto Tryptic Soy Agar with Yeast Extract and sodium thiosulfate (mTSAYE), modified for the selective and differential recovery of *Salmonella* (Appendix A, Hildebrandt et al., 2016; Wei et al., 2020; Lau et al., 2021). All plates for the thermal treatments were incubated at 35°C for 24 h.

3.2.4. Analysis of the Thermal Inactivation Kinetics of *Salmonella*

The *D*-value, or decimal reduction value, is the amount of time required (min) to achieve a 10-fold or 1-log reduction of the bacterial population at a constant temperature (°C). The thermal inactivation kinetics of *Salmonella* in the NFDM and MPC-85 were modeled with the log-linear model to obtain the *D*-values by plotting the reduction of the bacterial population, taking the inverse of the slope, and comparing them.

The z-value is defined as the change in temperature required to either raise or reduce the *D*-value by 90% and was determined for each powder and water activity level by plotting the log *D*-values against the temperature and taking the inverse of the slope. The R^2 values were used to evaluate the linearity of the *D*- and z-values. To evaluate the effect experimental parameters (a_w, milk powder, and temperature) had on the *D*- and z- values for *Salmonella*, analysis of variance (ANOVA) and Tukey's Honest Significant Difference (HSD) Test were performed. All

statistical analyses were performed in RStudio (RStudio v. 4.0.3, Boston, MA) and results with $p < 0.05$ were considered statistically significant.

3.3. Results

3.3.1. Inoculum Stability in Nonfat Dry Milk

Preliminary work investigated the stability of *Salmonella* spp. in NFDM over a period of 5 days at 23°C and a_w 0.30 and 0.20. The a_w 0.30 NFDM showed a higher *Salmonella* population over the course of the 5 days compared to the a_w 0.20 NFDM for both TSA and XLD (8.6±0.30 and 8.1±0.37 vs. 8.1±0.32 and 7.8±0.36, respectively).

3.3.2. *D*- and z-values of *Salmonella* in Nonfat Dry Milk and Milk Protein Concentrate with 85% Protein

Thermal inactivation data for *Salmonella* in NFDM is shown in Table 3.2. For the present study, the *D*-values of *Salmonella* in NFDM at a_w 0.20 were 16.21±1.34, 7.14±1.82, and 3.24±0.52 min at 75, 80, and 85°C, respectively, and the z-value was 13.24±1.78°C. At a_w 0.30, the *D*-values of *Salmonella* in NFDM were 13.25±5.40, 7.93±0.98, and 3.36±0.78 min at 75, 80, and 85°C, respectively, and the z-value was 18.18±8.65°C. *Salmonella* thermal inactivation in MPC-85 data is shown in Table 3.4. The *D*-values for *Salmonella* at a_w 0.20 were 31.92±6.01, 16.57±2.54, and 7.70±2.40 min at 75, 80, and 85°C, respectively, with a z-value of 16.60±5.76°C. At a_w 0.30, the *D*-values for *Salmonella* were 25.82±7.90, 11.85±2.52, and 4.34±1.03 min also at 75, 80, and 85°C, respectively, with a z-value of 12.58±2.95°C.

ANOVA results showed that temperature had a statistically significant effect on the *D*-values of *Salmonella* ($p < 0.0001$), indicating that the lower the treatment temperature, the higher

the reported *D*-values (and vice versa). However, the effect of water activity on *Salmonella D*-values was not found to be statistically significant ($p > 0.05$). Furthermore, there was no interaction between temperature and water activity, so they were considered independent factors ($p > 0.05$). However, when the powders were analyzed separately, the water activity did have a significant effect on the *D*-values for MPC-85, but not NFDM. Results from a Tukey's HSD showed that the *Salmonella D*-values from 75-80°C and 75-85°C were significantly different, however, the *Salmonella D*-values from 80-85°C were not significantly different, regardless of water activity or powder type. These differences are illustrated in Figure 3.1. There is not considerable overlap on Figure 3.1 for the MPC-85 *D*-values like there is with the NFDM *D*-values. Results from ANOVA on the z-values showed that water activity did not significantly affect the z-values of *Salmonella* ($p > 0.05$).

3.4. Discussion

The *D*-values for *Salmonella* found in this study were within the range of those reported in other studies for NFDM (Liu et al., 2019; Wei et al., 2020; Wei et al., 2021). The z-values for *Salmonella* in the present study were similar to those reported in Wei et al. (2020) for the same a_w. However, the differences in NFDM z-values reported in this work for a_w 0.20 and 0.30 (13.24±1.78°C and 18.18±8.65°C, respectively) versus the study performed by Wei et al. (14.90±0.10 and 15.02±1.45, respectively) may be attributed to the different inactivation methods that were employed. Wei et al. used custom-designed thermal-death-time sandwiches (TDT sandwiches) for their inactivation studies, which was a different thermal cell than the thermal disks used in the present study (Lau and Subbiah, 2020; Wei et al., 2020). The choice of thermal inactivation methodology has been shown to affect thermal inactivation kinetics. A

cross-laboratory study performed by Hildebrandt et al. demonstrated that differences in various factors such as how CUT is defined and the type of inactivation vessel can result in differences in thermal resistance values (2016b). A case study performed by Lau et al. comparing three methods for determining *Salmonella* thermal inactivation in whole milk powder (WMP; 2021) illustrated differences in inactivation kinetics. The methods compared were TDT disk in water bath, aluminum pouches in a water bath, and the TDT Sandwich, and the results from each method suggested that there were no significant differences in *Salmonella* thermal inactivation kinetics (Lau et al., 2021). However, the TDT Sandwich method resulted in less variation (smaller standard deviations) in the $D_{80°C}$-values and z-values of *Salmonella* in WMP compared to the TDT disks and the aluminum pouches (Lau et al., 2021). The results of the present study using TDT disks highlight this greater variation in inactivation kinetics when compared to the NFDM results reported by Wei et al. using the TDT Sandwiches.

Previous studies have shown that reduced a_w plays a considerable role in increased *Salmonella* thermal resistance and survivability in LWAF (Laroche et al., 2005; Santillana Farakos et al., 2013; Rachon et al., 2016; Smith et al., 2016; Syamaladevi et al., 2016; Tadapaneni et al., 2017; Jin et al., 2018; Wei et al., 2020). Although the present study demonstrated higher *Salmonella D*-values with decreased a_w, the a_w 0.2 and 0.3 levels were not found to have a statistically significant effect on *Salmonella D*-values in all instances.

The mechanisms for how reduced water activity may increase thermal resistance are not yet fully understood. A study by Lee et al. (2020) applied the glass transition phenomenon to explain how *Salmonella* may be able to persist in a desiccated state and exhibit greater thermal resistance at lower water activities. The authors hypothesized that with decreasing a_w, *Salmonella* enters a “glassy” state characterized by decreased molecular movement which may

lead to increased resistance to other external stressors such as desiccation and heat stress. The authors found that there was an inverse relationship between glass transition temperature (T_g) and a_w. They found that a decrease in a_w was accompanied by an increase in T_g. The lowest a_w reported in the study was 0.43, which corresponded to T_g around 80°C (Lee et al., 2020). Since 80°C is well above room temperature (23°C), the *Salmonella* cells would likely be in the glassy state at room temperature, which is a standard processing condition for LWAF such as milk powders. Based on these results, one would expect the T_g for the present experimental conditions (a_w 0.20 and 0.30) to be considerably higher than room temperature, and possibly even higher than the thermal treatment temperatures (75, 80, and 85°C), which would lead to increased thermal resistance of *Salmonella* due to the cells being vitrified at the treatment temperatures.

The composition of the two powders may have influenced the *D*-values in that the MPC-85 had a higher fat content than the NFDM (1.23±0.00 vs 0.52±0.09, respectively). Previous studies have shown that fat content may provide a protective effect on *Salmonella* in LWAF (Werber et al., 2005; C. Li et al., 2014; Syamaladevi et al., 2016). Werber et al. hypothesized that the fat contained in chocolate may provide a protective coating around the *Salmonella* cells, which would allow it to be protected from the gastric acid in the stomach and therefore able to colonize the intestines and cause illness (Werber et al., 2005). Increased fat content has been shown to lead to increased thermal resistance of *Salmonella*, however the trend hasn't always been consistent, indicating that a variety of factors are at play, and that a single factor is not likely solely responsible for indirectly increasing the thermal resistance of *Salmonella*. For example, a study conducted by C. Li et al. (2014) found that the thermal inactivation of *Salmonella* was drastically reduced in peanut butter (a high-fat food) inoculated with a peptone water suspension of *Salmonella* which resulted in a higher water activity. Whereas the peanut

butter samples inoculated with a corn oil suspension of *Salmonella* resulted in higher thermal resistance due to a protective effect resulting from the high fat and low-water activity environment (C. Li et al., 2014). This study showed that it is not just simply the formulation of a food that can lead to increased thermal resistance, but how the *Salmonella* is introduced into the food can also factor into the thermal resistance of *Salmonella*. These studies, along with the presented results highlight that it is not any one factor, rather it is a balance of factors such as increased fat content, reduced water activity, and inactivation methodology that all play an important role in *Salmonella* inactivation in LWAFs.

3.5. Conclusions

The results of this study support previous research in that thermal resistance of *Salmonella* does appear to increase with decreasing a_w. However, the factor of water activity was not found to be statistically significant for the present study. What was significant, however, was the temperature at which the experiments were conducted. This increased thermal resistance was most likely attributed to a variety of factors such as the varied composition of the powders, especially in fat and protein content, which may have conferred a protective effect on the pathogen.

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3.7. Tables

Table 3.1. *Salmonella* strains used in the present study and their associated outbreaks.

Microorganism	Source	Associated Outbreak	Reference
Salmonella Agona 447967	FDA, ORA Regional Laboratory (Jefferson, AR)	Rice and wheat puffed cereal	CDC, 1998
Salmonella Mbandaka 698538	FDA, ORA Regional Laboratory (Jefferson, AR)	Sprouts	Jackson et al., 2013
Salmonella Montevideo 488275	FDA, ORA Regional Laboratory (Jefferson, AR)	Black and red pepper	CDC, 2010
Salmonella Reading Moff 180418	FDA Culture Collection (Bedford Park, IL)	Alfalfa sprouts	CDC, 2016
Salmonella Tennessee K4643	University of Georgia (Athens, GA)	Peanut butter	CDC, 2007

Table 3.2. Average *Salmonella* $D_{75\text{-}85°C}$-values in nonfat dry milk (NFDM) and milk protein concentrate with 85% protein content (MPC-85) at a_w 0.20 and 0.30.

Milk Powder	Water Activity (a_w)	Temperature (°C)	*D*-value (min)	R^2
NFDM	0.20	75	16.21 ± 1.34^a	0.95±0.02
		80	7.14 ± 1.82^b	0.96±0.03
		85	3.24 ± 0.52^b	0.93±0.05
	0.30	75	13.25 ± 5.40^a	0.81±0.16
		80	7.93 ± 0.98^b	0.99±0.004
		85	3.36 ± 0.78^b	0.92±0.08
MPC-85	0.20	75	31.92 ± 6.01^a	0.81±0.10
		80	16.57 ± 2.54^b	0.90±0.07
		85	7.70 ± 2.40^b	0.88±0.07
	0.30	75	25.82 ± 7.90^a	0.76±0.15
		80	11.85 ± 2.52^b	0.80±0.17
		85	4.34 ± 1.03^b	0.79±0.12

Letters of significance indicate statistically different D-values.

3.8. Figures

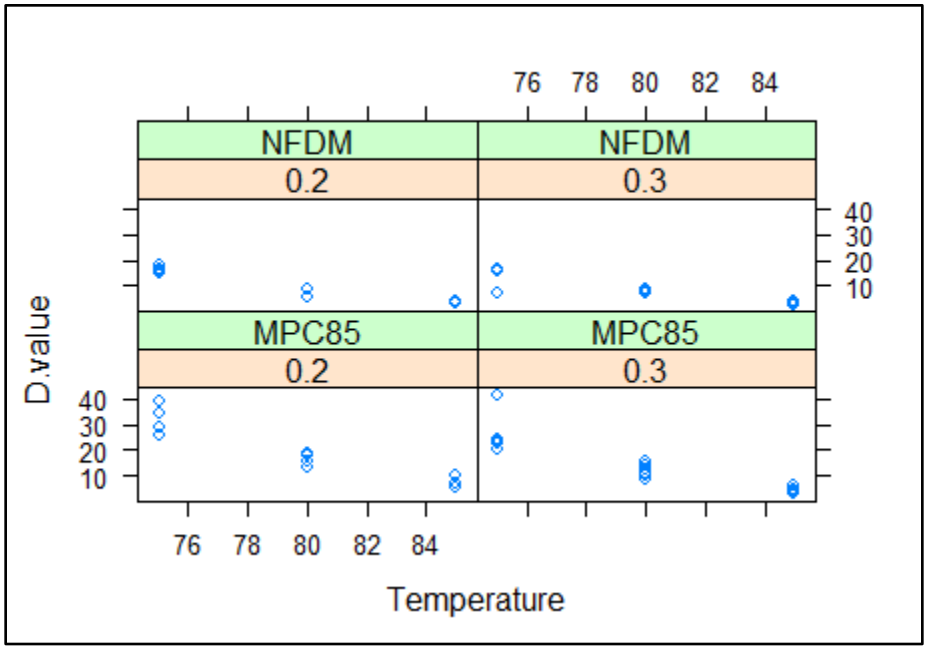

Figure 3.1. Differences in *D*-values of *Salmonella* across each milk powder, temperature, and water activity.

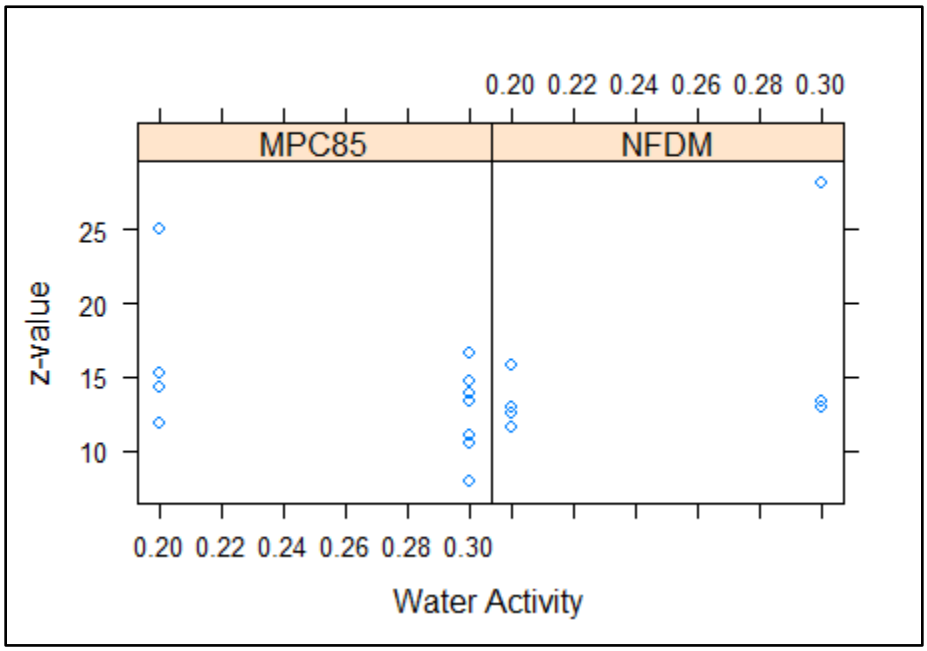

Figure 3.2. Differences in z-values of *Salmonella* across each milk powder and water activity.

Chapter 4: General Conclusions and Future Directions

The work presented in this thesis served to better understand the thermal inactivation kinetics of *Salmonella* in two different milk powders by means of evaluating the sorption behavior of the two powders and evaluating the effect the milk powder type and water activity had on the thermal inactivation of *Salmonella*. Evaluation of the sorption behavior allowed for a more intimate understanding of the powders and the various changes they undergo under certain different temperatures. Although not used as a metric of microbiological safety, the isotherms do serve a purpose in the processing environment as moisture and microbial spoilage is a factor in determining shelf-life and packaging decisions. Even though most pathogenic microorganisms are unable to grow below $a_w < 0.85$, processors tend to use $a_w \leq 0.65$ when making product decisions as to completely exclude the possibility of microbial growth. Sorption behavior is heavily related to the food matrix. The food matrix also plays a role in *Salmonella* thermal resistance, and the alteration of that matrix as well as the presence of certain components, such as fat, plays a considerable role in not only sorption behavior of milk powders but also thermal resistance of *Salmonella*.

The thermal inactivation kinetics of *Salmonella* were determined at two different water activity levels (a_w 0.20 and 0.30) and in two milk powders (NFDM and MPC-85) to evaluate if either temperature, water activity and/or powder type influenced the thermal inactivation kinetics of *Salmonella*. Although the present study showed that water activity was not statistically significant on its own, there were differences in the *D*-values between the water activity levels influenced by milk powder type. However, had even lower levels had been tested, such as a_w 0.10, water activity may have proved to be a more influential factor.

The proposed research initially included evaluating *Enterococcus faecium* NRRL B-2354 as a potential surrogate for *Salmonella* in these powders at the specified temperatures and water activities. However, due to extenuating circumstances with equipment malfunctions and inconsistencies with published results, the experiments with *E. faecium* were removed from the scope of this thesis. Equipment maintenance will be addressed and new *E. faecium* cultures will be acquired to further address this valuable piece of information that we strive to provide industry partners.

Appendix

Table A.1. Media Recipes for the recovery of *Salmonella* and *E. faecium* after thermal treatments.

Media	Purpose	Ingredients
mTSAYE	Modified tryptic soy agar for the selective and differential isolation of *Salmonella* spp.	1. 40.0 g Tryptic Soy Agar 2. 6.0 g Yeast Extract 3. 0.5 g Ammonium Iron Citrate 4. 0.3 g Sodium Thiosulfate 5. 1.0 L Deionized Water
eTSAYE	Modified tryptic soy agar for the selective and differential isolation of *Enterococcus faecium*.	1. 40.0 g Tryptic Soy Agar 2. 6.0 g Yeast Extract 3. 0.5 g Ammonium Iron Citrate 4. 0.25 g Esculin Hydrate (97%) 5. 0.2 g Sodium Azide 6. 1.0 L Deionized Water

Both mTSAYE and eTSAYE are autoclaved at 121°C for 15 minutes.

R Code for the statistical analyses of *Salmonella D*-value comparisons:

```
##############################################
# 07-01-2022 coded by Jung Ae Lee
# Erika Kadas Thesis
# Split plot design analysis
##############################################


## set working directory on ELK computer


data1 = read.csv("data1.csv")
data2b = read.csv("data2b.csv")
```

```
data3b = read.csv("data3b.csv")

summary(data3b)
data4 = data3b[data3b$Microorganism == "Sal",]
# use data4 for the rest of analysis!

# Summary statistics

aggregate(D.value ~ Milk.powder, data4, plyr::each(mean, sd, length))

aggregate(D.value ~ Water.activity,data4, plyr::each(mean, sd, length))

aggregate(D.value ~ Temperature, data4, plyr::each(mean, sd, length))

aggregate(D.value ~ Temperature+Water.activity+Milk.powder, data4, plyr::each(mean, sd,
length))




# RCBD with replications: X1 = treatment combination

  with(data4, table(X1))

  mod2 = lm(D.value ~ X1, data = data4)  #one-way ANOVA for 12 treatment combinations on
the response of Sal ONLY!

  anova(mod2)  # treatment combination is significant but not specific about which combinations
```

```
# Separate dataset already made data4



########################################
# Split-plot design with RCBD
########################################
# Block = Milk power (no testing)
# Whole plot factor = Water activity
# Sub-plot factor = Temperature

# Experimental Unit for WP = 4
with(data4, table(Milk.powder, Water.activity))
data4$EUw = paste(data4$Milk.powder, data4$Water.activity, sep=":")
table(data4$EUw)

# Experimental Unit for sub = 12
with(data4, ftable(Milk.powder, Water.activity, Temperature, rep))

# What EU matter? Source of (random) variation

library(nlme)
sp = lme(fixed = D.value ~ Milk.powder + factor(Water.activity) + factor(Temperature),
         random = ~ 1 | EUw/Temperature,
         #random = ~ 1 | Milk.powder/Water.activity/Temperature,
         data = data4,
         method = "REML",
        # weights = varIdent(~1|Milk.powder),
        # correlation = corSymm( form = ~ 1 |EUw/Temperature),
```

```
        control = list(maxIter = 1000, opt= "optim"))



sp  # check the units

# anova(sp) # don't use it since it is type 1 sum of squares

library(car) # use type 3 SS for unbalanced design
Anova(sp, type=3)

intervals(sp, fixed = TRUE)

# Temperature is significant
# interaction (Water x Temperature) is Not significant

# Diagnotics:
par(mfrow=c(2,2)) #set graphical parameter
qqnorm(sp$residuals[,3]); hist(sp$residuals[,3])

shapiro.test(sp$residuals[,3])

  # Normality assumption may violate - it is the right tail values

# Multiple comparisons
library(emmeans)
lsmobj.1 = lsmeans(sp, "Temperature", by ="Water.activity")
```

```
 lsmobj.2 = lsmeans(sp, "Water.activity", by ="Milk.powder")

  pairs(lsmobj.1, adjust = "tukey")
  pairs(lsmobj.2, adjust = "tukey")

  library(multcomp)
  cld(lsmobj.1)

  #-----------------------------------
  # Plot
  #-----------------------------------

  library(lattice)
  xyplot(D.value ~ Temperature |
         factor(Water.activity) + factor(Milk.powder), data = data4)
```

R Code for the *Salmonella* z-value statistical analysis:

```
##############################################
# 07-01-2022 coded by Jung Ae Lee
# Erika Kadas Thesis
# RCB
##############################################

## set working directory on ELK computer

data1 = read.csv("data1.csv")
data2b = read.csv("data2b.csv")
data3b = read.csv("data3b.csv")
```

```
#Summary statistics
# --- use data4z

data4z = data4[!is.na(data4$zval), ]  # newid is the unit of z-value

aggregate(zval ~ Milk.powder, data4z, plyr::each(mean, sd, length))

aggregate(zval ~ Water.activity, data4z, plyr::each(mean, sd, length))

aggregate(zval ~ Temperature, data4z, plyr::each(mean, sd, length))

aggregate(zval ~ Water.activity+Milk.powder, data4z, plyr::each(mean, sd, length))


## RCBD with replications: X1 = treatment combination (i.e., temp, powder, aw)
data4z$X1 = paste(data4z$Milk.powder, data4z$Water.activity, sep="-")
with(data4z, table(X1))


mod5 = lm(zval ~ X1, data = data4z) # One-way ANOVA for 4 treatment combinations on the
response of Sal only!

anova(mod5) # treatment combination is NOT significant!

with(data4z, table(Milk.powder, Water.activity))
## RCB with replications - Block factor = Milk.powder, treatment factor = Water.activity
lmobj = lm(zval ~ Milk.powder * factor(Water.activity), data = data4z)
anova(lmobj) ## milk powder and water activity did not significantly affect the z-values of Sal
```

```
## there is an interaction between milk powder and water activity, but not at the 0.05 level of significance


##Interaction plot


with(data4z, interaction.plot(Water.activity,Milk.powder, zval,
                    col=c(2,3), lwd=2, xlab = "Water Activity", ylab = "Mean z-value",
                    main = "Interaction Plot of Water Activity and Milk Powder \non z-value",
                    cex.main=0.8))


## check for normality
hist(data4z$zval, col = "blue")
qqnorm(lmobj$residuals)
shapiro.test(lmobj$residuals)
## it is okay to assume normality due to small observations


#-----------------------------------
# Plot
#-----------------------------------


library(lattice)
xyplot(zval ~ Water.activity |
       factor(Milk.powder), data = data4z,
      xlab = "Water Activity", ylab = "z-value",
      main = "Differences in Salmonella z-values on the basis \nof Water Activity and Powder Type",
      cex.main = 0.3)


library(emmeans)
```

```
lsmobj = lsmeans(lmobj, "Water.activity", by = "Milk.powder")
lsmobj
pairs(lsmobj)
```