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Selection and Use of Pantoea dispersa strain JFS as a Non-Pathogenic Surrogate for Salmonella Typhimurium Phage Type 42 in Flour

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ABSTRACT

Selection and Use of *Pantoea dispersa* Strain JFS as
a Non-Pathogenic Surrogate for *Salmonella*
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Salmonella, a common food pathogen, costs more than any other pathogen in the United States in terms of health care costs and loss of work due to the illnesses it causes. Low-moisture foods, especially flour, are susceptible to being contaminated by *Salmonella*. Food producers want flour to be pathogen-free but to also retain the same functionality of non-treated flour. Heat treatment is the most common method employed for lowering the concentration of pathogens in food. However, heating can result in the loss of the flour's functionality. *Pantoea dispersa* strain JFS has been isolated from flour as a nonpathogenic bacterial surrogate that closely matches the D-value of *Salmonella* in flour. Flour samples were subjected to dry heat (70, 75, and 80°C) and heat tolerance was determined by plating out at least four different time points for each temperature. The death rate of *P. dispersa* strain JFS was similar to ($p<0.05$) *Salmonella.* This strain of *P. dispersa* was then used as a surrogate for *Salmonella* in a continuous and batch heat treatment processes to determine the amount of kill achieved by each. The continuous process was conducted using varying levels of four independent variables: temperature, residence time, use of steam, and manipulation of initial water content. All 15 runs resulted in a reduction of at least 1.5 logs of the surrogate, with the greatest reduction being 2.5 logs. The batch process was conducted using one independent variable, temperature. All runs for the batch process resulted in a reduction of at least 2.5 logs of the surrogate, with the greatest reduction being 4.3 logs at 170°F. Both processes could be used to reduce any *Salmonella* present in flour.

Keywords: *Salmonella*, *Pantoea dispersa*, flour, thermal processing, continuous process, batch process

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Abstract

Salmonella is a common pathogen which has been the cause of foodborne illness outbreaks in a variety of commodities, including low-moisture foods such as flour. *Salmonella* costs more than any other pathogen in the United States in terms of health care expenses and time of lost work. Heat treatment can be used to reduce *Salmonella* and other pathogens in flour to safe levels. However, in low-moisture foods, process times must be increased to achieve adequate lethality, possibly resulting in changes in the flour's functionality such as changes in the gluten quality, vitamin content, and the level of starch gelatinization. There is a need to determine the minimal heat treatment required to achieve desired lethality in flour and other low-moisture foods, with the goal of retaining the flour's functionality. Currently there is no published data about a nonpathogenic bacterial surrogate for *Salmonella* in flour. In this study, a surrogate, which closely matches the thermal death rate of *Salmonella* in flour, has been isolated. The surrogate was identified following an evaluation of thermal death curves of ten different strains of bacteria isolated from heat-treated flour and two nonpathogenic surrogates used in other commodities. Flour samples were inoculated with *Salmonella* or one of the twelve bacterial isolates, and then subjected to heat (70, 75, and 80°C) for 12-60 minutes. The heat tolerance for each organism was determined by plating out at least four different time points for each temperature and comparing the death curve to those from *Salmonella*. The death curve from *Pantoea dispersa* was not statistically different ($p<0.05$) than the death curve of *Salmonella*. This strain of *Pantoea dispersa* (strain JFS) can be used as a conservative, slightly more heat resistant, surrogate for

Salmonella. It can be used to verify the combination of heat and time necessary kill *Salmonella* in flour using a commercial heat-treatment process.

Introduction

Eliminating or reducing pathogens to safe levels is one of the most important safety considerations when producing a new food product or process. However, a food manufacturer struggles with the difficult balance of applying adequate heat treatment to reduce the microbial load without resulting in excessive structural, functional, and sensory changes in the treated product. In low-moisture foods, heat transfer is slower and heat efficiency, with respect to lethality, is lower than in higher moisture products, resulting in a longer processing time and a higher chance of altering essential attributes of the product (Denyer et al., 2011). This change in heat transfer efficiency is due to the low amount of water in low-moisture products and the increased ratio of air to water in these products (Jay et al., 2005). This has made the development of a suitable thermal process especially difficult for low-moisture foods.

The ability to better test ingredients has brought to light the fact that pathogens are found in both high- and low-moisture ingredients. While high-moisture foods may contain more pathogens, low-moisture foods--defined by Jay et al. (2005) as foods having a water activity of <0.70, have recently been the source of several foodborne disease outbreaks around the world (Izurieta and Komitopoulou, 2012; McCallum et al., 2013; Neil et al., 2012; Zhang et al., 2007). For example, *Salmonella* Typhimurium phage type 42 (PT 42) was recently isolated from an outbreak in New Zealand. One of the factors that made this outbreak unique is that the suspected *Salmonella* strain was isolated from flour found in the consumers' homes (McCallum et al., 2013). Outbreaks such as this one have caused companies to start designing and using methods to lower the microbial load of specific pathogens in their low-moisture products.

One area of research that has received little attention is the development of heat treatment options for flour. Heat treatment is one way to ensure the safety of low-moisture foods. The implementation of the Food Safety and Modernization Act has mandated that food companies develop a plan to validate the efficacy of all food-processing methods in reducing pathogens to acceptable limits. Another problem that has prolonged the development of heat treatment processes for low-moisture foods is the notion that because bacteria cannot grow in low-moisture foods, they are not as much of a concern as in high-moisture foods. This misconception ignores the reality that low-moisture foods are often ingredients in high-moisture products, where conditions are suitable for growth. Complicating the process, microorganisms also exhibit a higher heat resistance in low-moisture foods. One of the original observations of the phenomenon was made by Goepfert et al. (1970) with *Salmonella*. This phenomenon has also been observed by several others (Fine and Gervais, 2005; Laroche et al., 2005; Villa-Rojas et al., 2013). This increase in heat resistance prohibits simply using the same surrogates for high-moisture and low-moisture products. These surrogates cannot be used without further testing to validate they exhibit the same increase in heat tolerance as the pathogens of interest. These realities—requirement to validate the process, the fact that pathogens are present in low-moisture foods, and since all surrogates should be validated in the product currently being tested, the use surrogates that are employed to validate the process for high-moisture products is not good practice—has created a need for a non-pathogenic surrogate in low-moisture products. Surrogates have been used to verify the safety of food-processing systems for many years.

Salmonella is often a target pathogen of heat treatment processes. Of the fourteen most common pathogens, *Salmonella* has exhibited the greatest impact in terms of illness, loss of quality of life, number of hospitalizations, and number of deaths each year (Batz et al., 2012).

One potential non-pathogenic surrogate organism for *Salmonella* in flour is *Enterococcus faecium. E. faecium* has been evaluated as a potential surrogate for *Salmonella* in both a high-moisture food (beef) and a low-moisture food (almonds) (Bianchini et al., 2012; Jeong et al., 2011; Ma et al., 2007). Another potential surrogate is *Escherichia coli* which has been used as a surrogate in high-moisture foods (Eblen et al., 2005).

While there have been numerous studies conducted to find surrogates for *Salmonella* in high-moisture foods (such as chicken and beef), there have been relatively few studies conducted in the area of low-moisture foods—and none regarding flour. This study will fill the current void of research that exists in the area of heat-related lethality of *Salmonella* in flour. The purpose of this study was to test nonpathogenic organisms as potential surrogates for *Salmonella* Typhimurium PT 42 in flour.

Materials and Methods

Bacterial strains

Salmonella Typhimurium PT 42 was received from The Institute of Environmental Science and Research (Porirua, New Zealand), an isolate from a New Zealand outbreak of salmonellosis in flour in 2008 (Izurieta and Komitopoulou, 2012). Once received, the *Salmonella* stock cultures were maintained at -40°C. Cultures used during the experiment were maintained on Columbia agar plates (Hardy Diagnostics, CA; USA*). Escherichia coli* and *Enterococcus faecium* were used from stock cultures kept at -40°C. The flour isolates were collected from flour that was subjected to seven minutes of heat treatment at 75°C. Once subjected to heat-treatment, the samples were plated on Columbia agar and incubated at 35°C for 48 hours. After 48 hours, the colonies were classified by colony morphology and gram stain. Once classified, the cultures

were maintained on Columbia plates for the duration of the experiment. Stock cultures were also made and frozen at -40°C.

Preparation of cultures

Twelve different strains of bacteria, comprising *Escherichia coli*, *Enterococcus faecium*, and ten different isolates from flour (identified as A-J) were evaluated as potential surrogates for *Salmonella*. Cultures were streaked onto Columbia agar and incubated 24 hours at 37º C to obtain a uniform lawn containing the maximum amount of stationary phase colonies. (Martinez et al., 2003; Smith and Marks, 2015). The bacterial lawn was then harvested by scraping the lawn plate. The cells were then suspended in 3mL of warmed Maximum Recovery Diluent (MRD, 1% peptone 8% NaCl per liter) (Komitopoulou and Penaloza, 2009), and vortexed for 10 seconds to homogenize the inoculum.

Procedure for inoculating flour

There was no noticeable population of *Salmonella* in flour samples used for the test by plating the samples on XLD agar. Samples were plated at the 10^{-1} dilution. The dilution was made with MRD and the plates were incubated for 48 hours at 35°C. No noticeable growth was observed on the plates after incubation.

A modified method from Bookwalter et al. (1980) was used to inoculate the flour. The flour for this study was irradiated at a level of 10 kGy. Irradiated flour (120g) was mixed for 30 seconds on the lowest setting of a kitchen stand mixer in a cooled autoclaved bowl. Inoculum (3mL) was pipetted into the flour and allowed to mix on the lowest setting for 5 minutes. Inoculated flour was then placed in sterile quart size ceramic grinding mill jars (US Stoneware, OH; USA) with 950g of 6.35 mm ceramic beads in each jar; the jars were then rolled for 30 min on a jar mill (US Stoneware, OH; USA) at 60 rpm. After mixing, the contents of the jars were

screened through a sterile nr 4 sieve to remove mixing beads from the flour. The flour was packaged into sterile sample bags and stored at 4°C for one week to allow for stabilization of the bacteria (Beuchat and Scouten, 2002; Jeong et al., 2012).

Heat treatment

A modified method from Izurieta and Komitopoulou (2012) was used for heat treating the samples. Samples of 1.2g inoculated flour were weighed into individual sterile 2mL screw top vials containing a septum in the cap then placed in the center of an agitation water bath. Type-T thermocouples monitored the heat of the flour during treatment to ensure a constant temperature. Thermocouples were placed in the geometric center of each vial. An inoculated flour sample representing each strain of bacteria was tested at 75°C for 0-40 minutes in ten minute increments. The D-values of the samples were compared to the D-values obtained for *S.* Typhimurium PT 42. After comparison of the D-values at 75°C, flour isolates A and F were selected and tested against *S.* Typhimurium PT 42 at 70° and 80°C for 0-60 minutes depending on the temperature. Three replicate runs were performed at each temperature. The timing of the treatments began when the slowest heating vial reached target temperature (Izurieta and Komitopoulou, 2012). Once heat treatment was finished, the vials were removed from the water bath and placed on ice until completely cool.

Comparison of *Salmonella* and potential surrogate survival

Once cooled, the flour was unloaded from vials and placed into sterile 18 x 150 mm test tubes. MRD was used to make serial dilutions, which were then plated onto either bilayered spread plates of XLD (Hardy Diagnostics, CA; USA) and Columbia or onto Columbia spread plates. Bilayered plates were created by layering 17 mL of XLD agar on top of 30 mL of Columbia agar. *Salmonella* tests were plated on the bilayered medium (Kang and Fung, 2000).

The bilayered plates were used to confirm that the bacterium grown was *Salmonella*. Kang and Fung (2000) showed that there was no substantial difference between counts of heat treated bacteria grown on bilayered plates and heat-treated bacteria grown on pure nutrient agar plates. All other potential surrogates were plated on plates containing 20 mL of Columbia agar. All plates were then incubated for 24 hours at 35ºC.

Determination of D-values

Plate counts recorded after 24 hours of incubation were used to determine D-values using the method of Izurieta and Komitopoulou (2012). The time needed to kill 90% of the microorganisms was calculated by taking the negative inverse of the slope from the linear regression of the treatment time and the log of the number of survivors.

Sequencing and Identification of 16S rRNA PCR product

The 16s rRNA was amplified, for the purpose of identifying the bacterial isolates, using the polymerase chain reaction and 27F and 1492R primers. The PCR product was then analyzed using gel electrophoresis to ensure the desired 16s cDNA had been amplified. The cDNA was then cleaned using ExoSAP (Affymetrix, Santa Clara, CA; USA) reagent following manufacturer recommendations. The DNA was prepared for sequencing by using the 63F and 1387R primers and then sequenced using Fisher Sequencing on an Applied Biosystems 3730xl DNA Analyzer (Life Technologies, CA; USA) at the DNA Sequencing Center at Brigham Young University. The DNA sequence was analyzed using the National Center for Biotechnology Information BLAST system (NCBI, Bethesda, MD; US).

Statistical Analysis

A hierarchal linear model using SAS version 9.3 was used (2010 Cary, NC; USA). This allowed each run to have a slope and used the average of those slopes as the estimated slope for

each organism. The comparisons of slopes were used to determine the statistical similarity or dissimilarity. The independent variables were time and temperature. The dependent variable was the log count of the surviving organisms.

Results/Discussion

Viability of bacteria in inoculated flour

Salmonella Typhimurium phage type 42 (PT 42), when added to flour at room temperature and stored at 4°C, underwent an initial reduction of viable cells (die-off) as the cells adapted to the low-moisture environment of flour. On average, the decline resulted in a 2 log reduction in the amount of viable organisms per gram after one week of storage. The remaining *S.* Typhimurium PT 42 colonies remained stable with very little drop in viability for at least 3 months (data not shown). This trend of an initial loss followed by the ability to survive for an extended time, was also observed by Beuchat and Scouten (2002) and Janning et al. (1994). The stabilized flour contained a final *S.* Typhimurium PT 42 level of 10^7 colony forming units per gram (CFU/g). This level allowed for a considerable reduction during the heat treatment experiments while still providing quantifiable, reliable results. This same stabilization process was used for all organisms evaluated, achieving a similar level of viable CFU/g at the starting point (data not shown).

The *S.* Typhimurium PT 42 was the most adept at acclimatizing to flour: *S.* Typhimurium PT 42 samples showed less of a decline in viable organisms than other isolates tested using the same method. This may be due to differences in the organism's ability to survive desiccation. The recent realization that *Salmonella* can survive in flour has heightened the level of concern regarding pathogens in flour. There was also a distinct difference in the growth rate of some of the organisms used for this test. The isolates exhibited very different growth rates in the

preparation of the different vials of inoculum. This was determined by the relative amount of bacteria present on the plate and the size of the colonies. *Salmonella* exhibited one of the quickest growth rates, while some of the flour isolates took up to 48 hours to show comparable growth to *Salmonella* at 24 hours. This was taken into account in the preliminary testing. Plates from the initial tests for all potential surrogates were incubated for 48 hours. Once the isolates that most closely matched the D-value of *Salmonella* were selected, the incubation time was decreased to 24 hours. This was done because there was no significant increase in colony counts from 24 hours to 48 hours.

Surrogate Selection

The first temperature selected for testing was 75°C. This temperature was selected as a starting point as it is currently used by a commercial processing facility. Figure 1 compares potential surrogates to *S.* Typhimurium PT 42 at 75°C. After comparing all the surrogates against

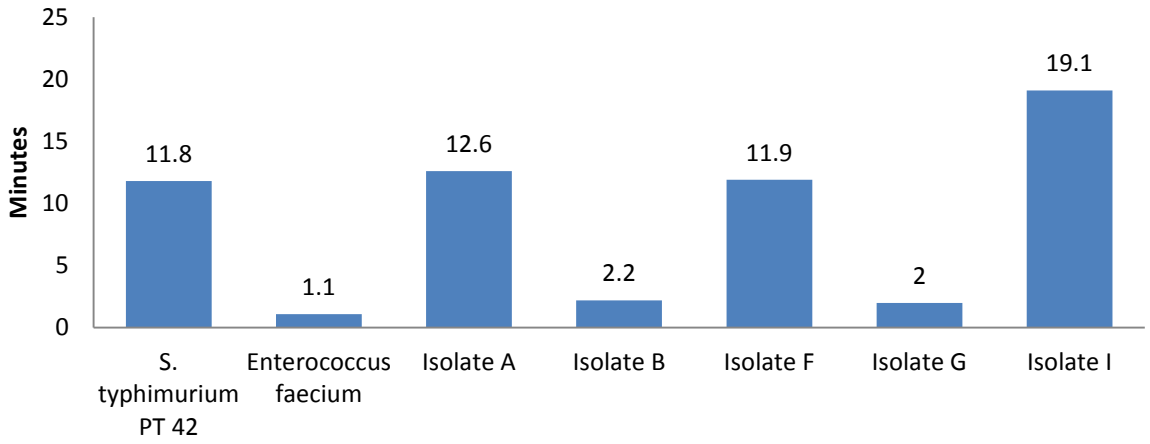

Figure 1 D-values of *Salmonella* Typhimurium PT 42 and Select Surrogates at 75°C

Salmonella at 75°C, flour isolate A, later identified as *Pantoea dispersa*, and flour isolate F were selected as potential surrogates for further testing against *S.* Typhimurium PT 42. After testing at

different temperatures (results not shown), flour isolate F resulted in a z-value that was much higher than *S*. Typhimurium PT 42 (results not shown).

Enterococcus faecium resulted in a D-value that was about one tenth of the D-Value of *S*. Typhimurium PT 42. The lack of similarity between the *E. faecium* and *S.* Typhimurium PT 42 in this study was surprising, as several studies have shown *E. faecium* to be more heat resistant than different strains of *Salmonella* in almonds (Jeong et al., 2011). This observation highlights the challenging task of needing to validate an organism as a proper surrogate for each pathogen in the desired food. A proper surrogate should be evaluated in all unique food environments. *Escherichia coli* is very closely related to *Salmonella* in the phylogenetically and has been used as a surrogate in pilot plant studies for *Salmonella* (Eblen et al., 2005), yet it did not closely match the death curve of *S*. Typhimurium PT 42 in flour. The *E. coli* strain used for this study proved to be completely susceptible to heat treatment (data not shown). The flour containing *E. coli* exhibited no growth once heated to 75°C. This was surprising as previous studies showed it to exhibit a similar heat resistance. Several tests were conducted to confirm this finding and each test resulted in no growth.

The lack of data about *Salmonella* survival in flour and specifically concerning the heat treatment of flour warrants further research into the activity of all types of *Salmonella* in flour. This is true especially for *S*. Typhimurium PT 42 as it is the only known strain of *Salmonella* isolated from flour and implicated in a foodborne disease outbreak.

D-Value Determination of *S.* Typhimurium PT 42 and *Pantoea dispersa*

The D values for *S.* Typhimurium PT 42 and *P. dispersa* JFS at the three different temperatures are shown in Table 1. The higher resistance of *P. dispersa* JFS at 75°C and above makes this organism a suitable surrogate for heat treatment of *S.* Typhimurium PT 42 in flour at

these two temperatures. The somewhat greater heat resistance of *P. dispersa* JFS at 75°C and higher would ensure the process would achieve at least the observed reduction of *P. dispersa* JFS

Table 1 D-Values of *Salmonella* Typhimurium PT 42 and Pantoea dispersa

Temperature (ºC)	*S.* Typhimurium PT 42	Pantoea dispersa	P Value
	Minutes		
70	20.73±2.82	15.04±2.88	0.105
75	11.6±1.48	11.74±1.51	0.611
80	4.78±1.51	7.22±1.66	0.159

in any possible *Salmonella* population in the flour. Using *P. dispersa* to set the time and temperature parameters to achieve the desired log reduction would inherently have an extra measure of safety due to the higher heat resistance of *P. dispersa*.

S. Typhimurium PT 42 exhibited a fairly linear death curve when subjected to heat treatments above 75°C (shown in Figure 2). Studies have shown there are several different survival curves when testing *Salmonella*. Du et al. (2010), Shachar and Yaron (2006), and Archer et al. (1998) reported a non-linear curve when reporting the D values of *Salmonella* subjected to heat treatments. These results were found in almonds, peanut butter, and flour. The

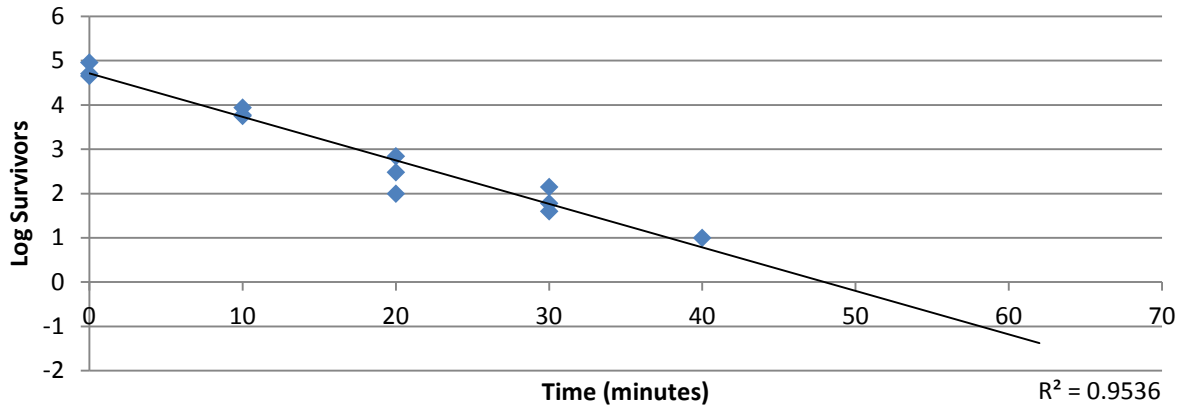

Figure 2 Typical Death Curve of *S.* Typhimurium PT 42 at 75°C

existence of a non-linear biphasic death curve poses an especially difficult hurdle to developing heat treatment options as the heat resistance of the organism appears to increase with exposure to continued heating. Heat treating flour that contained *S.* Typhimurium PT 42 resulted in a linear survival curve. *S.* Typhimurium PT 42 also exhibited a fairly linear death curve when subjected to heat treatments at 70 and 80°C. As this is the only strain of *Salmonella* that has been isolated from flour implicated in a foodborne disease outbreak, the linear model was applied in this study; the use of a biphasic model was unnecessary. The shape of the curve has been shown to be dependent on the strain of *Salmonella* and the food matrix tested (Gould, 1989).

Water activity of the flour also plays a pivotal role in the D value of *Salmonella*. Laroche et al. (2005) found that *Salmonella* in flour is most heat-resistant when the water activity is 0.4. Fine and Gervais (2005) found that both bacteria and spores were most heat-resistant with water activity levels of 0.3-0.5. Flour samples often exhibit in these water activity ranges. The samples run in these tests were between 0.32-0.42. Thus, these tests were run under conditions that resulted in the most heat resistant attributes of *S.* Typhimurium PT 42. *P. dispersa* JFS closely matched the heat resistance of *S*. Typhimurium PT 42 in flour. *P. dispersa* JFS is an affective surrogate for *S.* Typhimurium PT 42 in flour and can be used which may aid in any future selection of surrogates in other low-moisture foods.

Identification of Flour Isolate A

Isolate A is an isolate from heat treated flour. During the initial testing, the identity of the organism was unknown. It was distinguished by the distinct yellow colonies produced when grown on Columbia agar. To identify the isolate, 16s rRNA was amplified and sequenced. The sequence produced was analyzed by the NCBI BLAST database using the nucleotide blast option with the 16s rRNA database. The results from the analysis produced a sequence match for *P.*

dispersa that covered 94% of the sequence length and had an 85% match. Further analysis of the sequence and correction of ambiguous bases resulted in a query cover of 97% and an identity match of 93%. The flour isolate was designated as *P. dispersa* JFS.

A search of the literature showed that *P. dispersa* is an opportunistic pathogen that can cause infections when a large amount of the bacteria are introduced directly into the blood stream. However, there have been no reported foodborne disease outbreaks attributed to *P. dispersa*. There have been several food enzymes isolated from *P. dispersa* (Kulkarni et al., 2013; Wu and Birch, 2004). *P. dispersa* produces a highly efficient sucrose isomerase. It has also been used to detoxify alcibidin phytotoxins which cause leaf scald in sugar cane (Zhang and Birch, 1996). These applications help to establish *P. dispersa* JFS as safe for use in food manufacturing facilities.

P. dispersa is a genus and species that was recently designated from a reclassification of *Enterobacter agglomerans* (Gavini et al., 1989). The authors isolated *P. dispersa* from humans, plant surfaces, seeds, and the environment. *P. dispersa* JFS like other *P. dispersa* strains produces a yellow colony on Columbia agar and a salmon colored colony on Hecktoen Enteric agar. These attributes were used to differentiate *P. dispersa* JFS from other organisms when testing flour.

P. dispersa strain JFS was compared to the closest returned matches from BLAST with the creation of a phylogenetic tree. Figure 3 shows the phylogenetic tree highlighting the relationship between the submitted sequence, *P. dispersa*, and other closely related bacteria. *P. dispersa* belongs to the Enterobacteriaceae family. This family is comprised of gram negative rod bacteria that include several food pathogens. Some of the food pathogens that are included in this family are *E. coli* and *Salmonella*. Several genera of non-pathogenic bacteria from the

Enterobacteriaceae family are closely related to *P. dispersa* JFS including; *Erwinia*, *Cronobacter*, and *Flavobacterium*.

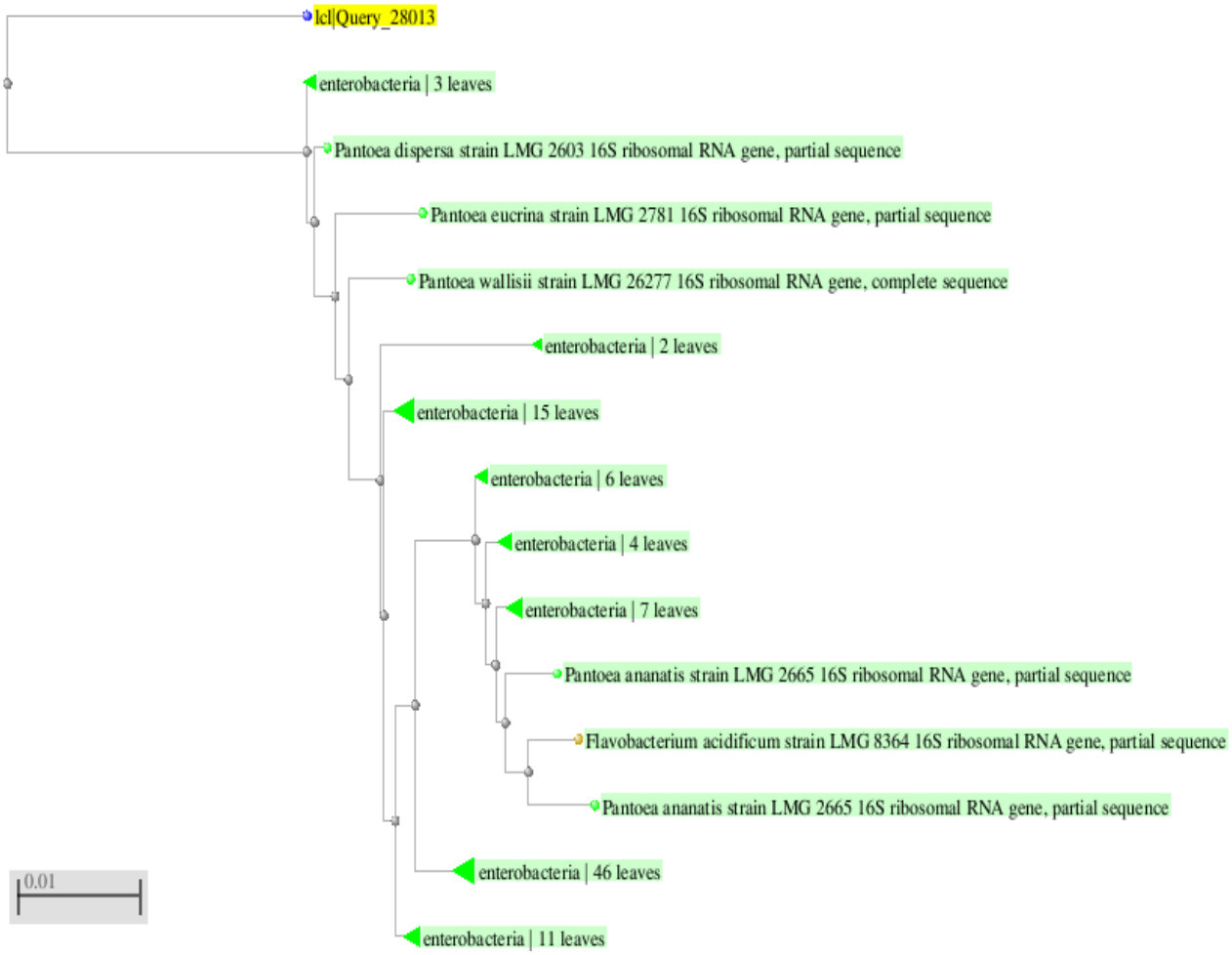

Figure 3 Phylogenetic Trees of *Pantoea dispersa*

Fig. A phylogenetic tree containing the selected surrogate (*P. dispersa*) and other bacteria that closely match the 16S rRNA sequence of the surrogate

Conclusion

Pantoea dispersa JFS has been identified as a suitable surrogate for *Salmonella* in wheat flour. It should be used in processes that heat the flour to a minimum of 75°C. *P. dispersa* was isolated from flour and is not a pathogen in food. This is the first surrogate suggested as a test organism in flour. The use of this organism will aid in the development of future heat-treating

methods for flour. It should aid in the design of heat treatment options that optimize bacterial kill, while limiting the treatment's effects on the flour's functionality.

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Manuscript Two: Quantifying the Lethality of a High-Temperature Continuous Thermal Process using the *Salmonella* surrogate *Pantoea dispersa* strain JFS in Flour

Abstract

Pantoea dispersa strain JFS, a surrogate for *Salmonella*, was used to determine the level of kill achieved by a continuous thin-film thermal process using pastry flour. The use of surrogates to estimate pathogen kill by heat treatment in commercial facilities is a common practice, as the use of pathogens is not a viable option outside of a research laboratory. The continuous process evaluated contained four independent variables; temperature (85, 95, 105°C), residence time (2, 4.5, 8, and 10.7 minutes), use of direct steam injection (0 or 5%), and manipulation of initial water content (10 or 13%). 15 runs were conducted using varying levels of the different independent variables. A statistical design was created and carried out varying the levels of each independent variable across the 15 different process runs. All runs resulted in a reduction of at least 1.5 logs of the surrogate with the greatest reduction being 2.5 logs. The best reduction was achieved when a combination of the highest temperature (105°C), maximum residence time (10.7 minutes), use of direct steam injection, and higher moisture (13%) was used to process the flour. The recommendation would be to process at 105°C, increase the initial moisture content of the flour, and to use steam in the SolidAire® chamber.

Introduction

Studies have recently begun to focus on the heat-treatment or pasteurization of low-moisture food products (Cheon and others 2015; Neill and others 2012; Izurieta and Komitopoulou 2012; Jeong and others 2011; Fine and Gervais 2005). This focus is due to the increasing amount of low-moisture products that are being recalled for the presence of pathogens. There are several pathogens of importance when focusing on low-moisture products.

These pathogens include: the obvious spore-formers *Bacillus cereus* and *Clostridium botulinum,* and surprisingly the gram-negative rods *Salmonella* and *E. coli*. The presence of *Salmonella* and *E. coli* on this list is surprising as gram negative bacteria are generally thought of as poor survivors during the desiccation process. Several studies and foodborne illness outbreaks have shown that for these two bacteria that is not the case. *Salmonella* and *E. coli* do survive well in low-moisture products (McCallum and others 2013; Rose and others 2012; Zhang and others 2007). Due to the increased awareness of these pathogens in low-moisture products, food companies have desired viable verified options for the reduction of pathogens in low-moisture products. The goal of this study was to evaluate a continuous heating process using *Pantoea dispersa* JFS as a non-pathogenic surrogate for *Salmonella*. The data provided by this study can be used by both researchers and food processors as a starting point in the evaluation of currently available heat treatment options for low-moisture products.

Using a surrogate to verify the lethality of a heat treatment process is common practice in the food industry. Food producers use data from process verification studies to establish critical limits for temperature and time, which ensure the desired lethality is achieved each time. While the process of verification and monitoring of the kill step is regulated and mandated for all meats and juices, there are currently no mandated regulations for low-moisture foods. Identification of an appropriate surrogate would make verification possible.

Pantoea dispersa JFS, the surrogate used for this study has a thermal death rate (D-value) that is close to, or higher than *S.* Typhimurium PT 42 in flour at the tested temperatures (Fudge and others 2015). Fudge and others found that *P. dispersa* JFS exhibited a slightly higher heat resistance at temperatures above 75°C. Any decrease in *P. dispersa* JFS can be safely correlated to a potential reduction in *S.* Typhimurium PT 42 due to the slightly higher heat resistance of *P.*

dispersa JFS over *S.* Typhimurium PT 42 (Fudge and others 2015). *P. dispersa* JFS is a surrogate that can be identified easily when plated on Columbia or Hektoen enteric agar, due to the characteristic colony color on each agar (Fudge and others 2015). *P. dispersa* JFS is a non-pathogenic bacterium and can be used in food-process environments. This is vital, as no commercial facility will knowingly allow nor are they allowed by law to knowingly allow a pathogen into the facility.

Salmonella was the target of this process: as it causes the most bacterial foodborne infections each year, and has resulted in several low-moisture food recalls in recent years (Batz and others 2012). The CDC reports that *Salmonella* causes an estimated 1,000,000 food-borne disease incidents, 19,000 hospitalizations, and 380 deaths each year (Scallan 2011). It has recently been implicated in several foodborne illnesses from low-moisture foods (McCallum and others 2013; Zhang and others 2007; Shachar and Yaron 2006). Due to the recent recalls and the prevalence of foodborne diseases attributed to *Salmonella* each year the target organism for the heat processing study was *P. dispersa* as a surrogate for *Salmonella*.

One challenge considered in the planning of this project was that most bacteria exhibit increased heat resistance in low-moisture environments (Izurieta and Komitopoulou 2012; Laroche and others 2005; Beuchat and Scouten 2002; Goepfert and others 1970). Studies have shown that *Salmonella* is particularly resistant to heat treatment when present in low-moisture products (Villa-Rojas and others 2013; Shachar and Yaron 2006; Laroche and others 2005; Janning and others 1994; Izurieta and Komitopoulou 2012; Archer and others 1998). Goepfert and others (1970) were some of the first to note this increased heat resistance with decreased water activity. While Goepfert and others (1970) did not evaluate the water activity level of low-moisture ingredients (<0.60) Beuchat and Scouten (2002) found that both *Salmonella* and

Escherichia coli O157:H7 required the longest heat treatment to achieve the same lethality achieved at a higher water activity level when the samples exhibited a water activity level between 0.4-0.6. The water activity of flour is typically in this range. This means that any *Salmonella* present in flour will be in the most heat resistant environment possible from a water activity standpoint. Because of this high level of heat resistance, the heat treatment must be high enough to overcome this increase in heat tolerance.

One factor that needs to be considered when evaluating any heat treatment process is the effect that this process has on the functionality of the product. This is especially true in low-moisture products as they often require a harsh heat treatment to achieve the desired level of lethality. One aspect of functionality that is of particular concern in flour is the effect the heat treatment has on gluten. If the process destroys the gluten and it may render the flour useless. Most applications of flour rely on the development of gluten. If the gluten quality is altered this may pose a particular problem for that heat treatment process. The use of heat treatment on low gluten flour, soft flour, has been proven to enhance the functionality of the flour (Neill and others (2012). The attributes tested were the texture, moistness of the crumb, and the taste of the cakes. All of these attributes were noticeably improved in the cakes that were made with heat treated soft wheat flour. The cakes also had a longer shelf life than the cakes made with non heat treated flour. Other functionality aspects to consider are the effect of the heat treatment on the gelatinization of the starch, shelf life, sensory attributes, and appearance. Any potential heat treatment process must maintain the functionality of the flour and lower the bioburden of microbial pathogens in the flour.

Methods

Preparation of Inoculated Flour

Cameo® pastry flour (kindly donated by General Mills©) was inoculated using the method of Fudge and others (2015). The inoculation procedure was repeated until 1.8 kg of flour were inoculated with *Pantoea dispersa* JFS at a level of 10^7 CFU/g. Inoculated flour was then dispersed in 181.4 kg of virgin (non-inoculated) pastry flour. This was done with a ribbon blender model IMHS-16 (Bepex, Minneapolis, MN, USA) set at a rate of 49 rpm. The mixing was performed for 15 minutes. The flour was then emptied into three plastic-lined barrels. The barrels containing the flour were emptied back into the ribbon blender and the mixing/emptying cycle was repeated two more times, for a total of three mixing stages. After the third mixing cycle a 25 gram sample was taken from each barrel to test the homogeneity of the mix and to determine the amount of viable bacteria in the flour. The 25 gram sample was diluted with 0.1% peptone water (3M®, Minneapolis, MN, USA). Serial dilutions were performed up to 10^{-4} CFU/g. Aerobic Petrifilm® Count Plates (3M®, Minneapolis, MN, USA) were used to sample the 10^{-2}-10^{-4} dilutions. The Petrifilm® was incubated at 37°C for 48 hours. The mixing produced a 1:100 dilution. This was confirmed when the plates were analyzed and the plate counts indicated a microbial bioburden of 10^5 CFU/g in each barrel.

Design of Experiment

The experiment was designed with a fractional factorial design to optimize the amount of variables tested while staying within the amount of time allotted by the test facility and the amount of product availability. The design is shown in Table 1. The table is organized based on temperature.

Table 1 Settings for All Variables for Each Run

Run	RPM (minutes)	Temperature (°C)	Initial Moisture	Steam
Run 2	1	85	12%	0%
Run 5	1	85	16%	0%
Run 8	10.7	85	12%	5%
Run 11	3.5	85	16%	5%
Run 3	3.5	95	16%	0%
Run 4	11	95	16%	0%
Run 6	3.5	95	16%	0%
Run 10	3.5	95	16%	5%
Run 12	10.7	95	12%	5%
Run 13	3.5	95	12%	5%
Run 1	3.5	105	12%	0%
Run 7	11	105	16%	0%
Run 9	1	105	16%	5%
Run 14	9	105	12%	5%
Run 15	9	105	16%	5%

Equipment Set-up

The equipment was set up as shown in Figure 2. The equipment was arranged and sanitized two days before performing the first tests. Two volumetric feeders were used to measure in the two types of flour (inoculated and virgin). The virgin and inoculated flour were mixed using a paddle blender (Turbulizer®, Bepex, Minneapolis, MN), to achieve a 1:10 dilution, of the inoculated flour, resulting in a 10^4 CFU/g level of *P. dispersa* JFS in the flour. The blender was run at a rate of 2000 rpm and, when desired, culinary water was added at this point to achieve a specific initial moisture levels in the flour. The addition of city water was regulated by a liquid rotometer (flowmeter) at a rate of 180 cubic centimeters per minute. Once mixed, the flour was conveyed by auger and then fed into a SolidAire® thin film thermal process

unit (Bepex, Minneapolis, MN) using a volumetric feeder (Acrison, Moonachie, NJ, USA). The SolidAire® has an outer jacket filled with steam and the product moves through the inner tube.

Figure 1 Schematic of SolidAire®

The SolidAire® (see Figure 1) creates a thin film of product against the heated face of the inner tube using centrifugal force created by the rapid spin of paddles inside the inner tube. The paddles inside the inner tube are adjustable and are set to different angles to obtain the desired flow and residence time of the product in the SolidAire®. Steam can also be injected into the inner tube of the SolidAire® via small holes in the paddles. The SolidAire® was set to a rate of 800 rpms for all runs.

Product from the SolidAire® continuously emptied into a heat-jacketed screw conveyor (KWS, Burleson, TX, USA), intended to maintain the temperature of the product for a specific period of time (residence time), dictated by the rpms and flow rate through the screw conveyor. The product exiting the screw continuously fed into a cyclone to slightly cool the product, before being deposited into barrels.

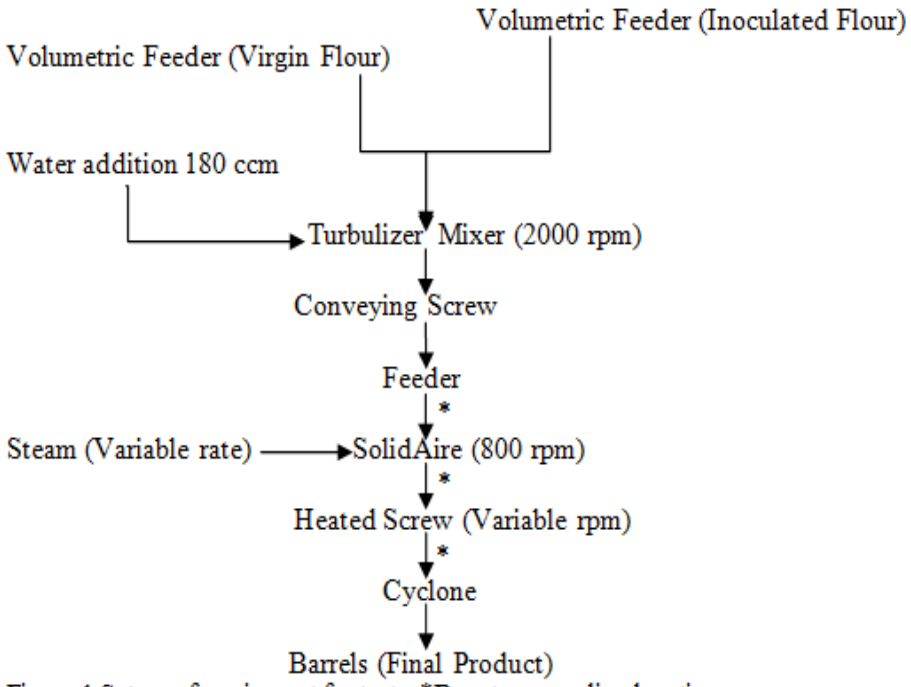

Figure 1 Set up of equipment for tests. *Denote a sampling location.

Figure 2 Process Flow of Equipment

Variables Tested

The residence time for the process was determined by the flow rate through the SolidAire® unit and the speed of the auger in the heated screw. An rpm of 12 on the heated screw would result in a residence time of 1 minute. Likewise rpms of 4, 2.7, 2.2, and 2.1 resulted in residence times of 3.5, 8, 9.7, and 10 minutes respectively. The slight variance in rpms around 2 was caused by difficulty moving the product. When the residence time in the heated screw was too long and moisture was added to the flour via steam injection or manipulation of the initial moisture content, the flour would build up in the screw and block the system. This issue was overcome by slightly increasing the speed of the auger in the heated screw. The time in the SolidAire® was not manipulated. It was kept at a constant of 1 minute regardless of temperature or direct steam injection in the SolidAire® chamber.

Manipulation of the initial moisture content of the runs was done to determine the effect of initial moisture content on the lethality of the process. The average beginning moisture content of the flour that was not manipulated was 10%, and the moisture content after addition of culinary water in the paddle blender was 13%. Thus, this variable resulted in a 3% increase in the initial moisture content of the flour entering the SolidAire®.

Sampling During Runs

Samples were collected at three different locations during the test. The locations are designated by asterisks in Figure 1. They are as follows: the feed into the SolidAire®, the exit from the SolidAire® before entering the heated screw, and the exit from the heated screw. The equipment was allowed to stabilize and equilibrate for 15 minutes, after which four 100 g samples were collected at 5-minute intervals from each location during each run. The samples were cooled quickly by the addition of 20°C diluent as described below to prevent any additional kill.

Analysis of Samples

Twenty grams of flour were weighed into a sterile bag. Sterile peptone water (160 g) was used to dilute the sample, resulting in a 1:10 dilution. The samples were then plated onto 3M® Aerobic Count Petriflim (Minneapolis, MN). Each sample was assayed by plating the 10^{-1}, 10^{-2}, and 10^{-3} dilutions. Each dilution was plated in duplicate. The plates were then incubated at 37°C for 48±2 hours. Once removed from the incubator, a total plate count was conducted and a representative of each type of colony was plated on Columbia agar for differentiation. *P. dispersa* produces a yellow colony on Columbia agar and this indicator as well as a gram stain was used to identify the *P. dispersa* colonies. Once the morphology of *P. dispersa* was confirmed, the Aerobic Count Petriflim® was recounted selecting only the *P. dispersa* colonies.

Statistical Analysis

The data was analyzed using a multiway analysis of variance. The dependent variable was log number of CFU/g. The independent variables were temperature, residence time, steam injection, and initial moisture. Following the analysis, pairwise means were compared using a Tukey adjustment for multiple comparisons. The initial data structure was based on a fractional factorial arrangement that only allowed for the comparisons of the main effects.

Results and Discussion

The level of kill achieved during each run was determined and is shown in Figure 3. Overall, all the tests resulted in about a 2 log reduction. This is surprising as all the different

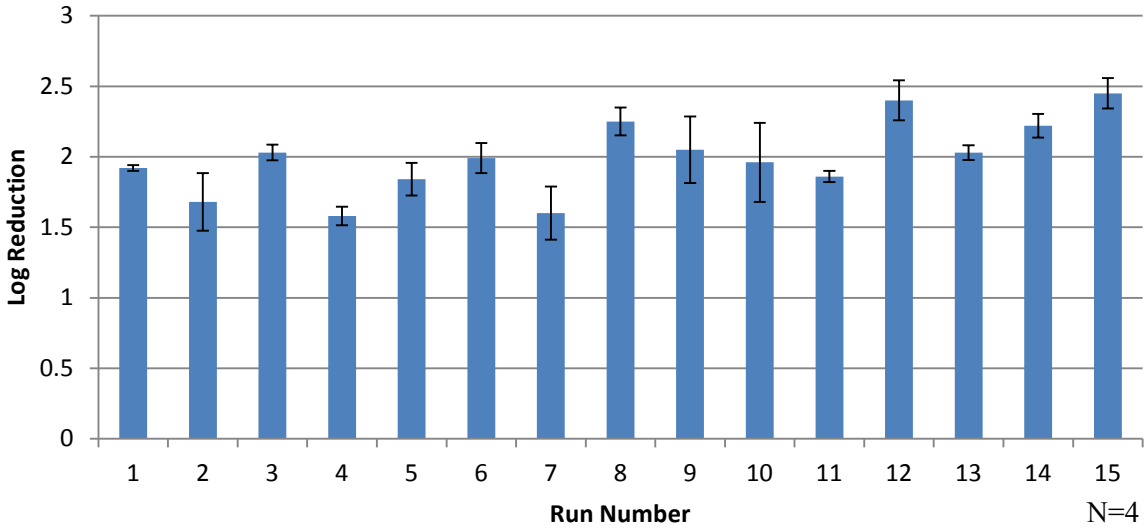

Figure 3 The Cumulative Lethality of P. dispersa JFS in Each Run of the Continuous Process in Flour

Figure 3-This figure displays the total or cumulative lethality achieved during each run of the continuous process. The bars on each column represent the standard error. The total lethality ranged from 1.5 logs up to 2.5 logs.

variables had the potential to greatly enhance or diminish the lethality of each distinct run. It was predicted that the combination of variables in runs 14 and 15 would have produced a much

higher lethality than all other runs. Runs 14 and 15 were both conducted at the highest temperature (105°C) tested and used the longest residence time possible (9 minutes) in the heated screw. It was thought that this combination would result in a much higher loss than the runs conducted at the lower temperatures or at much lower residence times, up to 9 times the amount of time in the heated screw compared to the runs that had the lowest residence time. While a specific combination of variables did not stand out as clearly causing the most kill, general trends were observed and, if implemented, may help to increase the overall effectiveness of the system. There were no statistically significant difference ($p \geq 0.05$) between any of the runs, but there was a one log increase in the level of kill between the lowest lethality observed for run four and the highest lethality for runs twelve and fifteen. The reduction of any of these runs would greatly lower the amount of any potential *Salmonella* in flour. This is especially true in a low-moisture product like flour where the initial concentration of *Salmonella* will usually be well below 10^2 CFU/g (Best 2010). It is also important to remember that the infectious dose for *S.* Typhimurium is in the range of 10^4 CFU/g. This means that a reduction of 1.5 to 2 logs would greatly lower the likelihood that the product contained a high enough level to cause Salmonellosis.

The run that resulted in the highest kill was run 15. Run fifteen was performed at the highest temperature tested, as well as the longest residence time for the holding screw. This run also used the combination of steam in the SolidAire® with the addition of water to raise the initial moisture content of the flour. The flour from run 15 had an initial water content of 13.11% as the product entered the SolidAire® and a water activity of 0.65. This was reduced to a moisture content of 8.56% and a water activity of 0.37 in the SolidAire® chamber. There was a fairly large amount of moisture loss in the SolidAire®, especially considering the addition of

steam in the SolidAire®. Moisture loss occurred since the SolidAire® is an open system, and the temperature was above 100°C.

It was also observed that a majority of the kill happened during the first stage of the heat treatment, in the SolidAire® itself (see Figure 4). In some cases, up to two logs of kill were observed in product exiting the SolidAire®. The use of the heated screw to extend the residence time resulted in very little increase in death. The initial drop in water activity and moisture content agrees with the observation of Archer and others (1998) when studying *Salmonella* in flour. Archer and others (1998) found that in the first few minutes, regardless of the initial moisture content of the flour, the water content would decrease to a level of about 8%. This was also seen in this study during the heating of the samples at the 105°C temperature. In the samples, regardless of whether water was added or not, the moisture content would end up around 8%. Archer and others (1998) also found that during the rapid loss of water there was an exponential loss of the bacteria, but with continued heating of the product there was very little additional death of the organism. This would help to explain the lack of lethality in the heated screw.

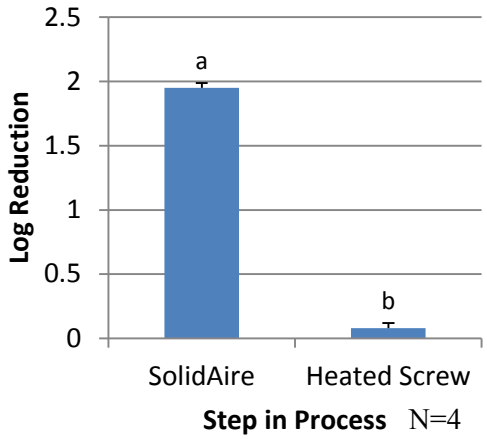

Figure 4 Comparisons of the Lethality on *P. dispersa* JFS Achieved by the Two Heating Steps from the Continuous Process

The kill achieved from the SolidAire® was more than 24 times the kill achieved in the heated screw. Extending the time in the SolidAire® is possible, and may be done to increase the lethality of the system. The use of the heated screw may not be necessary if the residence time in the SolidAire® chamber can be increased by a factor of 2-3 times. The residence time in the

SolidAire® was one minute, meaning a six log reduction may potentially be achieved in 3 minutes if the target moisture can be maintained in the screw. This process is much faster than other heat treatment options that are currently available.

Another finding shown in Figure 5 is that the addition of water, to increase the initial moisture content, enhanced the level of kill. The initial water activity for run 15 was 0.65. At this water activity, water is more accessible for heat transfer. The rate of heat transfer is much higher with water than with air (Singh 2001), meaning that the temperature rises quicker around the bacteria and may be delivered in a more lethal fashion to the microorganisms when more water is present. One way the higher water activity may increase this lethal effect of heat is that the denaturing of proteins occurs at a faster rate in water than in air. This may be due to the formation of free –SH groups which, in turn, increase the water binding capacity of the proteins (Jay and others 2005). This may be one explanation for the observed decrease in heat resistance of *P. dispersa* with an increase in water activity. This finding also agrees with the first finding, that the majority of the kill happened in the SolidAire®. The biggest lost of moisture occurred in the SolidAire®. This loss of moisture may also decrease the heat transfer rate as previously discussed.

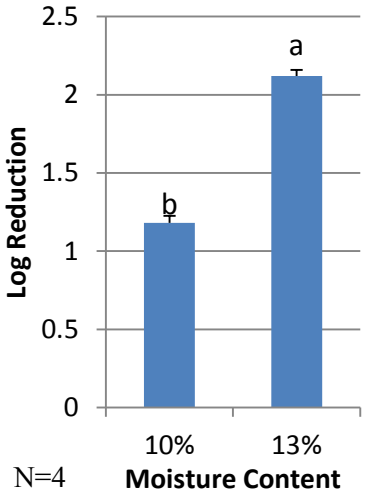

Figure 5 Comparison of the Log Reduction of *P. dispersa* JFS Due to the Initial Moisture Content of Pastry Flour

There was statistically more kill when steam was directly injected within the inner chamber of the SolidAire® (Figure 6). As previously discussed, the location where the greatest lethality was observed was in the SolidAire® and the effectiveness of the SolidAire® was greatly enhanced by the application of steam.

The use of steam did result in an increase in moisture in the end product when the processing temperature was less than 105°C (data not shown). Generally, the product would increase in moisture by 3% when steam was used. The final product was also noticeably moister as clumps formed in the product and were observed during the sampling process. If steam is combined with water addition to maximize the lethality, the system may require additional processing steps to dry the flour after it leaves the SolidAire® or heated screw. The moisture content at the end of the runs that combined water addition and steam usage were between 14-15% moisture content. The water activity at these levels of moisture content was around 0.8. A water activity of 0.8 would greatly increase the risk of microbial spoilage. This would necessitate further processing steps to dry the flour once again to make the product stable and avoid microbial growth. Drying of the flour may also increase the lethality of a process.

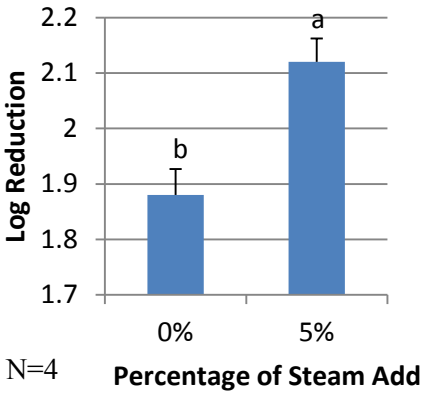

Figure 6 Comparison of the Log Reduction of *P. dispersa* Due to Steam Usage in SolidAire® Chamber

The increase in moisture observed at 85 and 95°C was in contrast to the observed decrease in moisture when the SolidAire® was run of 105°C. Even with the addition of steam, at 105°C there was an overall decrease in moisture when comparing the product entering the SolidAire® and the product leaving the heated screw. On average the water content would decrease by 4.5%. Therefore processing at temperatures at 105°C or higher using this SolidAire® system would need no additional drying step. To ensure the desired level of moisture is maintained in the flour, the recommendation would be to process at 105°C and add water to the flour before processing in the SolidAire® with steam. The addition of water would serve the

dual purpose of enhancing the lethality of the system and ensuring the customer's desired moisture content is maintained in the product.

The role that temperature plays in the process was also determined. Figure 7 illustrates that an increase in temperature causes greater lethality. This trend was anticipated. The fact that an increase of 20°C only resulted in an increase in lethality of 0.26 logs was surprising. A greater increase in lethality was anticipated when moving from 85-105°C. The lack of a greater increase in lethality may be accounted for by the large loss of water at 105°C and the decrease in heat transfer at the lower water activity.

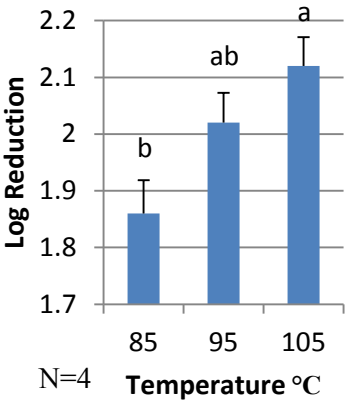

Figure 7 Comparison of the Log Reduction of *P. dispersa* Due to Temperature of Flour in °C

The effect of residence time of flour in the heated screw on lethality is shown in Figure 8. As discussed previously, there was little lethality achieved in the heated screw over all. The data shown in the graph is the cumulative data for the process including the kill from the SolidAire®. As the time in the heated screw increases, the kill increases. Running the screw for 10.7 minutes resulted in the most kill. There is a statistical difference between the holding time of 9.7 minutes and all other times. The heated screw proved to be a very ineffective addition to the processing procedure. The extra expense of the heated screw would not be justified in terms of added lethality.

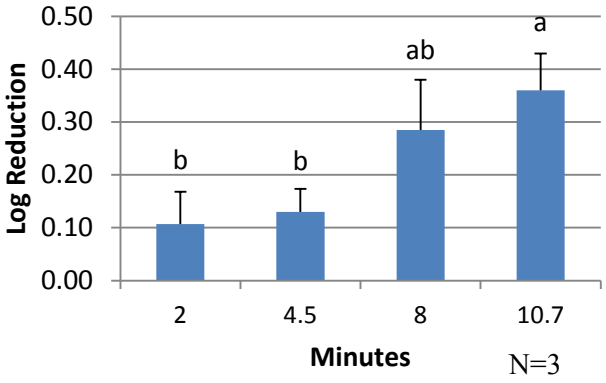

Figure 8 Log Reduction of *P. dispersa* Due to Residence Time of Flour in Heated Screw

Conclusion

The continuous process tested resulted in an average reduction of 2 logs. The kill can be enhanced primarily by increasing the time in the SolidAire® chamber. Increasing the moisture of the flour entering the SolidAire® and the use of steam would also increase the kill. This continuous heat treatment processing method is one possible means of heat-treating flour to reduce the bioburden of pathogens in the flour. This study concluded that processing the flour at 105°C, at a moisture content of 13%, and using steam in the SolidAire® chamber resulted in the highest kill. The heated screw added little lethality to the system and should not be used in future work or studies. Future studies that determine the effects of the different parameters on the functionality of the flour will be used to determine exactly which levels of the test parameters should be used to obtain both a functional and safer product.

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Manuscript Three: Quantifying the Lethality of a Thermal Batch Process using the *Salmonella* surrogate *Pantoea dispersa* strain JFS in Flour

Abstract

Pantoea dispersa strain JFS, a surrogate for *Salmonella*, was used in a batch process to determine the amount of kill achieved by the process. The independent variables were temperature, tested at levels of 65.6, 71.1, and 76.6°F, and residence time, tested at levels of the amount of time to reach the target temperature and holding the sample at the target temperature for one hour. The dependent variable was the lethality achieved by the system. This was measured by plating and counting the colonies that produced the characteristic yellow color of *P. dispersa*. All runs resulted in a reduction of at least 2.9 logs of the surrogate, with the greatest reduction being 4.3 logs at 76.6°F. This process also resulted in a general reduction of the bioburden in flour.

Introduction

Currently there is a lot of interest in the area of heat-treatment on low-moisture products. This is evident by the fact that several studies have recently focused on this topic (Cheon and others 2015; Neill and others 2012; Izurieta and Komitopoulou 2012; Jeong and others 2011; Fine and Gervais 2005). The reason several studies are currently focusing on this topic is the fact that there have been several product recalls due to contamination with *Salmonella* (McCallum and others 2013; Zhang and others 2007; Shachar and Yaron 2006). While there are several pathogens of concern in low-moisture products, *Salmonella* has become a particular problem in low-moisture products. *Salmonella* is one of the most common causes of foodborne illness worldwide (Scallan 2011). With this in mind the target organism for this research is *Salmonella*, specifically *Salmonella* Typhimurium PT 42.

There are several methods to lower the microbial load of food products. Each of these methods employs a different lethal action on the bacteria. Due to the varying ways of acting on the microorganisms and the distinct differences between different food matrixes surrogates must be selected for each unique food system and treatment method. For example, bacteria such as *Salmonella* exhibit an increased heat resistance in low-moisture products (Villa-Rojas and others 2013; Shachar and Yaron 2006; Laroche and others 2005; Janning and others 1994; Izurieta and Komitopoulou 2012; Archer and others 1998). These studies show that a difference of 0.1 can result in drastically different D-values for *Salmonella*. The category of low-moisture products range from a water activity of 0.7 to 0.0, that is a much bigger range than high moisture foods, therefore it is vital to not clump all low-moisture products together.

The non-pathogenic surrogate bacterial strain selected for this study was *Pantoea dispersa* strain JFS. This surrogate was selected because it is the only surrogate tested against *Salmonella* in flour and it is also the only surrogate tested against a *Salmonella* strain isolated from flour (Fudge and others, 2015). This strain of *Salmonella* is *S.* Typhimurium PT 42 which was isolated from flour (McCallum and others 2013). This surrogate is also distinct from other bacteria due to the yellow colony it produces on Columbia agar.

The goal of this study is to provide data about a batch process using *Pantoea dispersa* JFS as a non-pathogenic surrogate for *S.* Typhimurium PT 42. This data can be used by both researchers and food producers as a starting point in the evaluation of currently available heat treatment options for low-moisture products.

Methods

Bacterial strains

Pantoea dispersa JFS was used as a surrogate for *Salmonella*. The justification of this strain as a surrogate was previously studied by Fudge and Others (2015). *P. dispersa* was maintained on Columbia agar for the duration of the study.

Procedure for inoculating flour

Cameo® pastry flour (kindly donated by Honeyville Grain Inc©) was inoculated using the method of Fudge and others (2015). This process was repeated until 2.25 kg of flour was inoculated at a level of 10^9 CFU/g. Inoculation of flour for all samples used during this test were performed on the same day. This flour was stored at 5°C for one week prior to the test. After one week of the storage the total microbial population of *P. dispersa* JFS was 10^7 CFU/g.

Heat Treatment Chamber

The heat treatment chamber was the size of a tractor trailer. The chamber consisted of four heating zones. Each zone contained a set of three zones were hot air was pushed out of the chamber on one wall and sucked out of the chamber on the opposite wall. Each entrance for air contained diffusers to spread out the hot air as it entered the chamber.

Experimental Design

The experimental design contained three temperatures; 65.6, 71.1, and 76.6°C. The design also contained two residence times at each temperature. The target residence time for each temperature was 0 minutes at the target temperature, or removing the bag once the center of the bag reached the target temperature, and also 60 minutes after the center of the bag reaches the target temperature. Due to operating procedures the bags held for 60 minutes did increase in temperature during the 60 minute time frame. This is due to the fact that changing the large oven

takes time and was not practical under normal processing procedures. The change in temperature over the 60 minutes was less than 3°C.

Placement of Inoculated flour bundles

Mesh bundles of previously inoculated flour were inserted into the geometrical center of ten individual 22.7 kg bags of Cameo® pastry flour (General Mills, Minneapolis, MN, USA). Bundles were made by placing flour containing *Pantoea dispersa* JFS into a fine mesh net of polypropylene and then securing with twist ties. Incisions were made with a sterile scalpel in the center of the unopened 22.7 kg bag. A hole big enough to accommodate the bundle was made in the flour, and the bundle was inserted and covered with flour, then the incision was covered with heat resistant tape. Ten inoculated bags were prepared for each run.

Placement of Thermocouples

Once at the test facility, bags were placed in an alternating layout on vertical metal racks so no bag was directly above or below or next to another bag to allow for maximum heat penetration. The bags were numbered one to ten, top to bottom. Each bag was then probed with a type T thermocouple probe. The probe was placed in the geometrical center (cold point) of the 22.7 kg bag next to the inoculated sample.

Traveling Control

The term traveling control refers to a control sample that was taken through the whole process of inoculation, preparing the samples, and the placement of other samples, traveling to the facility, placement of the thermocouples, and loading of the chamber along with the actual test samples. The difference is that the traveling control is not placed in a bag and returned to the lab and tested directly after the chamber was loaded. The traveling control was used to track any loss in inoculum level of test samples that was due to the process of placing the samples,

traveling to the test facility, and loading of the samples in the heat chamber instead of the heat treatment.

Heat treatment

The bags of inoculated flour were exposed to a dry heat treatment ranging from ten to forty hours depending on desired temperature. The chamber was set at a temperature of 110°C and the three target temperatures tested were: 65.6, 71.1, and 76.6°C. The process was repeated for a total of five runs for each temperature. Five of the ten bags were exposed until they reached the targeted internal temperature. The other five bags of each run were held at the target internal temperature for an hour before being removed from the heating chamber. The holding of the bags in the chamber did cause the internal temperature to continue to rise. This rise resulted in up to a 3°C change in temperature. When desired the internal temperature of the product in each bag was verified using a digital thermometer.

Recovery of inoculated samples

Once the internal temperature and holding times was reached the bag of flour that had reached the targeted temperature and time parameters was removed from the heating chamber. The tape covering the initial incision was peeled back and the inoculated bundle was dug out. The inoculating bundle was immediately removed and placed in a sterile sample bag and allowed to cool on a stainless steel table. Both the sample and the 22.7 kg bag of flour were labeled with the date, temperature, residence time, total time in heating chamber, and bag number.

Determination of survival rate

Samples of flour from the heated bundles were serially diluted, using Maximum Recovery Diluent (MRD, 1% peptone 8% NaCl per liter); to 10^{-3} then all dilutions were plated in duplicate on both 3M® Petrifilm® (Minneapolis, MN, USA) and Columbia agar spread plates.

Colonies were counted at both 24 and 48 hours for the Columbia plates; the Petrifilm® plates were counted at 48 hours.

Statistical Analysis

A mixed models analysis blocking on Traveling Control amounts, and runs nested in Traveling Control amounts, was used to analyze the data. This was analysis was performed by SAS version 9.3 (2010, Cary, NC). The dependent variable was lethality and the independent variables were temperature and time.

Results and Discussion

Inoculum Levels of Flour

Lowering of the levels occurred during the equilibrium process as the bacteria endured the desiccation process going from a high moisture media to a low moisture environment, flour. This tendency is normal and has been observed by others (Beuchat and Scouten 2002; Janning and others 1994). An additional lowering of the levels of *P. dispersa* JFS occurred during the placement of the samples into the 22.7 kg bags of pastry flour and during the transportation of the 22.7 kg bags from the lab to the process facility. To quantify the effect of transporting the prepared bags from the lab to the process facility, a traveling control was prepared and accompanied the samples through this process. Once the samples were placed into the heating chamber, the traveling control was sampled to determine the initial count.

As tests were run on different days and were prepared at different times, the tracking of the sample proved to be vital. Some control samples underwent no die off and contained the full 10^7 level when the test began, while other samples lost 3 logs during the transport of the samples to the facility and back to the location. Due to this variance the statistical analysis was blocked by the level of *P. dispersa* JFS in the traveling control sample. The loss of inoculum level did not

affect this test as there were tests still contained a high enough level of bacteria to quantify the drop in *P. dispersa* JFS.

Heat Treatment of Flour

The results for each run were averaged and the total lethality of each testing parameter was determined. Figure 1 shows the averages for each tested parameter. There was no statistical

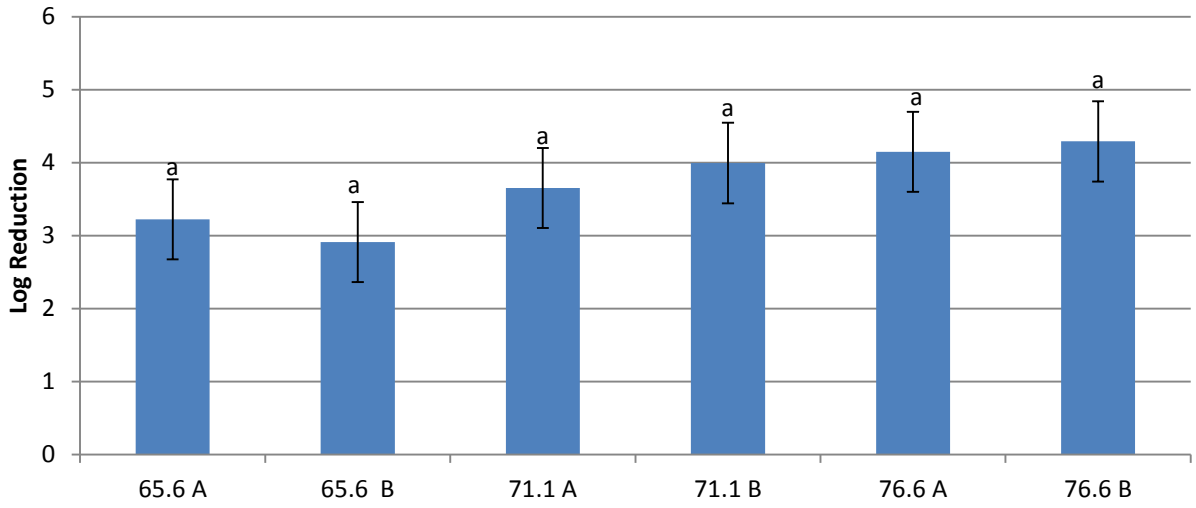

Figure 1 Average Lethality of P. dispersa in Flour for Each Tested Temperature and Holding Time

N=13

Fig. 1-150, 160, and 170 represent the corresponding temperatures in Fahrenheit. Samples with an A were removed once the samples reached the target temperature. Samples with a B were held at the target temperature for one hour.

difference between any of the runs due to the variance in the heating profiles of the flour. On average there was a 3.2 log decrease at 65.6, a 3.75 log decrease at 71.1, and a 4.2 log decrease at 76.6°C. The large amount of variance in the heating profiles of the flour highlights one of the challenges experienced with this processing method. The time needed to achieve the target temperature is not always predictable. The large capacity of the unit, the density of the product, the density of pastry flour is different than the density of bread flour, and the ambient temperature of the unit. The unit contains minimal insulation so the ambient temperature plays a big role in the starting temperature of the unit. The process was replicated a total of five times for

each temperature. From one replicate to the next there was up to a four hour difference in the time needed to achieve the target temperature. This time played a large role in the lack of statistical difference between any of the tested parameters.

On average the unit achieved at least a 2.5 log reduction in the flour. This level of reduction while small would add a large amount of safety to the flour. The normal microbial bioburden in flour is around 10^3 CFU/g. Reducing this level by 2.5 logs would result in a great reduction in any bacteria present in the flour. It is also important to remember that the infectious dose of *S.* Typhimurium is around 10^4 CFU/g with normal healthy adults. The reduction of 2.5 logs would greatly increase the amount of raw flour that would need to be consumed to cause illness in a normal healthy adult. Especially since the level of *Salmonella* in flour would rarely exceed a level of 10^2 CFU/g and most likely would be on the level of 10^1 CFU/g (Best 2010). Reducing the bioburden of the flour by 4 logs would greatly reduce the likelihood of the presence of any pathogens in the flour. Since the overall bioburden of flour is around 10^3 this process removes most of the bacteria from flour. There are some thermo-tolerant bacteria in flour and they were observed on both plating methods used for this study. The samples that underwent the highest level of treatment, 76.6°C, still exhibited growth of some bacteria at the 10^1 dilution.

While there is a debate about how much of a reduction is needed in a low-moisture product to ensure the safety of the product (Best 2010), the functionality of the product must be maintained. Therefore reducing the bacterial load must always be countered by a potential loss of functionality. The flour from this study will be tested to determine any change in functionality in the flour. Another important factor to conserve during heat treatment is the appearance of the product. There were no observable changes in the appearance of the product after heat treatment. There was no discoloration or change in the flow of the product. While other tests are needed to

ensure there was no change in the functionality of the product, there was no observable change in the product during sample collection and analysis of the product from all runs performed for this study.

Benefits and Challenges of Batch Process

The use of a batch process allows for an extra measure of protection due to the fact that the product is contained in its final packaging. This helps to avoid any contamination after heat treatment. However, the added packaging does require more time in the heat treatment process as the heat has to penetrate through the packaging material before reaching the product. Another added benefit to this system is the fact that almost any product can be used in the chamber. Unlike a continuous process where the system needs to change if the material changes, this process can accept most any product that is packaged in large 22.7 kg bags.

The loss of moisture is a large factor in this process. The resulting product lost up to 5% of the original moisture (data not shown). This would require the education of end-users of the product. Bakers are some the biggest users of flour and they are accustomed to working with flour that has a specific water content. If the water content is changed, their formulas may need to be altered as well. This loss of water in the final product does pose a challenge; the consumer would need to be educated about using the new flour. Further research would need to be conducted to determine if the water loss causes any real change in the use of this flour compared to non-heat treated flour.

Conclusion

This heat treatment method provides a very high level of reduction of total bacteria in flour. It resulted in a 4 log reduction of *P. dispersa* JFS at 76.6°C. This reduction is high enough to ensure an extra measure of safety in any flour processed using this system. The combination

of the lethality achieved during the heat treatment process and the fact that the product is in the final packaging make this a viable option for reducing the bioburden of pathogens in flour. However, further work is needed to address the functionality of the end product.

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APPENDICIES

Appendix A Expanded Literature Review

Food safety, specifically related to microbiology, in the past has been a reaction to foodborne disease outbreaks. (Best 2010) An outbreak would occur and the company would then focus on fixing the process to make sure it did not happen again. This antiquated approach resulted in a very retroactive approach to food safety. In a constant effort to produce safer food the food industry has been changing their approach to food safety. There has been a desire to have a more proactive approach to food safety and to prevent any foodborne illnesses from occurring at all in the US. This study is focused on a possible proactive approach to the current issue of managing the risk of *Salmonella* in flour.

Pathogens Found in Flour

Over the past several years *Salmonella*, *Bacillus cereus*, and *Escherichia coli* have all be isolated from flour and have been implicated as causative agents in foodborne illness. (Eglezos 2010; Berghofer and others 2003; Sperber and North American 2007). Recently Shiga toxin producing *E. coli O157:H7* was isolated from ready-to-bake cookie dough in the United States in 2009. (Neil and others 2012) This was one of the first times an outbreak of *E. coli* was traced back to the consumption of a ready-to-bake product. Another outbreak due to flour was an outbreak of Salmonellosis in the year 2005. The outbreak was due to the consumption of "raw" cake batter in ice cream.(Zhang and others 2007) The *Salmonella* was originally undetectable in the cake mix used for the ice cream but upon further testing *Salmonella* was isolated from the samples. The isolating of *Salmonella* from samples requires extra steps. This is due to *Salmonella* being in flour at such low quantities in the samples that if normal procedures are followed *Salmonella* will most likely not be isolated. (Zhang and others 2007; McCallum and others 2013) These extra steps are needed for the isolation of all pathogens from flour. These

extra steps included sampling a much bigger sample of the product when trying to isolate the bacteria. In isolating *Salmonella* from flour instead of using 25 gram samples which are typical in food testing the authors had to test samples that were up to 300 grams in size. This sample is 12 times the typically tested size of most food products. Because of the need to increase sample size for low levels of bacteria there may be a need for the development of new ways of testing for pathogens in this class of products.

Source of Contamination in Flour

While few studies and reports have focused on the microbial content of wheat the reports that have focused on this show that the microbiological quality of flour has improved. This has been accomplished by way of lowering the microbiological counts of flour (Sperber and North American 2007). While the microbial counts on flour have been reduced there are still about 10^6 microorganisms on typical flour. The most common families of bacteria that are found in grains are *Pseudomonas, Micrococcaceae, Lactobacillaceae,* and *Bacillaceae.* The main causes of microbial contamination in grains are the dust, water, soil, fertilizer, animal feces, and infected plants that surround the grains as they are growing (Laca and others 2006).

While few studies address pathogens in wheat and flour the awareness of *Salmonella* in flour has increased as foodborne illnesses have occurred. One way in which *Salmonella* is uniquely suited to exist in flour is that it has developed surface epitopes that allow for the attachment of the *Salmonella* to plant structures (Warriner and Namvar 2010). These epitopes give *Salmonella* a competitive advantage over other bacteria when attaching to plants. Once attached to the plants *Salmonella* has developed systems that allow it to metabolize the nutrients contained in the apoplastic fluid of plants. This also gives *Salmonella* a competitive advantage when attaching to plants because other pathogens, such as *E. coli O157:H7*, which also have the

epitopes to attach to the plants have not yet evolved to utilize the nutrients from the apoplastic fluid in plants.

Salmonella in Flour

In a study of 4,000 samples concentrating on both the growing season and the protein content of the wheat and then testing the flour made from these wheat crops for the presence of pathogens Richter and others (1993) found that *Salmonella* was most prevalent in soft red winter wheat and least prevalent in durum wheat. The high likelihood that *Salmonella* will be present in spring wheat as opposed to winter wheat may be due to the fact that winter wheat is planted in the late summer. The wheat will then sprout in late summer but will not be ready to harvest until after winter. This produces a plant above ground that *Salmonella* can come in contact with during fall and winter. This extended time where the plant is exposed may be one reason that winter wheat is more likely to harbor *Salmonella* than spring or durum wheat.

Soft wheat would most likely be used for pastries and cakes. These products are the products of most concern when *Salmonella* is present in flour. These products are of most concern because they are the products that would most likely be consumed raw by the consumer. Most other products that contain flour are fried, baked, or cooked in some way which would result in the death of any *Salmonella* in the flour. This is a concerning statistic, as the flour that is most likely to be contaminated with *Salmonella* is also the classification of flour that consumers will most readily eat raw.

While the most commonly required test for a pathogen in flour is a qualitative test for *Salmonella* (Sperber and North American 2007), there is rarely any test conducted on flour for the presence of pathogens. This is due to the very low level of *Salmonella*. The low level of *Salmonella* also complicates the taking a representative sample of the flour. Since there is such a

low level in flour it is hard to take a sample and with confidence say that there is no pathogen in the flour. The extra steps needed for the isolation of *Salmonella* from cake batter mix would be necessary; namely taking a much larger sample size and taking more samples. These sampling measures would result in the loss of a large of amount of product which would require a large space with more technician time dedicated to running the tests. Because of these demands and the fact that even with these extra steps there is no guarantee that pathogens are not present in the flour, most facilities do not run these tests.

Current State of Treatments for Flour

The heat resistance of *Salmonella* in low-moisture foods has been studied in the past. There are several studies that have compared the heat resistance of different serotypes of *Salmonella* in low-moisture foods even testing them in flour. (Archer and others 1998) One thing all these studies lack is the ability to test a strain of *Salmonella* that has actually been isolated from flour.

One novel approach presented by Laca and others (2006) showed that by removing part of the bran before processing into flour would greatly reduce the amount of the micro flora of flour. This is due to the fact that most of the microorganisms found in flour are found on the wheat before it is processed. Most of the microorganisms in wheat are found in the outer layers of the bran. Laca and others (2006) found that by removing 75 mm of the bran you could reduce the CFU/g by 2 logs. This approach offers a way that the microbial load could be drastically reduced and functionality would be unaffected as bran imparts no functionality to the flour. As the bran is removed as part of the milling step changing the amount that is removed could help to lower the amount of heat treatment needed to ensure a safe product. Since the removal of bran removes a portion of the microorganisms, but not all, the heat treatment would still be needed.

Flour is often found to have a water activity from 0.3-0.5. Laroche and others (2005) found that *Lactobacillus plantarum* and *Saccharomyces cerevisiae* were the most resistant to heat treatment, had the highest D value, when the environment was at a water activity of 0.4. This value falls directly into the range of water activity for flour. Fine and Gervais (2005) also found that the heat resistance of *Bacillus subtilis* spores were greatly enhanced when the spores were heat treated in a product with a water activity between 0.3 and 0.5. This means that once in flour *Salmonella* may adapt to become more heat resistant than previously seen. Any heat treatment developed for flour will need to be prepared for killing the microorganisms in their most heat stable state.

Surrogates for *Salmonella*

Several studies have sought to find a surrogate for *Salmonella*. Kim and Linton (2008) sought to find a surrogate for *Salmonella* in fruit processing using chlorine dioxide. They took a different approach in trying to find one surrogate for several different pathogens. The pathogens of concern in fruit are *Salmonella*, *Escherichia coli O157:H7*, and *Listeria monocytogenes*. Kim and Linton sought to identify a single surrogate that could be used for all three of the pathogens of concern in fruit. They identified *Hafnia alvei*, which they isolated from mushrooms, as a suitable surrogate for the three pathogens of concern in fresh fruit.

Another study conducted by Eblen and others (2005) determined that *E. coli B-14573*, *ECRC 97.0147, ECRC 97.0152, ECRC 97.0190,* and *ECRC 99.0512* were suitable surrogate for both *E. coli O157:H7* and *Salmonella* when used on pilot plant equipment. The tests were focused on recovery media used and attachment to the pilot plant equipment.

Ma and others (2007) found that *Enterococcus faecium B-2354* was a suitable surrogate for *Salmonella* Senftenberg 775W, a known outlier for *Salmonella* species when subjected to

heat, in ground beef. E. faecium was significantly more resistant to heat treatment than *S.* Senftenberg.

NRRL B-2354: *Enterococcus faecium* was investigated as a surrogate for *Salmonella Enteritidis phage type 30*. The study found that the log reductions in *E. faecium* were 0.6 logs lower than SE ST30 at 100 °C when tested on the surface of almonds. (Jeong and others 2011) *Enterococcus* was also tested as a surrogate in ground beef. Once again the heat resistance *of E. faecium* was higher, by 4.4 to 17.7 times, than *Salmonella*. (Ma and others 2007)

A potential surrogate that is closely related to *Salmonella* would be *Escherichia coli*. The strain of *E. coli* used would be one of the several nonpathogenic strains. Eblen and others (2005) found that the less heat resistant strains of *E. coli* closely matched the heat resistance of *Salmonella*.

Effect of heat treatment on flour functionality

There is a debate about how close a surrogate should be to the actual death curve of a pathogen. There has been little consensus on the topic. It would appear that the determination should be made on a product specific basis. Some products such as ground beef lose no additional functionality by being processed for significantly more time, but other products such as flour may lose their functionality entirely. The loss of functionality entirely would render flour useless to the consumer.

In spite of the fact that heating proteins causes denaturation of the proteins and loss of functionality; there are studies that say the heat treatment of soft wheat flours can improve functionality. Neill and others (2012) found that the heat treatment of soft wheat, low protein, flour enhanced the attributes of high ratio cakes. The attributes tested were the texture, moistness of the crumb, and the taste of the cakes. All of these attributes were noticeably improved in the cakes that were made with heat treated soft wheat flour. The cakes also had a longer shelf life

than the cakes made with non heat treated flour. Neil and others found that the optimum heat treatment for soft wheat flour in their system was 120-130°C for 30 minutes with an initial 12.5% moisture content for the flour.

D value determination for *Salmonella*

The heat treatment of products to determine the processing time has been done in the food industry for years. Determining the d value, decimal reduction time, of in low-moisture products is a fairly new concept. The determination of d values has previously been focused on high-moisture foods, such as canned foods, where the possibilities of pathogens growing and producing its toxin are the highest. Since low-moisture foods inhibit proliferation of microorganisms there has been relatively little research conducted to determine the d values for *Salmonella* in low-moisture foods.

Recent studies have begun to fill in the void where no data existed previously. Izurieta and Komitopoulou (2012); Archer and others (1998) determined the d value for *Salmonella* in cocoa and hazelnut shells and flour respectively. These products represent two low-moisture products that have been implicated in a foodborne outbreak of salmonellosis. As testing procedures improve and bacteria adapt to survive in low-moisture foods more research is needed to ensure a safe food supply.

Du and others (2010) used a new approach to determining the time necessary to kill *Salmonella* in low-moisture foods. They reported times necessary to kill a certain amount of logs in almonds. The determination of d values usually greatly discounts the amount of time it takes to bring the product up to the processing temperature known as come up time. The approach used by Du and others allowed for the use of come up time. This time may be very important in low-moisture foods as there is a significant loss of viable organisms in the first few minutes of

processing. Archer and others (1998) found that there was a significant loss of microorganisms in the first 5 to 10 minutes of heating. This is most likely due to the fact that the water activity lowers from the starting level down to about 0.2 in low-moisture products during the first few minutes of heating. Traditional methods for heat treatment require a significant amount of time to come up to temperature. This means that the time when the death is most exponential is not fully utilized. In low-moisture foods where there is no proliferation of the microorganisms and the heat transfer from conduction is slower due to the presence of less water accounting for the loss during the first few minutes while the product is heating, may enable a more accurate processing time.

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Appendix B Detailed Methods for Inoculating Flour

Procedure for Inoculating Flour

1. Autoclave the kitchen aid bowl, mixing paddle, spoon, sieve, pie tin, small ceramic jar and 950 grams of ceramic beads at 121°C for 15 minutes on the dry setting.

2. Turn on biosafety cabinet

3. Set up kitchen aid and jar mixer in the biosafety cabinet

4. Allow the autoclave to finish the complete cycle

5. Removed autoclaved materials from the autoclave and cool in biosafety cabinet

6. While wearing gloves weight out 100 grams of flour into the kitchen aid bowl

7. Add the kitchen aid bowl and mixing paddle to the mixer using new/clean gloves

8. Turn the mixer on to the slowest speed and allow the mixer to run for approximately 30 seconds

9. Using a 1 ml mechanical pipette to spray 3 ml of inoculum into the flour

10. After all 3M®l are injected into the flour allow to mix on the slowest speed for 5 minutes

11. Using an autoclaved spoon to guide, put the inoculated flour into the cooled autoclaved ceramic jar (IF THE JAR IS TOO HOT IT WILL KILL THE BACTERIA)

12. Add the 950 grams of ceramic beads to ceramic jar

13. Using the dial on the front of the jar mixer set the speed to 65 rpm

14. Turn on the jar mixer

15. Begin timing once the jar mixer is started and allow to run for 30 minutes

16. Turn off after 30 minutes

17. Place sieve on pie tin

18. Pour the contents of the jar recently removed from jar mill into the sieve

19. Shake the sieve and pie tin until all of the flour is in the pie tin

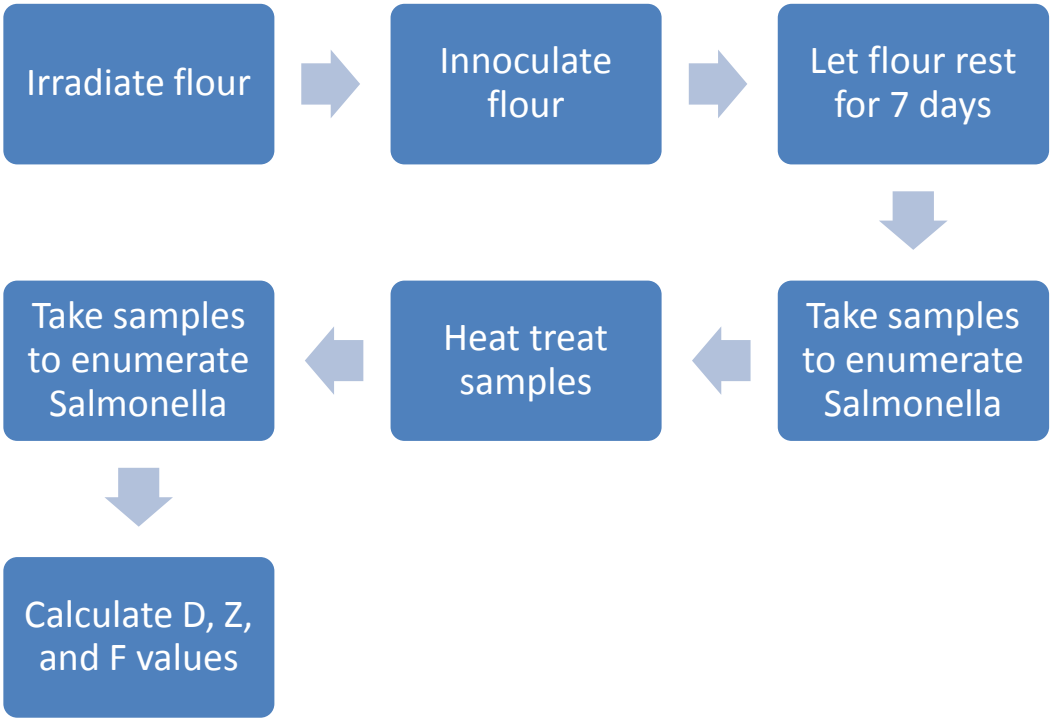
20. Pour the flour from the pie tin into a new sterile whirl pak bag.

21. Label the bag with the inoculum, date, time, and your initials

22. Place bag into the small fridge for 1 week to all for equilibration between the bacteria cells and the flour

Appendix C Expanded Methods

Surrogate Study



The organisms that will be used in this study will be; *Salmonella* Typhimurium Phage Type 42 isolated from flour implicated in a foodborne outbreak of Salmonellosis (McCallum and others 2013), *Escherichia coli* ATCC 11229, *Enterococcus faecium*, and ten isolates from thermally processed flour.

A. Irradiate Flour

Flour samples were to Sadex Co. for irradiation to eliminate all resident microbes. The flour samples were packaged into pre measured plastic bags. The flour was irradiated at a level of 10kGy.

B. Preparation of inocula (Komitopoulou and Penaloza 2009)

The test bacteria were grown up on Columbia agar to obtain uniform lawns. The plates were then scrapped with a sterile inoculating loop. The organisms from the plate were then suspended in 3 mL of sterile Maximum Recovery Dilutant (MRD).

C. Inoculation of flour samples (Modified method from Bookwalter and others (1980))

The 3M®L of MRD containing the organism was pippetted directly into the pre-measured flour with low speed mixing. Once all the MRD had been distributed the mixer continued for 5 minutes at the low speed. The flour was then transferred to a 500 mL jar with ceramic beads, and tumbled at 65 rpm for 30 minutes.

D. Let flour rest for 7 days (Beuchat and Scouten 2002)

The flour samples were then allowed to rest for 7 days at 5°C.

E. Pre-Samples taken

Samples were taken to enumerate the initial *Salmonella* concentration. This was done by plating the samples on APC Petri film.

F. Heat treatment of samples (Izurieta and Komitopoulou 2012)

The samples were placed in a glass vial with a type T thermocouple probe and then placed in a water bath. (Izurieta and Komitopoulou 2012) The water activity of all inoculated flour sets were taken and recorded before the flour was placed into the vials. 1.2 grams of flour was added to each HPLC vial. Triplicate vials were used for each test. The vials were sealed and placed in a

test tube holder, and then tapped against the counter to allow the flour to settle. The vials were exposed to, 70°C, 75°C, or 80°C. Each temperature will be run in triplicate.

G. Post-Samples taken (Izurieta and Komitopoulou 2012)

After heat treatment, the samples were cooled on ice and plated. (Izurieta and Komitopoulou 2012) *Salmonella* was plated on XLD agar with a thin layer of Columbia agar on top. Potential surrogates were be plated on Columbia agar using the spread plate method (Kang and Fung 2000) All plates were incubated at 37°C for 48 hours. (Izurieta and Komitopoulou 2012) The plates were counted at 24 hours and 48 hours

H. Calculate D, Z, and F values (Patashnik 1953; Izurieta and Komitopoulou 2012)

Death curves were created by plotting the time points against the $\log_{10}$ number of survivors determined from each plate count. Using SAS, the inverse of the slope of these death curves was calculated as the D value. The D values from each of the three vials were averaged to determine the mean D value at each time point. The $\log_{10}$ average D values from each temperature were plotted using Excel to obtain the slope of the line, which was used to determine the Z value. Both values were determined by taking the inverse of the slope of the lines created by Excel.

I. Statistical Analysis (Izurieta and Komitopoulou 2012)

Seven time points were collected for the temperatures 70°C and 75°C and six time points for 80°C. These seven time points constituted a run at each temperature. Triplicate runs at each temperature were conducted to determine variance and repeatability. In each run, three replicate vials were used for each microorganism at each time point.

To determine the baseline for each microorganism, three vials containing one of the different microorganisms were taken out of the water bath once all the vials in the water bath reached the correct temperature.

Continuous process

The continuous process was conducted at Bepex International in Minneapolis, MN.

Each run took 30 minutes with the flour pasteurizer set at a rate of 300 lbs per hour. Each run began with the machine equilibrating for 15 minutes. After the machine is equilibrated three samples of the flour were pulled from three distinct locations of the process. One sample was pulled from the flour about to enter the SolidAire®, one flour sample was pulled at the exit of the SolidAire®, and one sample was pulled from the exit of the heated screw. This sampling pattern was repeated every 5 minutes. This resulted in 15 samples being collected; three samples each at times 15, 20, 25, and 30 minutes for each run. Each sample size was 100 grams.

From each sample of 100 grams a subsample of 25 grams was used for plating. The samples taken were plated on differential agar using a spread plate method. Serial dilutions were made and the 10^{-1}, 10^{-2}, and 10^{-3} dilutions were plated. Each dilution was plated in duplicate.

The plates were incubated for 48 hours. Counts were recorded and results were analyzed for statistical significance.

To mix the inoculum into all the flour, a small portion (4 lbs.) of the flour was inoculated with 10^{7} cfu/g of *Pantoea dispersa* strain JFS at Brigham Young University using the methods previously outlined for the surrogate study. These 4 lbs. of inoculated flour was mixed into 400 lbs of flour at Bepex with a ribbon blender. This flour was then fed into each run using a high speed dispersion mixer.

The test design was as follows:

Run	Residence Time	Temperature	Initial Moisture	Steam
Run 1	4.5	105	10%	0%
Run 2	2	85	10%	0%
Run 3	4.5	95	13%	0%
Run 4	11	95	13%	0%
Run 5	2	85	13%	0%
Run 6	4.5	95	13%	0%
Run 7	11	105	13%	0%
Run 8	10.7	85	10%	5%
Run 9	2	105	13%	5%
Run 10	4.5	95	13%	5%
Run 11	4.5	85	13%	5%
Run 12	10.7	95	10%	5%
Run 13	4.5	95	10%	5%
Run 14	9	105	10%	5%
Run 15	9	105	13%	5%

Batch Process

The batch process was conducted at Honeyville Grains.

Temperature	Time of First Pull	Time of Second Pull
150	When bag reaches 150	After one hour above 150
160	When bag reaches 160	After one hour above 160
170	When bag reaches 170	After one hour above 170

Flour was inoculated for the batch process method using the previously outlined methods for the surrogate study. Inoculated flour for the convective chamber was packaged a small pouch made of polypropylene and placed in the center of standard 50-lbs bags of pastry flour. The process was evaluated using 12 "test" bags (containing inoculated pouches of 26 grams of flour in a mesh sack made of spun bonded polypropylene). Thermocouples were placed in the geometric center of each test bag. The flour was subjected to convection heating. Bags were removed for analysis when the cold-point temperature (bag center) reached either 150, 160, or 170°F or after being held for one hour at the target temperature. Samples were returned to Brigham Young University for analysis. The process was repeated 7 times. The samples were assayed using 3M® Petrifilm® for aerobic plate counts. They were also plated on Columbia agar (*Pantoea* produces yellow colonies). The 10^{-1}-10^{-4} dilutions of each sample were plated. The results were analyzed after 48 hours of incubation. The results were then evaluated to determine any statistical difference in processing temperature or time and also to determine any testing parameters that resulted in an increase of more than 0.5 logs in lethality.

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