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Evaluation of thermal inactivation parameters of *Salmonella* in whole wheat multigrain bread

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ABSTRACT

This study was conducted to validate a simulated commercial whole wheat multigrain bread baking process at 375 °F (190.6 °C) oven temperature for 35 min to inactivate *Salmonella*, and to determine the thermal inactivation parameters of a 7-serovar *Salmonella* cocktail in whole wheat multigrain bread dough. A ≥5-log CFU/g reduction in *Salmonella* population was achieved by 15 min, and no viable *Salmonella* was detected after enrichment plating by 16 min. The a_w of the bread crumb (0.96) after baking and 60 min of cooling was similar to that of pre-baked bread dough, whereas the a_w of bread crust decreased to 0.81 at the end of baking and cooling. The D-values of the *Salmonella* cocktail in bread dough were 59.6, 20.0 and 9.7 min at 50, 52 and 55 °C, respectively; and the *z*-value was 6.5 °C.

1. Introduction

Salmonella serovars are critical bacteriological hazards because of their ability to survive in diverse food matrices and processing environments for extended periods, even under adverse conditions. The potential of *Salmonella* to survive under desiccated state for a long period of time in low water activity (a_w) foods such as flour (McCallum et al., 2013), spices (Hara-Kudo et al., 2006), nuts (Zhang et al., 2017), spray dried dairy products (Miller et al., 1972; Jung and Beuchat, 1999), dried egg products (Cahill et al., 2008), and on equipment (Grasso et al., 2015) makes it one of the most challenging foodborne bacterial pathogens for food manufacturers around the world (Finn et al., 2013). According to the U.S. Centers for Disease Control and Prevention (CDC), *Salmonella* was a predominant confirmed bacteriological agent associated with foodborne outbreaks and illnesses in 2013 and 2014. *Salmonella* was associated with 149 outbreaks and 3553 illnesses in 2013 (CDC, 2015); whereas in 2014, *Salmonella* was responsible for 140 outbreaks and 2395 illnesses (CDC, 2016). *Salmonella* serotypes Enteritidis, Typhimurium, Newport, Javiana and Heidelberg were the most common serotypes associated with these foodborne outbreaks (CDC, 2015, 2016).

The U.S. Food and Drug Administration's Food Safety Modernization Act (FSMA), enacted in January 2011, aims to ensure the safety of the U.S. food supply by focusing on preventing food contamination instead of responding to process failures (FDA, 2016a; King and Ades, 2015). To achieve this goal, the FSMA requires food processors to establish and implement Hazard Analysis and Risk-Based Preventive Controls (HARPC) plans. These HARPC plans for specific processes should include written and validated preventive controls for the critical processing steps that can prevent and/or significantly reduced the identified hazards (FDA, 2016b; King and Ades, 2015).

The baking process in food manufacturing is generally considered to be an effective step in controlling foodborne pathogens; however, there are very few published studies on the validation of baking processes against pathogens in different bakery products (Channaiah, 2016; Lopez, 2014). Further, the diversity of bakery products that are commercially manufactured and their range of intrinsic and extrinsic properties necessitate multiple process validation studies to adequately demonstrate effective pathogen control capabilities. Pre-bake product mixes (batters or doughs) can become contaminated with pathogens from the commonly used raw ingredients in the bakery industry, such as flour, sugar, milk powder, cocoa powder, nuts and chocolate (Akins, 2011; GMA, 2009; Saranraj and Geetha, 2012).

In earlier studies, we demonstrated that hamburger bun (Channaiah

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et al., 2016) and plain muffin (Channaiah et al., 2017) baking process were efficient in reducing prebaking contamination of *Salmonella* (≥5-log reduction). However, it is important to understand the survival and the heat resistance kinetics of *Salmonella* in large sized bakery products such as bread to optimize the baking process parameters to achieve food safety. Therefore, the main objectives of this study were to validate the simulated commercial baking process to inactivate *Salmonella* in whole wheat multigrain bread prepared from contaminated flour, and to determine the thermal inactivation parameters (D- and *z*-values) of *Salmonella* during heating of bread dough.

2. Materials and methods

2.1. Experimental design and statistical analysis

This research was conducted to validate the effectiveness of a simulated commercial whole wheat multigrain bread baking process to eliminate *Salmonella*, and consisted of the following studies: 1) validation of baking at 375 °F (190.6 °C) oven temperature for 35 min as a kill-step to eliminate a 7-serovar *Salmonella* cocktail in whole wheat multigrain bread, and determination of the breakpoint (baking time at which no viable *Salmonella* cells were detected after enrichment) of the inoculated *Salmonella* cocktail in the bread; 2) determination of oven temperature and humidity ratio, and pH and a_w of the crumb and crust of whole wheat multigrain bread during baking; and 3) determination of D- and *z*-values of the 7-serovar *Salmonella* cocktail during heating of whole wheat multigrain bread dough.

For the baking validation study, whole wheat multigrain bread dough was prepared from inoculated (*Salmonella* cocktail) whole wheat multigrain flour comprised of a coarse and fine flour mix. The dough was baked using a 190.6 °C oven temperature for 35 min followed by 60 min of ambient air cooling (B + C), with product sampling at predetermined times during baking and B + C to enumerate surviving *Salmonella* population levels. This study used a randomized complete block design with three replications as blocks and twelve sampling times as treatments (inoculated flour; pre- and post-proof dough; 10, 11, 12, 13, 14, 15, 16 and 17 min of baking; and B + C). For the temperature and humidity ratio monitoring, and pH and a_w determination studies, non-inoculated whole wheat multigrain flour mix was used to prepare bread dough. For the temperature and humidity ratio monitoring, baking was conducted for 35 min without opening the oven door; whereas for the pH and a_w determination part of the study, oven door was open at regular intervals for no longer than 5 s. This study was also designed as randomized complete block (three replications) with eleven sampling times as treatments (inoculated flour; pre- and post-proof dough; 5, 10, 15, 20, 25, 30 and 35 min of baking; and B + C) for pH and a_w. The statistical analyses of microbial, pH and a_w data were conducted by analysis of variance at $P \leq 0.05$ using SAS version 9.3 (SAS Institute, Cary, NC). An additional bread baking was conducted using non-inoculated flour mix to collect samples during the baking and at B + C for the proximate analyses.

D-values of the *Salmonella* cocktail in whole wheat multigrain bread dough were determined using thermal-death-time (TDT) disks and temperature-controlled water baths. The mean D- and *z*-values were calculated from the linear regression graphs plotted using Microsoft Excel 2011 (Microsoft Corp., Redmond, WA) for each replication separately. This study utilized a randomized complete block design with three replications and microbial plating was done in duplicate.

2.2. Salmonella cultures and inoculum preparation

Three *Salmonella* serovars were obtained from the American Type Culture Collection (ATCC; *S.* Senftenberg 775 W 43845, *S.* Newport 6962 and *S.* Typhimurium 14028), and *Salmonella* Tennessee and three dry pet food isolates were obtained from Richter International, Inc. (Columbus, OH). *Salmonella* Senftenberg 775 W was selected in this study because of its greater heat resistance in high moisture food matrices compared to other *Salmonella* serovars (Ng et al., 1969; Quintavalla et al., 2001; Bayne et al., 1965), whereas *Salmonella* Newport and Typhimurium were included because of their frequent association with foodborne outbreaks in the U.S. (CDC, 2015). *Salmonella* Tennessee and three dry pet food isolates were also included to represent environmentally derived contamination of whole wheat multigrain flours.

All cultures were activated in brain heart fusion (BHI; Becton, Dickinson and Company, Sparks, MD) broth at 37 °C for 24 h, and then individually transferred and stored on protectant beads in glycerol (Microbank™ Bacterial and Fungal Preservation System, Pro-Lab Diagnostics, Round Rock, TX) at −80 °C. All cultures were individually reactivated and propagated as described by Channaiah et al. (2017). Briefly, frozen cultures were individually reactivated by transferring one bead of respective culture into 10 mL of BHI broth, incubated at 37 °C for 24 h, and then stored at 4 °C until use. Each culture from BHI broth was streaked on three BHI agar plates to grow lawns and incubated at 37 °C for 24 h. To harvest *Salmonella* cells, 1 mL of 0.1% peptone (Becton, Dickinson and Company) buffer was dispensed twice on the BHI agar surface and cells were dislodged using disposable L-shaped cell spreaders (Fisherbrand®, Fisher Scientific™, Pittsburg, PA). Harvested culture solutions were pipetted into 10 mL sterilized test tubes to obtain ~6 mL of individual culture solutions. All seven culture solutions were mixed in equal proportions in a 50 mL sterile conical tube to be used as a master inoculum for inoculating whole wheat multigrain flour mix.

2.3. Flour inoculation

The coarse (Stone Ground - EZ, ConAgra Mills, Omaha, NE) and fine (Stone Ground - EZ, ConAgra Mills) whole wheat multigrain flours were weighed (300 g each) into sanitized sealable plastic tubs (9.4 L, Rubbermaid, Atlanta, GA), mixed, spread into an even layer, and placed inside of a biosafety cabinet. Flour was then mist-inoculated by spraying the master inoculum as a fine mist (~1 mL per 100 g of flour mix) uniformly across the flour layer. The tubs were sealed and flour was mixed manually by shaking tubs inside of the biosafety cabinet for approximately 30 s. Inoculated flour was dried back to the original pre-inoculation weight by placing each tub with open lid into an incubator (Lab-Line®, Imperial III Incubator, Melrose Park, IL) at 37 °C for ~5 h. Dried, inoculated flour mix was mixed again using a spatula to break clumps and manually shaking the sealed tubs, and then stored in the sealed tubs at ambient temperature (~25 °C) until used (within 7 days).

2.4. Bread dough preparation

All ingredients and the recipe for preparing whole wheat multigrain bread were provided by AIB International (Manhattan, KS) (Table 1). The 12-grain mix (42025 Coarse 12 Grain Base, Dakota Specialty Milling, Fargo, ND) used in the recipe consisted of sunflower, wheat, corn, sesame seeds, barley, rye, triticale, brown rice, oats, millet, ground brown flax and buckwheat. The flour and other dry ingredients were weighed into a sanitized 18.9 L (20 quart) McDuffee mixing bowl and mixed with a spatula. After adding ascorbic acid and water, the bowl was placed into a Hobart A-20 mixer (Hobart, Troy, OH) attached with a fork agitator. The bowl and mixer were covered with a plastic bag to control biological aerosols, and only N-95 respirator approved laboratory personnel participated in ingredient preparation and dough mixing. Mixing was done first at low speed for 1 min followed by medium speed for 10 min. The dough was then divided into four pieces (~524 g each) and allowed to rest for 10 min for ambient temperature (~25 °C) proofing. Dough pieces were sheeted to ~12 mm thickness using a rolling pin, and hand rolled into semi-tight cylinders. Molded loaves were placed with seam side down into stainless steel bread pans (22.9 cm length, 11.3 cm width and 7.0 cm depth), which were pre-

Table 1
Ingredients used to prepare whole wheat multigrain bread.

Ingredient	Weight (g)
Flour, coarse whole wheat	525.0
Flour, fine whole wheat	525.0
12 grain mix	105.0
Vital wheat gluten	73.5
Sodium stearoyl lactylate	5.3
Mineral Yeast Food	1.8
Salt	21.0
Calcium propionate	2.7
Sugar	105.0
Shortening, all-purpose	42.0
Ethoxylated monoglyceride	5.3
Yeast, compressed	42.0
Ascorbic acid	2.3
Water	701.2

sprayed with pan releasing agent (DDA®, Dawn Distribution Advantage, Jackson, MI), and placed inside a proofing cabinet pre-set at 110 °F (43.3 °C) and 81.5% relative humidity (RH) for ~45 min, or until the dough rose to 90 mm height.

2.5. Bread baking

Due to the optimum mixer and oven capacities in the laboratory, only four bread dough loaves could be prepared and baked per batch. Therefore, for each baking replication (using inoculated or non-inoculated flour mix), two batches of bread dough were prepared to provide a total of eight loaves. These eight loaves were then randomly assigned to pre-determined sampling times during baking. Four pans were placed into an electric kitchen oven (Whirlpool®, 4.8 cubic feet, FlexHeat™, Dual Radiant Element, Benton Harbor, MI) pre-heated to 190.6 °C, and one pre-determined random bread loaf was assigned to monitor the internal bread crumb temperature during 35 min of baking and 60 min of ambient air cooling on a wire rack.

2.6. Temperature and humidity ratio measurements

For monitoring and recording the internal bread temperatures, fine-gauge type-T thermocouples (Omega Engineering Inc., Stamford, CT) connected to an eight-channel data logger (USB-TC with MCC DAQ software, Measurement Computing, Norton, MA) were inserted from the top of the loaves into their geometric centers. During preliminary baking trials, the geometric center of the loaves was determined to be the cold spot and the dough temperature increased faster on the surface compared to the various internal locations. One thermocouple was attached to the oven shelf to monitor the oven air temperature during baking.

As RH cannot be used as an accurate measurement to account for the amount of moisture present in the air at temperatures > 100 °C, humidity ratio (Kg of moisture per Kg of dry air) values were used to measure the amount of moisture inside the oven during baking. At temperatures > 100 °C, the RH is an inappropriate unit of measurement as air loses the capacity to hold moisture (Xue et al., 2004). Moreover, unlike RH, the humidity ratio measurements are independent of temperature. Therefore, measuring humidity ratio, instead of RH, is a common practice tin the bakery industry. Humidity ratio is simply expressed as the ratio of the actual mass of water vapor present in a moist air to the mass of the dry air.

$$x = m_w/m_a$$

where,

x = humidity ratio (lb_{water}/lb_{dry_air} or kg_{water}/kg_{dry_air})
m_w = mass of water vapor (lb or Kg)

Fig. 1. Humidity sensor enclosed in a thermal barrier.

m_a = mass of dry air (lb or Kg)

Humidity ratio of oven during 35 min of baking was monitored and recorded using SCORPION® 2 Humidity Sensor and data logger enclosed in the SCORPION® 2 HUTHB125 thermal barrier (Reading Thermal, Sinking Spring, PA; Fig. 1). Following the manufacturer's advice, the humidity sensor enclosed in the thermal barrier was preheated to ~65 °C inside an oven before using it for the humidity measurements during the baking process. The humidity sensor was placed on the lower shelf of the oven, whereas bread loaves were placed on the top shelf. Temperature and humidity ratio were recorded every second during monitoring.

2.7. pH, water activity and proximate analyses

During baking, bread samples were taken at 5, 10, 15, 20, 25, 30 and 35 min of baking, and at B + C for the pH and a_w determinations. At each sampling point, bread loaves were quickly removed from the oven and the oven door was shut within 5 s to maintain oven temperature and humidity conditions within. During the preliminary work, it was determined that opening the oven door for < 5 s for sampling did not affect the whole wheat multigrain bread temperature, and oven temperature and humidity significantly because bread temperature did not show any change, and oven temperature and humidity ratio values attained the original trend within 15 s of closing the oven door. The bread loaves were core sampled (2.5 cm diameter) from top to bottom at the center, and the bread crumb and crust were separated. For a_w determination, bread crumb and crust after sampling were immediately transferred separately into a_w cups (Decagon Devices, Inc., Pullman, WA), sealed, allowed to cool (~25 °C), and a_w was measured using AQUALAB Dewpoint 4 TE a_w meter (Decagon Devices, Inc.). For bread pH determination, 10 g of bread crumb and crust mixture (equal weights) was mixed in 90 mL of de-ionized water, and pH was measured by a calibrated pH meter (Accumet Portable, AP63 pH/mV/ion meter, Fisher Scientific). The pH meter was calibrated using pH 4.0 and 7.0 standard buffer solutions (Ricca Chemical Company®, Arlington, TX). An additional bread baking replication was conducted using non-inoculated whole wheat multigrain flour mix, sampled at the previously stated times, and sent to the analytical laboratory in the Animal Sciences and Industry Department at Kansas State University for the proximate analyses of moisture, fat, protein and starch. Briefly,

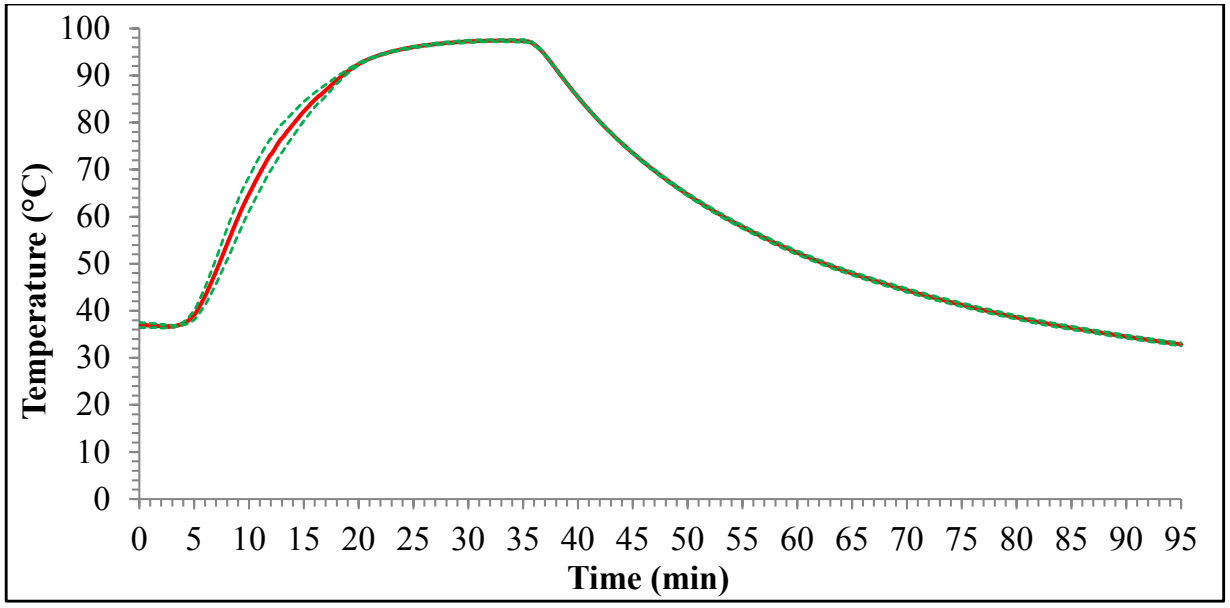

Fig. 2. Mean temperature (± SE, dotted lines) of whole wheat multigrain bread (geometric center) during 35 min of baking at 190.6 °C oven temperature and 60 min of ambient air-cooling.

moisture was determined using microwave based rapid moisture analyzer (CEM Corporation, Matthews, NC), fat was determined using nuclear magnetic resonance based fat analyzer (CEM Corporation, Matthews, NC), protein was determined using an automated nitrogen/protein analyzer (LECO, Saint Joseph, MI), and carbohydrate was determined using amyloglucosidase/α-amylase method.

2.8. Baking validation and breakpoint determination

For the baking validation study, bread loaves were sampled at 10, 11, 12, 13,14, 15, 16 and 17 min of baking. Each sample consisted of a composite of three 2.5 cm diameter cores (one taken through the loaf center and two from random locations near the loaf edges) to get total of ~100 g samples, placed into a filtered stomacher bag (31 cm × 18 cm; Fisherbrand®) containing 120 mL of 0.1% peptone solution at 4 °C to get an initial dilution of 2.2 in the stomacher bag. All sample bags were weighed to note the exact weights of the samples. During the preliminary work, it was determined that 120 mL of 0.1% peptone solution used in the bags was low enough to give the lowest possible detection limit but also large enough to completely immerse the samples. Each sample was immediately homogenized in the chilled buffer by hand to decrease the bread temperature to ~10 °C to arrest any further thermal lethality. The bread sample homogenates were stored at ~4 °C until they were analyzed (within 30 min of collection).

2.9. Determination of D- and z-values

The D- and *z*-values of the 7-serovar *Salmonella* cocktail in bread dough were determined using the method described by Channaiah et al. (2017). For each target temperature, ~10 g (into each TDT disk) of bread dough was transferred into five TDT disks (6.0 cm diameter and 0.5 cm height; University of Nebraska, Lincoln, NE) and sealed. A sixth TDT disk filled with bread dough was connected (geometric center of the TDT disk) to a fine-gauge type-T thermocouple and a data logger was used to monitor the dough temperature during thermal treatment. The TDT disks were placed into temperature-controlled circulating hot water baths (Model 2864, Thermo Fisher Scientific™, Marietta, OH), which were pre-set at the respective temperatures. After the target temperatures were attained, TDT disks were removed from the water bath at each sampling point and transferred into an ice water bath to quickly decrease the bread dough temperature to ~4 °C. Chilled bread dough samples were then transferred into stomacher bags (16 cm × 10 cm; Fisherbrand®) containing 10 mL of pre-chilled (~4 °C) 0.1% peptone buffer and the viable *Salmonella* population was enumerated. For each replication, regression graphs were plotted between the log *Salmonella* population and corresponding sampling time (min), and D-values were calculated as the absolute value of the inverse of the slope of the regression lines. The *z*-value was calculated as the absolute value of the inverse of the slope of regression lines plotted between the log D-values and the corresponding temperatures (°C).

2.10. Microbial enumeration

For the baking validation, the *Salmonella* population at defined processing points was enumerated on selective agar, as well as on injury-recovery media; whereas, only *Salmonella* population enumerated on injury-recovery media was used for D-value studies. Xylose lysine deoxycholate (XLD; Becton, Dickinson and Company) agar was used for the selective enumeration, whereas BHI agar overlaid (after 5 h of incubation at 37 °C) with XLD agar was used for the injury-recovery enumeration. The stomacher bags containing bread dough or bread samples in 0.1% peptone buffer were homogenized (Smasher™, bioMérieux Industry, Hazelwood, MO) for 1 min, serially diluted using 0.1% peptone, spread plated on the selective and injury-recovery media, and incubated at 37 °C for 24 h. For the baking validation study, the bags containing stomached samples were stored at ~4 °C to use for enrichment recovery when no visible *Salmonella* colonies on injury-recovery media were observed. For enrichments, 25 mL out of ~120 mL of the stored sample homogenates were transferred into 225 mL BHI broth and incubated at 37 °C for 24 h. Enriched samples were then streaked on XLD agar and incubated at 37 °C for 24 h to check for presumptive *Salmonella* growth. The sampling time during baking where no *Salmonella* growth was detected after enrichment was considered as the “breakpoint” indicating the elimination of the inoculated *Salmonella* by the baking process.

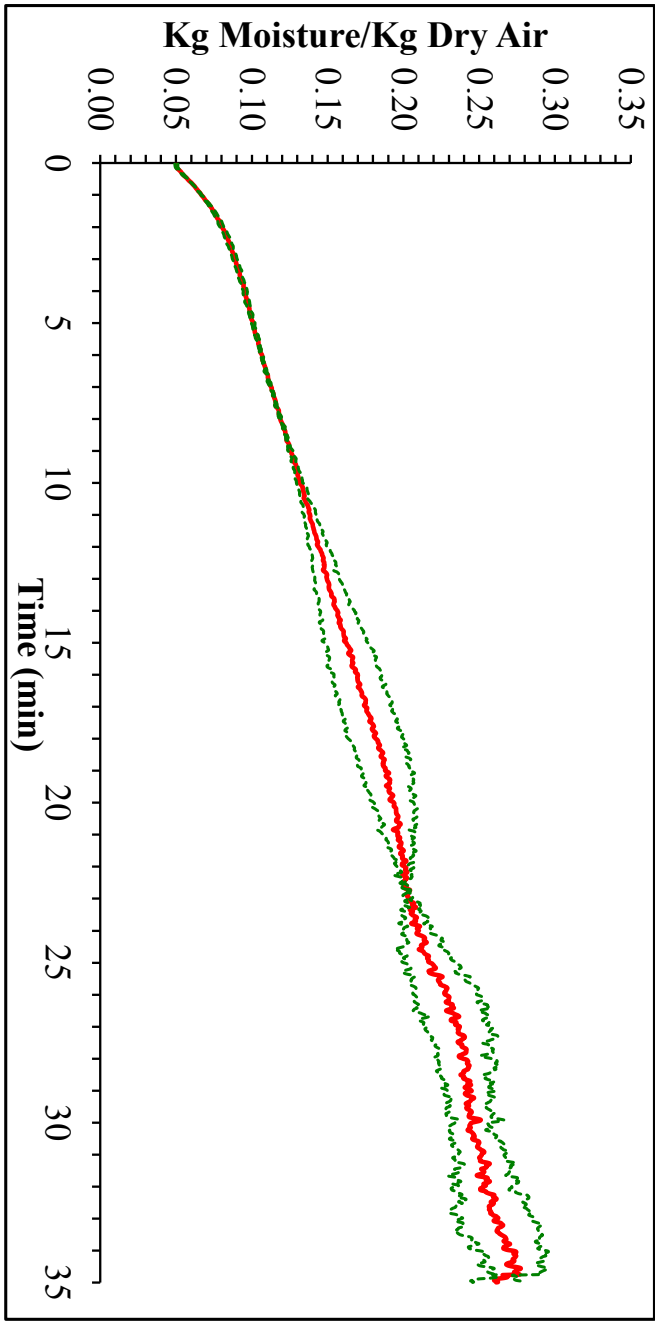

Fig. 3. Mean humidity ratio (Kg moisture/Kg dry air) (± SE, dotted lines) of oven during 35 min of baking at 190.6 °C oven temperature.

3. Results and discussion

3.1. Bread baking temperature and humidity ratio profile

The baking parameters for whole wheat multigrain bread used in this study were provided by AIB International. Bakery scientists at AIB International conducted several preliminary bread baking trials using an electric kitchen oven to simulate a typical internal temperature profile of bread during industrial baking. Baking whole wheat multigrain bread at 190.6 °C for 35 min in the kitchen oven followed by 60 min of ambient air cooling provided a representative temperature profile similar to industrial baking, and the final bread loaves met industrial quality parameters such as crust color, appearance, size and texture.

The internal temperature profile of the bread loaves at the geometric center during baking and ambient air-cooling is shown in Fig. 2. The initial internal temperature of bread at the start of baking was 37.0 ± 0.56 °C. The bread temperature increased to > 70 °C in 11.2 min and further increased to 97.0 ± 0.24 °C by 28.6 min, where it stabilized for the remainder of the baking period. Whereas, the humidity ratio of the oven at the start of baking just after placing bread loaves inside the oven and closing the oven door was

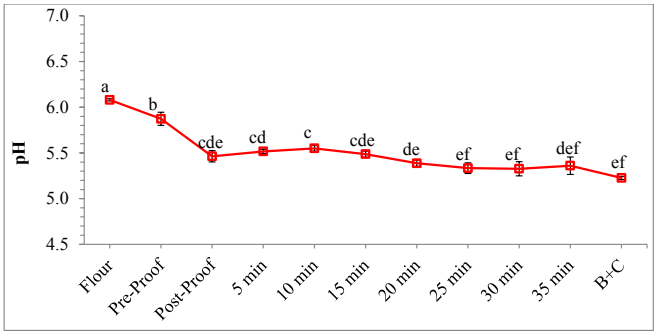

Fig. 4. pH (mean ± SE) of whole wheat multigrain flour mix, pre- and post-proofed bread dough, and bread during 35 min of baking at 190.6 °C and after 60 min of ambient air-cooling (B + C). **a-f:** Letters with different pH values are different ($P \leq 0.05$).

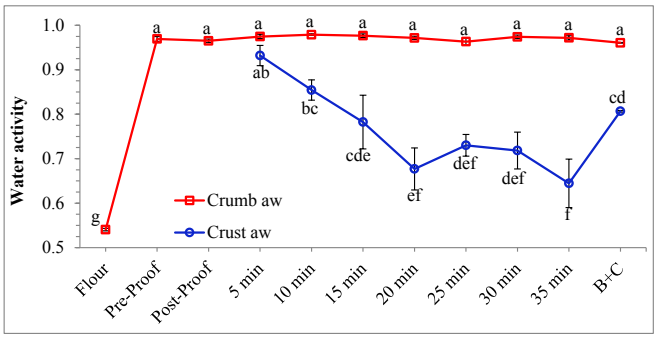

Fig. 5. Water activity (a_w; mean ± SE) of whole wheat multigrain flour mix, pre- and post-proofed bread dough, and whole wheat multigrain bread (crumb and crust separately) during 35 min of baking at 190.6 °C and after 60 min of ambient air-cooling (B + C). **a-f:** Letters with different a_w values are different ($P \leq 0.05$).

Table 2
Proximate analyses of whole wheat multigrain bread dough, and bread during 35 min of baking at 190.6 °C oven temperature and after 60 min of ambient air-cooling.

Sample		% Moisture	% Fat	% Protein	% Starch
Dough	Pre-proof	41.62	2.56	11.51	33.86
	Post-proof	41.68	2.71	11.93	36.19
5 min	Crumb	43.44	3.18	11.65	36.02
	Crust	36.70	3.55	12.31	41.09
10 min	Crumb	40.90	3.92	11.47	36.34
	Crust	32.11	3.71	13.23	41.50
15 min	Crumb	44.37	3.20	10.90	33.11
	Crust	25.67	3.83	14.26	44.92
20 min	Crumb	43.28	3.35	11.04	33.23
	Crust	21.61	4.23	15.34	46.43
25 min	Crumb	43.27	3.25	10.88	37.29
	Crust	20.24	3.85	15.25	47.76
30 min	Crumb	43.64	3.08	10.93	35.87
	Crust	21.37	3.99	15.19	47.24
35 min	Crumb	43.21	2.91	10.70	35.12
	Crust	14.88	3.11	16.18	53.04
B + C	Crumb	50.96	3.01	11.21	35.03
	Crust	40.96	3.47	14.38	42.15

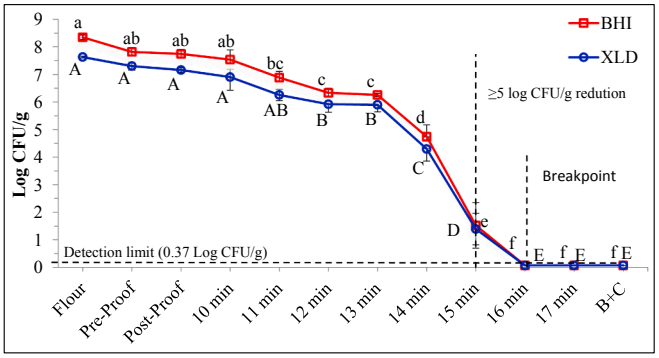

Fig. 6. Recovered *Salmonella* (7-serovar cocktail) populations (mean ± SE) in whole wheat multigrain bread during 35 min of baking at 190.6 °C and after 60 min of ambient air-cooling (B + C) as plated on selective [xylose lysine deoxycholate (XLD) agar] and injury-recovery [brain heart fusion (BHI) agar overlaid with XLD agar] media. **a-f:** Letters with different *Salmonella* counts plated on injury-recovery media are different ($P \leq 0.05$). **A-E:** Letters with different *Salmonella* counts plated on selective media are different ($P \leq 0.05$).

0.050 ± 0.0009 kg/Kg dry air (Fig. 3). As expected, the overall humidity ratio of oven increased during the baking process due to the steam generated from the moisture inside the bread loaves, and at the end of 35 min of baking, the humidity ratio was 0.261 ± 0.0158 kg/Kg dry air (Fig. 3). The increase in humidity ratio inside the oven during the baking process could be a potential safeguard in most baking processes against *Salmonella* on the bread surface. To the best of our knowledge, we are the first one to study and measure the humidity ratio at above 100 °C in a baking validation study. Therefore, there are no earlier literature to compare the humidity ratio results generated in this study. However, the humidity ratio of the oven during the whole wheat multigrain bread baking in the current study matches with the commercial ovens (personal communication with Richard Starke, Reading Thermal Company, Sinking Spring, PA, USA). During ambient air-cooling, the internal temperature of bread remained > 70 °C for 11.8 min, and at the end of 60 min of cooling had decreased to 32.8 ± 0.31 °C.

3.2. pH, water activity and proximate analyses

The pH of whole wheat multigrain flour mix, pre- and post-proofed bread dough, bread during baking and at B + C is presented in Fig. 4. The pH of whole wheat multigrain flour mix was 6.08 ± 0.01. During proofing, pH of bread dough decreased significantly from 5.87 ± 0.07 to 5.46 ± 0.06, and pH ranged from 5.52 ± 0.03 to 5.36 ± 0.10 throughout the 35 min of baking and reached 5.23 ± 0.02 after 60 min of ambient cooling (Fig. 4). The a_w of whole wheat multigrain flour mix was 0.54 ± 0.003 (Fig. 5). The a_w of pre-proofed bread dough was 0.97 ± 0.005, which remained similar to that of bread crumb throughout baking and at the end of 60 min of ambient cooling (0.96 ± 0.001). However, the a_w of bread crust significantly decreased from 0.93 ± 0.023 at 5 min of baking to 0.64 ± 0.054 at the end of 35 min of baking but increased to 0.81 ± 0.001 after 60 min of ambient air-cooling (Fig. 5).

The proximate analyses of whole wheat multigrain bread dough, and bread during baking and after ambient air-cooling are presented in Table 2. The chemical composition of bread dough did not change much during the proofing process. The moisture content of the bread crumb during baking remained similar (43.44 and 43.21% at 5 and 35 min of baking, respectively). However, as expected, the moisture content of the bread crust was lower than that of bread crumb throughout the baking. The moisture content of bread crust decreased from 36.70% at 5 min of baking to 14.88% at the end of 35 min of baking. However, at the end of 60 min of ambient air-cooling the moisture contents of the bread crumb and crust were 50.96 and 40.96%, respectively (Table 2).

The chemical composition, pH and a_w of variety of breads can vary based on the ingredients and baking parameters used. Fernandez-Salguero et al. (1993) reported that among the various food products purchased from the supermarkets across Spain during 1991 and 1992, the white sandwich bread had 0.929 a_w, 6.09 pH and 39.9% moisture. Whereas Feeherry et al. (2003) studied the growth kinetics of *Staphylococcus aureus* in ground bread crumb by adjusting the bread a_w from 0.836 to 0.909 at 5.2–5.5 pH and 35 °C. Feeherry et al. (2003) reported that the moisture content of these breads varied from 27.54 to 25.58%.

3.3. Baking validation and breakpoint

Sampling points for baking validation study were determined based on a preliminary study, whereby no viable *Salmonella* population was determined (after enrichment plating) on and after 16 min of baking or at B + C (data not presented). The 7-serovar *Salmonella* master inoculum used to inoculate the coarse and fine whole wheat multigrain flour mix was ~11 log CFU/mL. The dried, inoculated whole wheat multigrain flour mix demonstrated a *Salmonella* population of 8.35 ± 0.08 log CFU/g as enumerated on the injury-recovery media, which was similar to that of the *Salmonella* population in the pre-proofed (7.82 ± 0.16 log CFU/g) and post-proofed (7.74 ± 0.17 log CFU/g) bread dough (Fig. 6). During baking, the *Salmonella* population decreased by ≥ 5 log cycles at 15 min of baking (with a surviving

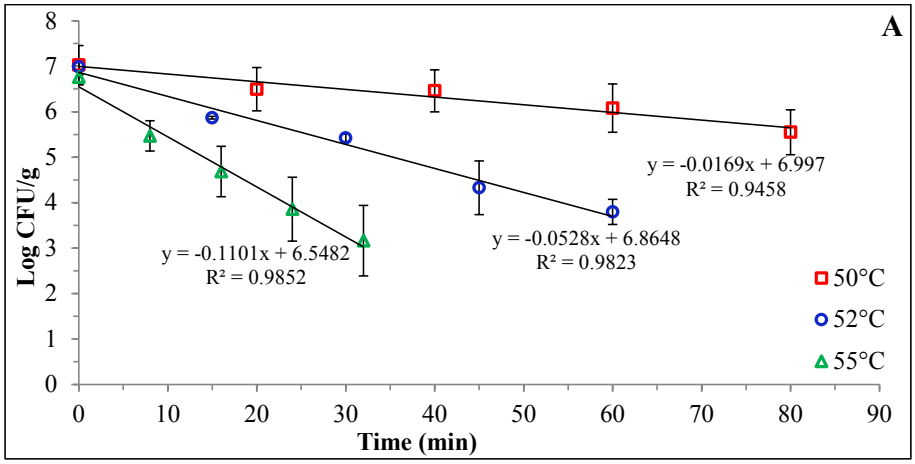

Fig. 7. Thermal inactivation graphs (mean ± SE) of a 7-serovar *Salmonella* cocktail in whole wheat multigrain bread dough at 50, 52 and 55 °C using injury-recovery media (brain heart infusion agar overlaid with xylose lysine deoxycholate agar).

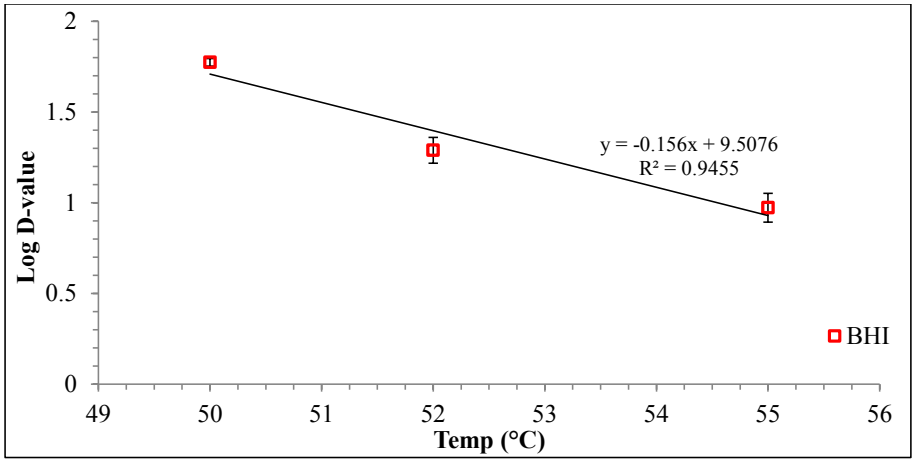

Fig. 8. Linear regression lines (log D-value *vs.* Temperature) of a 7-serovar *Salmonella* cocktail in whole wheat multigrain bread dough using injury-recovery media [brain heart infusion (BHI) agar overlaid with xylose lysine deoxycholate (XLD) agar].

Table 3
D and *z*-values (mean ± SE) of a 7-serovar *Salmonella* cocktail in whole wheat multigrain bread dough.

Temp. (°C)	BHI
50	59.56 ± 2.84
52	19.96 ± 3.02
55	9.70 ± 1.76
z-value	6.51 ± 0.57

Salmonella population of 1.52 ± 0.83 log CFU/g; Fig. 6). The break-point for *Salmonella* in whole wheat multigrain bread, defined as the baking time when no viable *Salmonella* cells were detected by enrichment plating, was at 16 min in the 190.6 °C oven. At 15 and 16 min, the internal whole wheat multigrain bread temperatures were 82.4 ± 2.04 and 84.8 ± 1.53 °C, respectively (Fig. 2). The *Salmonella* counts enumerated on selective media were comparatively lower than the counts on injury-recovery media before and during 15 min of baking. Selective media are not appropriate for the enumeration of injured and stressed bacterial cells. Therefore, lower *Salmonella* counts in inoculated whole wheat multigrain flour and dough indicates the presence of stressed cells as result of inoculation, drying and storage of flour. Whereas, lower *Salmonella* counts on selective media during baking indicates the survival of a sub-population of sub-lethally injured *Salmonella* cells during the baking process.

Channaiah et al. (2016) prepared hamburger bun dough from flour inoculated with *S.* Typhimurium (ATCC 14028), *S.* Newport (ATCC 6962) and *S.* Senftenberg 775 W (ATCC 43845). They reported that a > 5 log CFU/g reduction was achieved by 6 min (at ~80 °C internal bun temperature) of baking at 218.3 °C oven temperature for all three *Salmonella* serovars, and no viable *Salmonella* cells were detected after 9 min of baking. In a similar baking validation study, Channaiah et al. (2017) reported that the population of a 3-serovar *Salmonella* cocktail (Typhimurium, Newport and Senftenberg) in muffins decreased by 5 log CFU/g during baking at 190.6 °C oven temperature by 17 min (achieving ~98 °C internal muffin temperature). Similarly, Lathrop et al. (2014) reported that no viable *Salmonella* was detected in peanut butter cookies prepared from peanut butter inoculated with a 5-serovar *Salmonella* cocktail (Tennessee FSL-R8-5221, Tornow FSL-R8-5222, Hartford FSL-R8-5223, Typhimurium FSL-WI-030, and Agona FSLS5-517) at the end of 15 min of baking at 177 °C oven temperature.

Shrestha et al. (2016) also conducted a similar study in which they studied the inactivation of *Salmonella* in sesame-toped bread during the baking process using low oven humidity (mean 3% RH, and baking at 177 °C for 7 min) and high oven humidity (mean 20% RH, and baking at 204 °C for 9 min). They used a 6-serovar cocktail of *Salmonella enterica*, consisting of Enteritidis E8864 (cheesecake isolate), Enteritidis E40 (chicken ovary isolate), Enteritidis PT30 (raw almond isolate), Heidelberg S13 (human isolate), Typhimurium S9 (human isolate) and Typhimurium A1 (peanut butter isolate). Shrestha et al. (2016) reported > 5 log reduction in *Salmonella* population during baking at both low and high oven humidity; however, the *Salmonella* kill was faster at high humidity conditions of oven.

3.4. D- and z-values

The linear regression lines (log *Salmonella* population *vs.* Time) used to calculate the D-values of the 7-serovar *Salmonella* cocktail in whole wheat multigrain bread dough at 50, 52 and 55 °C are presented in Fig. 7. Regression lines (log D-values *vs.* Temperature) used to calculate the *z*-values obtained using injury-recover media are presented in Fig. 8. All regression lines had $R^2 \geq 0.93$. The D- and *z*-values of the *Salmonella* cocktail are presented in Table 3. The D-value temperatures were selected based on the preliminary work conducted to have at least 5 log CFU/g *Salmonella* population by the time samples inside the TDT disks achieve respective temperatures (time = 0 min for thermal inactivation graphs). At temperatures > 55 °C, *Salmonella* population were decreased to < 5 log CFU/g by the time respective temperatures were achieved; therefore, 50, 52 and 55 °C were used in the current study.

In previous studies, the D-values at 55 °C of the same 3-serovar *Salmonella* cocktail (Typhimurium, Newport and Senftenberg) in hamburger bun dough and muffin batter were reported as 28.64 and 62.16 min, respectively (Channaiah et al., 2016, 2017). Obviously, the differences in the D-values from this study and those from et al. (2016, 2017) are likely attributed to differences in the composition of bread dough, bun dough and muffin batter matrices. For example, the muffin batter contains high sugar (60%) and crude fat (8.8%) compared to 2% sugar and 2.71% crude fat in bread dough. Past research has clearly shown that *Salmonella* display the greater thermal resistance in high sugar and fat foods (Gibson, 1973; Corry, 1972).

4. Conclusions

Despite of several kill-step validation studies to demonstrate the effectiveness of a thermal process to destroy pathogen of concern in various food products, precise role played by various intrinsic and extrinsic factors such as fat, protein, pH, moisture content, water activity, sugar content, humidity, heat penetration and air current are yet to

understand. Nevertheless, the current study clearly demonstrated that baking whole wheat multigrain bread at internal crumb temperature of > 97 °C (and > 190 °C oven temperature) will effectively eradicate high levels of *Salmonella* contamination in whole wheat multigrain bread, if pre-baking contamination of bread dough has occurred through ingredients and/or handling. As for any validation study, it should be noted that each baking process should be individually validated for the specific baking parameter and bread recipe. The D- and z-values for *Salmonella* determined in whole wheat multigrain bread dough provides the basic information regarding *Salmonella* thermal resistance at the start of the baking process. The findings from this research could be useful as a first step towards supporting validation and optimization of commercial bakery operations for bread making. Water activity of the bread decreases significantly during the baking process. Therefore, to develop strong thermal inactivation predictive models, it is vital to conduct further research to determine the D- and z-vales of *Salmonella* in bread at multiple lower water activities corresponding to the actual bread water activities at different stages during the baking process.

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