Thermal inactivation of *Salmonella* Typhimurium and surrogate *Enterococcus faecium* in mash broiler feed in a laboratory scale circulated thermal bath

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ABSTRACT This study compares kinetic parameters of *Salmonella* and surrogate *Enterococcus faecium* in mash broiler feed during thermal inactivation. Two-gram samples of mash broiler feed were added into a filtered sample bag and inoculated with nalidixic acid (**NaL**, 200 ppm) resistant *S.* Typhimurium or *Enterococcus faecium*, followed by vacuum-packaging and heating in a circulated thermal water bath at 75°, 85°, and 95°C for 0 to 180 s. Counts of bacterial survival were analyzed on tryptic soy agar and bile esculin agar plus 200 ppm of NaL. Microbial data and thermal kinetic parameters (n = 8, Global-Fit and United States Department of Agriculture [**USDA**]-Integrated-Predictive-Modeling-Program software) were analyzed by JMP software. Heating mash broiler feed at 75°, 85°, and 95°C decreased ($P < 0.05$) *Salmonella* cell counts by >6 $\log_{10}$CFU/g after 180, 60, and 50 s, respectively. Heating *E. faecium* in feed at 75°, 85°, and 95°C for 180, 120, and 70 s achieved reductions of 3, 6, and >6.5 $\log_{10}$CFU/g, respectively. D-values of linear, Weibull models, and *z*-value of *Salmonella* at 75°, 85°, and 95°C were 1.8 to 11.2, 4.2 to 21.8, and 28.6 s, respectively, which were lower ($P < 0.05$) than those of *E. faecium* (3.7−18.1, 8.5−34.4, and 34.1 s). Linear with Tail, Linear with Tail and Shoulder, and Weibull with tail equations revealed that *E. faecium* were more resistant ($P < 0.05$) to heat than *Salmonella* as shown by longer "Shoulder-time" (26.5 vs. 16.2 s) and greater "Tail" effect (4.4−4.5 vs. 2.5−2.6 $\log_{10}$CFU/g). Results clearly suggested that *E. faecium* can be used as a surrogate for *Salmonella* to validate thermal inactivation during feed manufacture.

Key words: broiler feed, *Salmonella*, *Enterococcus faecium*, thermal inactivation, surrogate bacteria

INTRODUCTION

Salmonella is a major microbial hazard in animal feed. When animals, such as poultry consume contaminated feed, hazards exist for the animals as well as humans who may consume these animals for food (McIlroy, 1996; Jones, 2011). It is estimated that, in the United States, 1.35 million cases of salmonellosis, including 26,500 hospitalizations and 420 deaths occur annually (U.S.-Centers for Disease Control and Prevention, 2020). Surveillance data published by the US-Centers for Disease Control and Prevention (U.S.-CDC, 2020) found that poultry products were the number one food category related to *Salmonella* outbreaks. Broilers that consume feed that has been contaminated with *Salmonella* can become infected, increasing the potential for contamination of processing equipment in the plant (Jones and Richardson, 2004).

Numerous serotypes of *Salmonella* have been detected in feed mills with Braenderup, Orion, Heidelberg, Infantis, Tennessee, and Kentucky being found more frequently (Shariat et al., 2020). *Salmonella*'s ability to survive in dry environments allows the pathogen to remain in both raw ingredients and the feed mill equipment for extended periods of time thereby potentially contaminating multiple batches of feed (Jones, 2011). Thermal processing in the form of steam conditioning during the feed manufacture process can be manipulated by feed mill operators to reduce the pathogen load of feed as this can be viewed as the critical control point (**CCP**) during feed manufacture (Huleback and Schlosser, 2002; Boltz et al., 2019). Past research using differing steam conditioning temperatures, conditioning times, antimicrobial inclusion, and feed mill equipment have shown promise for reducing the bacterial load of

pelleted poultry feed (Boney et al., 2018; Boltz et al., 2019). Currently, there are no industry recommendations for feed manufacture but modeling could change this (Cutlip et al., 2008). Limited work has been conducted modeling pathogen inactivation in feed as these models could facilitate the development of industry feed safety standards (Boltz et al., 2021; Steghöfer et al., 2021).

Thermal processing of broiler feed, using steam, has yet to be validated in a pilot-scale feed mill because the feed mill environment is more difficult to control and more dynamic than a lab setting. However, due to the difficulty to obtain biosafety level II status and concern for contamination of non-research feed, the use of food-borne pathogens, such as *Salmonella,* in feed mills is uncommon. The use of surrogate organisms is a viable way to develop Hazard-Analysis-Critical-Control-Point (**HACCP**) plans for feed mills, including identifying the critical control points (**CCPs**) and critical limits (**CLs**) of conditioning temperatures and times. Therefore, it is important to first validate a potential pathogen surrogate candidate in a laboratory setting before applying it in a feed mill environment. Recent studies from West Virginia University (**WVU**) have utilized *Enterococcus faecium (E. faecium)* as a *Salmonella* surrogate during different feed manufacture conditioning times and temperatures as well as the use of standard or aggressive thermal pelleting of poultry feed (Boney et al., 2018; Boltz et al., 2019). However, *E. faecium* has not been evaluated with *Salmonella* during thermal processing of broiler feed to verify its use as a non-pathogenic surrogate.

Therefore, this study aims to conduct side-by-side studies of *Salmonella* verse *E. faecium* in mash broiler feed to compare their behavior in various thermal conditions and to calculate their thermal kinetic parameters using predictive microbial mathematical models.

MATERIALS AND METHODS

Feed Manufacture

All mash feed used in this study was batched at the WVU pilot feed mill located in Morgantown, WV as described by Boltz et al. (2021). The corn and soybean-based diet was formulated to meet the needs of broilers in the finisher phase (Table 1). A 136 kg of feed was batched, and 15 mash feed samples were collected in sterile WhirlPak sample bags (23 × 15 cm, Nasco, Modesto, CA) and stored at −7°C until physicochemical analyses and microbial thermal inactivation were performed. The physical and chemical characteristics of manufactured feed including pH, water activity, and moisture content were tested as described by Boltz et al. (2021). Water activity values were analyzed using an AquaLab 4TE water activity meter (Decagon Devices, Pullman, WA). Data is reported as average values of 12 samples. Sample cups were filled with enough mash feed to cover the bottom before placing into the calibrated meter with 0.200, 0.450, and 0.760 standard

Table 1. Diet formulation

Ingredient	% of Diet
Corn	60.2
Soybean meal, 44% CP	33.2
Soybean oil	3.95
Dicalcium Phosphate	0.78
Limestone	0.89
L-lysine-HCl	0.04
DL-methionine	0.24
L-threonine	0.02
Salt	0.33
Sodium bicarbonate	0.10
Choline 60	0.11
Vitamin and mineral premix [3]	0.25
Total	100
Calculated nutrient values (%)	
ME (kcal/kg) [1]	3,199
CP	19.5
Dig. Lysine [2]	1.02
Dig. Threonine [2]	0.68
Dig. Methionine [2]	0.53
Dig. TSAA [2]	0.80
Dig. Tryptophan [2]	0.22
Calcium	0.60
Non-phytate phosphorus [1]	0.20
Sodium	0.17

[1]Metabolizable energy and available phosphorus were based on Agristat values as suggested by M. Donohue. 2013. The Challenges in Feeding Broilers in Times of High and Volatile Feed Ingredient Costs: How to Cover the Costs?. 2013 Mid- Atlantic Nutrition Conference proceedings. A 2.2 ratio was maintained for Ca to AP.

[2]Digestible amino acids were based on the digestible lysine value (1.2%) suggested by P. B. Tillman and W.A. Dozier. 2013. Current Amino Acid Considerations for Broilers: Requirements, Ratios, Economics. www.thepoultryfederation.com for 8−14-d broilers. Digestible amino acid to digestible lysine ratios followed further minimum recommendations of this communication (0.54 methionine, 0.90 TSAA, 0.84 threonine, 0.19 tryptophan).

[3]Supplied the following per kilogram of diet: manganese, 0.02%; zinc, 0.02%; iron, 0.01%; copper, 0.0025%; iodine, 0.0003%; selenium, 0.00003%; folic acid, 0.69 mg; choline, 386 mg; riboflavin, 6.61 mg; biotin, 0.03 mg; vitamin B6, 1.38 mg; niacin, 27.56 mg; pantothenic acid, 6.61 mg; thiamine, 2.20 mg; menadione, 0.83 mg; vitamin B12, 0.01 mg; vitamin E, 16.53 IU; vitamin D3, 2,133 ICU; vitamin A, 7,716 IU.

solutions. Moisture content was determined by placing an aluminum weigh pan with a 2-g feed sample into an isotherm oven (Fisher Scientific, Hampton, NH) set at 105°C for 16 h of drying time, followed by placing drying samples into a desiccator for half an hour before being weighed for moisture loss. The percent of moisture was calculated as (weight before drying − weight after drying)/weight before drying × 100%. The pH of the feed samples was measured after plating of the microbial sample by using a digital pH meter with a glass electrode (Denver Instruments, Arvada, CO). The aerobic plate counts (**APCs**) and coliform/*Escherichia coli* of mash broiler feed were also tested in 3M APCs, *E. coli*/TCC Petri-films following the instructions from the manufacturers (3M Microbiology Products, St. Paul, MN)

Preparation of Bacterial Inoculum

The nalidixic acid (**NaL**) resistant *Salmonella* Typhimurium American Type Culture Collection (**ATCC**) 14028 and surrogate *Enterococcus faecium* ATCC 8459 were used in this study. Both bacterial strains were used

in previous studies investigating thermal inactivation of moisture-enhanced chicken patties (Jiang et al., 2021) and mash broiler feed (Boltz et al., 2019). The *Salmonella* and *E. faecium* strains were grown on tryptic soy agar with 200 ppm of NaL (Hardy Diagnostics, Santa Maria, CA) and maintained in refrigerated incubators for up to 3 wk before refreshment. Before the experiment, 2 single colonies of *Salmonella* were picked from tryptic soy agar with 200 ppm of NaL (Hardy Diagnostics) and 2 single colonies of *E. faecium* were picked from bile esculin agar with 200 ppm of NaL and grown in 10 mL of tryptic soy broth (**TSB**; Alpha Biosciences, Baltimore, MD) containing 200 ppm of NaL at 35°C for 24 h. The 24-h cultivated *Salmonella* or *E. faecium* solutions were centrifuged $(5,000 \times g)$ for 15 min (VWR Symphony 4417, VWR International, Radnor, PA) and washed triplicate in 0.1% of buffered peptone water (**BPW**, Hardy Diagnostics) followed by resuspension in 10 mL of 0.1% BPW. Initial inoculum level of *Salmonella* and *E. faecium* was adjusted to the final target concentration of ~8.0 $\log_{10}$CFU/mL by 1:9 and 1:6 serial dilution in 10 mL of 0.1% BPW.

Inoculation of Mash Broiler Feed

One hour before the experiment, 2-g samples of mash broiler feed were weighed and placed into a 7-oz (18 × 9.5 cm) filtered WhirlPak food sample bags with a total of 60 sample bags prepared for each experimental date. The 0.5 mL of the prepared *Salmonella* or *E. faecium* inoculum (0.5 mL) was added to the sample bags followed by a 30-s mixing in a blender (Microbiology International, Frederick, MD) to ensure uniform bacterial distribution in feed samples. Each sample bag was then vacuum packaged to ensure the feed was uniformly accumulated in the same corner of the sample bag before conducting thermal treatment.

Thermal Inactivation of Mash Broiler Feed

The 2-g mash broiler feed samples (one bag per time point processed) for both *Salmonella* and *E. faecium* were completely submerged into the circulated water bath with heating temperatures set at 75°C, 85°C, and 95°C for 0, 5, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 120, 150, and 180 s, respectively. The 2-g of feed samples were uniformly stacked onto the side of the filtered sample bags after vacuum packaging. A type-k thermocouple probe was inserted into the geometric center of the 2-g stacked feed samples and recorded at 10 s intervals using PicoLog software (Pico Technology Ltd., Cambridge, U.K.), which is uniformly done for each sample bag to monitor the temperature change of feed during thermal treatment.

Microbiological Analyses

Feed sample bags were immediately removed from the circulated water bath after heating and placed into an ice-water bath followed by adding 10 mL of refrigerated 0.1% BPW with 0.1% sodium pyruvate to recover heat injured cells (Jiang et al., 2021). Samples were then homogenized in a blender (Microbiology International) for 30 s followed by 10- or 100-fold serial dilution solutions and then plated onto tryptic soy agar or bile esculin agar with 200 ppm NaL to numerate survivals of *Salmonella* and *Enterococcus*, respectively. Plated agars were incubated at 35°C for 24 h and 48 h to recover *Salmonella* and *Enterococcus* cells, respectively. After incubation, colonies were manually counted to determine bacterial survival ($\log_{10}$CFU/g) with a detection limit of 0.3 $\log_{10}$ CFU/g.

Modeling of Bacterial Survivals

First, each individual temperature dataset was analyzed using an Add-in for Microsoft Excel GinaFit software (Geeraerd et al., 2005) which includes 7 bacterial survival equations (Linear, Linear + Shoulder, Linear + Tail, Linear + Shoulder + Tail, Weibull, Weibull + Tail, and Biphasic Linear; Table 2) to determine the fitness of each equation based on the calculated R^2 and root mean square error (**RMSE**) values (Geeraerd et al., 2005; López-Gálvez et al., 2012; Li et al., 2018). Since all data fit classic linear and Weibull models, the whole dataset was then processed using USDA-IPMP-Global fit software (Huang, 2017), which only includes Linear and Weibull models, to determine the D-values and *z*-values across all tested treatments (Table 2). Finally, the whole datasets fit for the other 5 models in Ginafit software were also analyzed individually as shown in Tables 4 and 5.

Data Analysis

This thermal inactivation study used a 2 × 3 × 10 −14 factorial structure with 2 different bacteria strains, 3 different heating temperatures, and 10−14 different heating times. The whole study was conducted with 2 replications. A total of 7 replications utilizing 2-g samples of feed were heated in the water bath at varying temperatures (95°, 85°, or 75°C) for a specified time ranging from 0 to 180 s. Survival of *Salmonella* and surrogate *Enterococcus* cells were analyzed using JMP software (SAS Inc. Carey, NC) with individual factors of temperatures and heating time, and their interactions. The calculated thermal inactivation kinetic parameters including D-value, *z*-value, shoulder, tail, P, f, K_{max1}, and K_{max2} values of each treatment were analyzed using JMP mixed model analysis with multiple comparisons. A pairwised *t*-test was used to compare parameter differences between *Salmonella* and surrogate *Enterococcus*. The parameter mean differences were considered

Table 2. Fitness (RMSE and R^2) of survival models in Ginafit software for the thermal inactivation of *Salmonella* Typhimurium and the surrogate *Enterococcus faecium* in broiler feed.

Temperature (°C)	Bacteria		Linear	Linear+ Shoulder	Linear+ Tail	Linear + Shoulder + Tail	Weibull	Weibull + Tail	Biphasic Linear
75	*S.* Typhimurium	RMSE	0.8717	N/A	0.6601	0.6647	0.8226	0.6569	0.6445
		R^2	0.8510	N/A	0.9283	0.9304	0.8775	0.9344	0.9345
	E. faecium	RMSE	0.6113	N/A	0.4265	0.3926	0.5514	0.3965	0.4234
		R^2	0.8250	N/A	0.9222	0.9410	0.8727	0.9377	0.9297
85	*S.* Typhimurium	RMSE	0.6959	0.5152	N/A	N/A	0.6041	N/A	N/A
		R^2	0.9402	0.9674	N/A	N/A	0.9587	N/A	N/A
	E. faecium	RMSE	0.7702	N/A	N/A	N/A	0.7813	N/A	0.7746
		R^2	0.7701	N/A	N/A	N/A	0.8838	N/A	0.8855
95	*S.* Typhimurium	RMSE	0.7852	0.5456	N/A	N/A	0.5464	N/A	N/A
		R^2	0.9905	0.9686	N/A	N/A	0.9690	N/A	N/A
	E. faecium	RMSE	0.7875	0.7444	N/A	N/A	0.7550	N/A	N/A
		R^2	0.9287	0.9441	N/A	N/A	0.9429	N/A	N/A

Abbreviations: RMSE, root mean sum of squared errors; R^2, coefficient of determination.
N/A, model is not suitable for this data.

significantly different if less than the significance level $\alpha = 0.05$.

RESULTS

Physical and Chemical Characteristics, and Microbial Quality of Feed

The tested pH, water activity, and moisture content of mash broiler feed samples were 6.31 ± 0.03, 0.628 ± 0.001, and $12.3 \pm 1.2\%$, respectively. The counts of APCs and total coliforms of feed samples were 4.94 ± 0.61 and 3.44 ± 1.10 $\log_{10}$CFU/g. No generic *E. coli* was detected in the mash broiler feed samples (detection limit is 0.3 $\log_{10}$CFU/g).

### Temperature Changes of the Mash Broiler Feed During Heating

Figure 1 shows the temperature changes in 2-g of mash broiler feed heated at 75°, 85°, and 95°C in a circulated water bath. The initial temperatures ranged from 22.4° to 25.2°C among all feed samples before heating (Figure 1). Internal temperatures of feed samples reached 73.7°, 84.3°, and 91.1°C after heating at 75°, 85°, and 95°C for 180, 140, and 110 s, respectively (Figure 1).

Survival of Bacterial Cells in Mash Broiler Feed After Thermal Treatments

Salmonella and surrogate *E. faecium* cell survival in mash broiler feed after heating at 75°, 85°, and 95°C for 0 to 180 s are shown in Figure 2. As expected, the bacterial cells in feed samples decreased ($P < 0.05$) with increasing heating time in a circulated water bath (Figure 2). Bacterial counts decreased at a greater rate with a higher target temperature (Figure 2). For *Salmonella*, heating feed at 75°, 85°, and 95°C decreased ($P < 0.05$) cell counts from 7.86 to 7.98 $\log_{10}$CFU/g to 1.79, < 0.3, and < 0.3 $\log_{10}$CFU/g after 180, 60, and 50 s, respectively (Figure 2). Compared to *Salmonella*, heating *E. faecium* in feed at 75° and 85°C for 180 and 120 s resulted in greater ($P < 0.05$) survival of 4.32 and 1.70 $\log_{10}$CFU/g (Figure 2). Heating at 95°C required a longer time (70 s, Figure 2) to reduce the surrogate cell populations below the detection limit (0.3 $\log_{10}$CFU/g). The reduction rate of *Salmonella* and *E. faecium* slowed down after 80 s heating at 75°C (Figure 2), suggesting a "Tail" effect with a less-heat susceptible subpopulation of the 2 tested bacterial cells which was more apparent in *E. faecium* inoculated samples than *Salmonella* (Figure 2).

Modeling of Thermal Inactivation of Bacteria in Mash Broiler Feed

As shown in Table 2, the R^2 and RMSE values of *Salmonella* and *E. faecium* calculated from the 7 bacterial
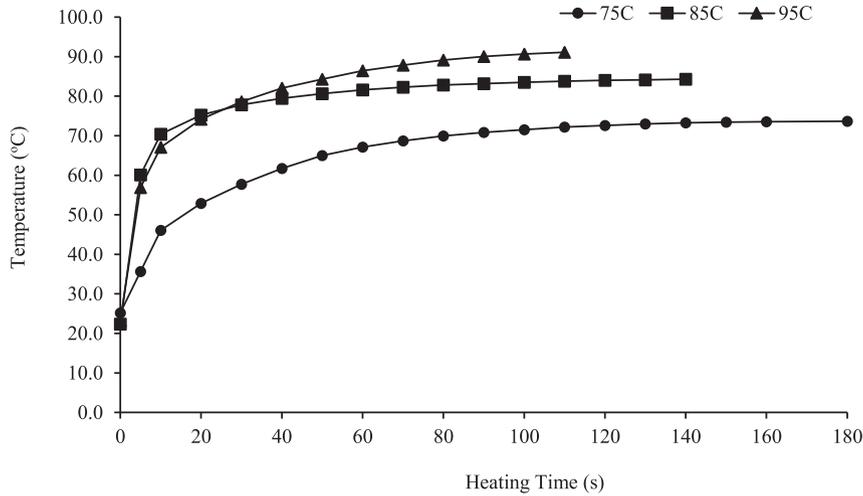
Figure 1. Time-temperature profiles of the geometric center of a 2-gram broiler mash feed sample during heating at 75, 85 and 95°C in a lab scale circulated thermal bath. Each data point is the average value of 3 replicates.

survival equations in the Ginafit software were used to determine which equation was best suited to the bacterial survival curves (Geeraerd et al., 2000, 2005; López-Gálvez et al., 2012; Li et al., 2018). Survival curves of *Salmonella* and *E. faecium* in feed samples heated at 75°, 85°, and 95°C fit the classic Linear (RMSE = 0.6113 −0.8717, R^2 = 0.8250−0.9905) and Weibull (RMSE = 0.5464−0.8226, R^2 = 0.8727−0.9690) models (Table 2). At 75°C, in addition to Linear and Weibull models, the survival data also fit Linear with Tail, Linear with Shoulder and Tail, Weibull with Tail, and Biphasic Linear models, with the RMSE and R^2 values ranged from 0.3926 to 0.8226 and 0.8727 to 0.9410, respectively (Table 2). At 85°C, the survival data of *Salmonella* and *E. faecium* can also be explained by Linear with Shoulder (RMSE = 0.5152, R^2 = 0.9674) and Biphasic Linear (RMSE = 0.7746, R^2 = 0.8855) models, respectively (Table 2). When heating temperature was increased to 95°C, survival data of the 2 bacteria only fit Linear with Shoulder (RMSE = 0.5456−0.7444, R^2 = 0.9441−0.9686, Table 2) besides classic Linear and Weibull models.

The IPMP-Global fit software (Huang, 2017), including classic Linear and Weibull models was used to compare the D- and *z*-values of *Salmonella* and *E. faecium* in mash broiler feed samples heated at 75°, 85°, and 95°C simultaneously. Based on the Linear model as shown in Table 3, D-values of *Salmonella* in feed samples heated at 75°, 85°, and 95°C were 11.2, 2.9, and 1.8 s, respectively, which were lower ($P < 0.05$) than the *E. faecium* inoculated samples (18.1, 8.4, and 3.7 s, Table 3). The Linear model calculated *z*-value and log D$_0$ value of *Salmonella* across all tested temperatures were 28.6 and 3.7 s, respectively, which were lower than (*z*-value, $P < 0.05$) or like (log D$_0$ value, $P > 0.05$) the *E. faecium* samples (Table 3). The Linear model, D-values of *Salmonella* calculated from Weibull models were 21.8, 6.9, and 4.2 s for samples heated at 75°, 85°, and 95°C, respectively, which are also significantly lower ($P < 0.05$) than those of *E. faecium* samples (34.4, 15.2, and 8.5 s, Table 3).

The detailed analysis of thermal kinetic parameters including "Shoulder time", "Tail", and D-values of Linear with Tail, Linear with Shoulder and Tail, and Weibull with Tail models for *Salmonella* and surrogate *E. faecium* at 75°C were shown in Table 4. The calculated "Shoulder time" of *Salmonella* from Linear with Shoulder and Tail was 16.2 s, a value which was lower ($P < 0.05$) than that of the *E. faecium* (26.5 s, Table 4). Similarly, the "Tail" values of *Salmonella* from Linear with Tail, Linear with Shoulder and Tail, and Weibull with Tail models ranged from 2.5 to 2.6 log$_{10}$ CFU/g, which were lower ($P < 0.05$) than those of the *E. faecium*, ranging from 4.5 to 4.6 log$_{10}$CFU/g (Table 4). D-values from Linear with Tail, Linear with Shoulder and Tail, and Weibull with Tail models of *Salmonella* in feed samples were 6.4, 5.7, and 26.5 s, which were also lower ($P < 0.05$) or similar ($P > 0.05$) to those of *E. faecium* (11.4, 7.6, and 38.1 s, Table 4). None of the f-, K$_{max1}$ and K$_{max2}$ values of *Salmonella* and *E. faecium* from the Biphasic Linear model differed significantly ($P > 0.05$).

As shown in Table 5, the "Shoulder time" and D-values of *Salmonella* at 85°C from the Linear with Shoulder model were 11.7 and 2.3 s, respectively. The f-, K$_{max1}$ and K$_{max2}$ values of *E. faecium* from the Biphasic Linear model were 0.98, 0.14, and 0.03, respectively (Table 5). Both shoulder time (6.0 s) and D-value (1.4 s) of *Salmonella* at 95°C from the Linear with Shoulder were lower ($P > 0.05$) than those of *E. faecium* (shoulder time = 12.45 s, D-value = 3.3 s, Table 5).

DISCUSSION

Water activity values (Aw) of the mash feed used in this study are similar to the previous study of Netto Teixeira et al. (2019) who reported Aw at 0.62. From a microbial feed safety point of view, Aw should be taken into consideration during the feed manufacture process of poultry feed. The low Aw value of pelleted poultry feed suppresses but does not eliminate *Salmonella* (Aviles et al., 2012). Although the addition of moisture
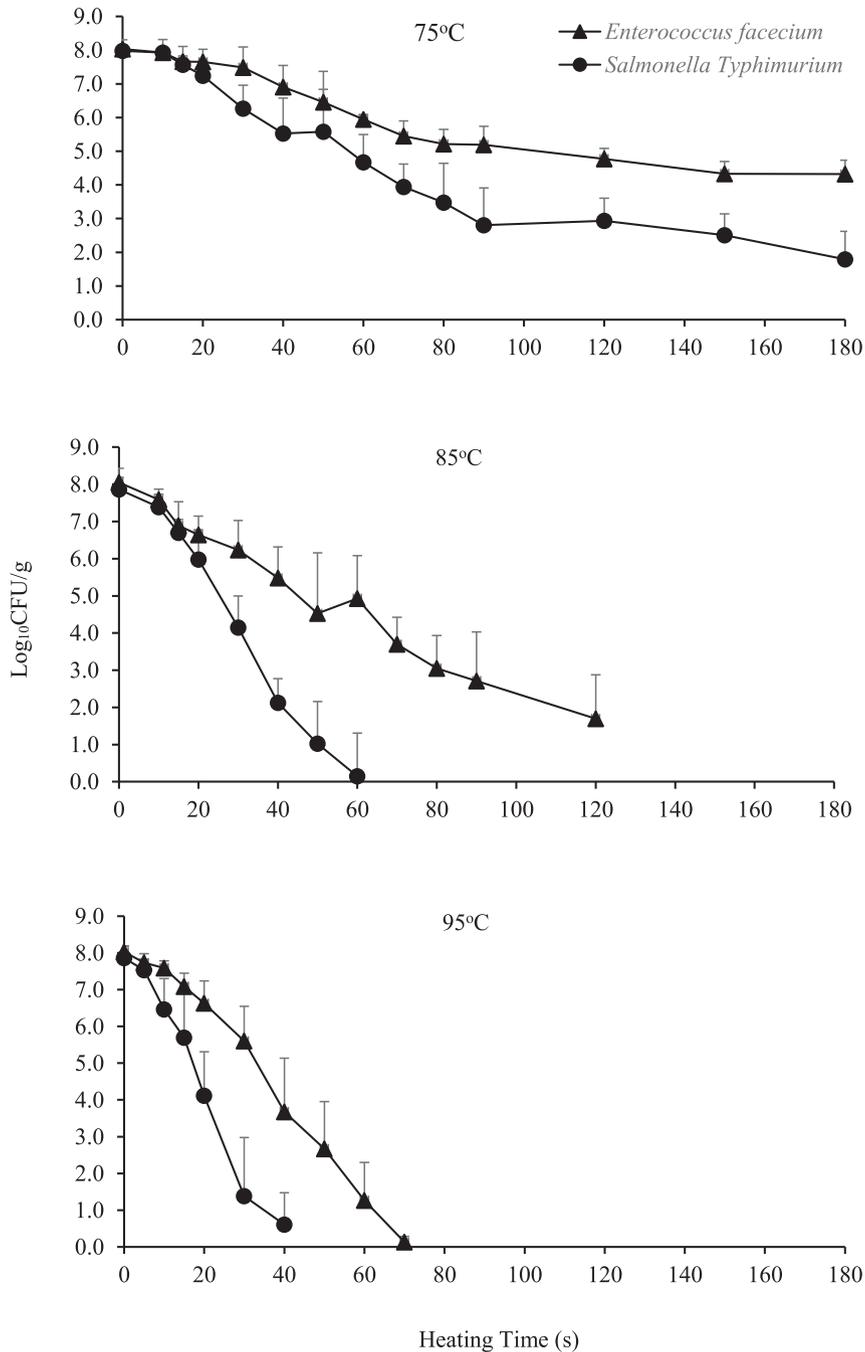
Figure 2. Survival-temperature profiles of *Salmonella* Typhimurium and the surrogate *Enterococcus faecium* in a 2-gram of broiler mash feed sample heated at 75, 85 and 95°C in a lab scale circulated thermal bath. Each data point is the average value of 7 replicates.

Table 3. D- and z-values (sec, Mean ± Standard Deviation) of *Salmonella* Typhimurium and *Enterococcus faecium* calculated from Linear and Weibull models from the USDA Integrated Pathogen Modeling Program for Predictive Microbiology - IPMP Global-Fit and Gina-Fit software.

Parameters	*Salmonella* Typhimurium	*Enterococcus faecium*
(A) Linear model (RMSE = 0.611 to 0.872; R^2 = 0.770 to 0.940)		
D, T95.0°C	1.8 ± 0.4[a,A]	3.7 ± 0.2[a,A]
D, T85.0°C	2.9 ± 0.3[a,A]	8.4 ± 1.9[b,B]
D, T75.0°C	11.2 ± 1.0[b,A]	18.1 ± 1.8[c,B]
z-value	28.6 ± 6.5[A]	34.1 ± 1.8[B]
Log D$_0$	3.7 ± 0.3[A]	4.1 ± 0.5[A]
(B) Weibull model (RMSE = 0.246 to 0.978; R^2 = 0.824 to 0.995)		
D, T95.0°C	4.2 ± 0.9[a,A]	8.5 ± 1.2[a,A]
D, T85.0°C	6.9 ± 1.6[a,A]	15.2 ± 0.8[b,B]
D, T75.0°C	21.8 ± 7.9[c,A]	34.4 ± 5.4[c,B]

[abc]Mean values with different letters within a column differ significantly ($P < 0.05$).
[AB]Mean values with different capital letters within a row differ significantly ($P < 0.05$).

Table 4. Thermal kinetic parameters (mean ± standard error) of linear with tail, linear with shoulder and tail, Weibull with tail, and Biphasic linear models for survival of *Salmonella* Typhimurium and the surrogate *Enterococcus faecium* at 75°C.

		Bacteria	
Model	Parameter	*Enterococcus faecium*	*Salmonella* Typhimurium
Linear with Tail	Tail ($\log_{10}N_{res}$)	4.4 ± 0.3[b]	2.5 ± 0.7[a]
	D-value (s)	11.4 ± 2.1[b]	6.4 ± 1.1[a]
Linear with Shoulder and Tail	Shoulder-time (s)	26.5 ± 17.6[b]	16.2 ± 9.6[a]
	D-value (s)	7.6 ± 3.6[a]	5.7 ± 1.2[a]
	Tail ($\log_{10}N_{res}$)	4.5 ± 0.2[b]	2.6 ± 0.7[a]
Weibull with Tail	Delta	38.1 ± 11.7[a]	26.5 ± 12.4[a]
	Tail ($\log_{10}N_{res}$)	4.5 ± 0.3[a]	2.5 ± 0.7[b]
Biphasic linear	f	1.00 ± 0.01[a]	1.00 ± 0.01[a]
	Kmax1	0.10 ± 0.02[a]	0.16 ± 0.04[a]
	Kmax2	0.02 ± 0.01[a]	0.02 ± 0.02[a]

[ab]Mean values with different letters within a row differ significantly ($P < 0.05$).

Table 5. Thermal kinetic parameters (mean ± standard error) of linear with shoulder and Biphasic linear models for survival of *Salmonella* Typhimurium and the surrogate *Enterococcus faecium* at 85 and 95°C.

			Bacteria	
Temperature (C)	Model	Parameter	*Enterococcus faecium*	*Salmonella* Typhimurium
85	Linear with Shoulder	Shoulder-time (sec)	N/A	11.7 ± 4.6
		D-value (sec)	N/A	2.3 ± 0.6
85	Biphasic linear	f	0.98 ± 0.05	N/A
		Kmax1	0.14 ± 0.03	N/A
		Kmax2	0.03 ± 0.04	N/A
95	Linear with Shoulder	Shoulder-time (sec)	12.5 ± 2.0[b]	6.0 ± 1.6[a]
		D-value (s)	3.3 ± 0.4[b]	1.4 ± 0.3[a]

[ab]Mean values with different letters within a row differ significantly ($P < 0.05$).N/A, model is not suitable for this data.

during feed processing may increase the sensitivity of *Salmonella* and *Enterococcus faecium* to thermal inactivation, at the same time, the added moisture may potentially increase the risk of pathogen growth. Therefore, the present study is important to microbial feed safety, which likely translates into increased food safety. Application of existing models to predict thermal inactivation of pathogens such as *Salmonella* in poultry feed is important for the development of industrial microbial safety standards and good manufacturing practices for poultry feed.

Although the microbiological safety risk of *Salmonella* spp. in feed mills has been well documented in several previous studies (Patterson, 1971; Franco, 2005; Shariat et al., 2020), limited studies have examined the thermal inactivation parameters for foodborne pathogens in poultry feed. Liu et al. (1969) reported that heating chicken starter feed samples in a water bath set at 73.9°C for 40 min reduced *Salmonella* Senftenberg by ~4.5 $\log_{10}$CFU/g. Steghöfer et al. (2021) recently found that heating conditioned broiler feed at 85°C for 30 s reduced *Salmonella* (5 serotypes) ranged from 1.9 to > 5.3 $\log_{10}$CFU/g. Hutchison et al. (2007) showed that heating cattle feed to 70°C for 20 or 120 s achieved the reductions of *E. coli* O157:H7 by 1.3 to 2.2 $\log_{10}$CFU/g. Our previous study found that heating *S.* Typhimurium in mash broiler feed in a water bath set at 95°, 90°, 85°, 80°, and 75°C achieved more than 7.0 $\log_{10}$ CFU/g reductions after 60, 70, 120, 120, and 180 s, respectively (Boltz et al., 2021). Results from this study found that heating mash broiler feed at 75°, 85°, and 95°C in a

circulated heated water bath achieved 6-7 $\log_{10}$ CFU/g reductions of NaL resistant *Salmonella* cells after 180, 60, and 50 s, respectively, which is slightly different compared to our previous study (Boltz et al., 2021). This discrepancy could be explained by the NaL-adapted *Salmonella* and circulated thermal water bath used in this study compared with the non-NaL resistant cells and the non-circulated static water bath applied in the previous study (Boltz et al., 2021).

The "Shoulder-time" is defined as the time required before appearing a log-linear decrease (Geeraerd et al., 2005) and "Tail" effect is defined as a submicrobial population not undergoing any significant subsequent inactivation (Geeraerd et al., 2005). The modeling suitability screening test of *Salmonella* from Ginafit shown in Table 2 suggested that heating mash broiler feed at the lower temperature of 75°C is not efficient to kill the pathogen immediately, which death was delayed for 16.2 s as shown by the "Shoulder-time" value from the Linear with Shoulder and Tail model. The pathogen could also generate a 2.5 to 2.6 $\log_{10}$CFU/g of subthermal resistant populations after a certain period of heating time. This is shown that the survival data fit the Linear with Tail, Linear with Shoulder and Tail, and Weibull with Tail models, which can be further verified by the 75°C thermal curves fit the Biphasic Linear model. The Biphasic Linear model indicates the existence of 2 or more subpopulations with different inactivation rates of K_{max1} and K_{max2} values (Geeraerd et al., 2005; Li et al., 2018). However, when the heating temperature increased from 75°, 85°, and 95°C, the "Tail"

effect disappeared although the "Shoulder time" still existed. These results are different from our previous study, which found that the "Tail" effect continue to appear until the heating temperature reached 95°C (Boltz et al., 2021). This discrepancy can be attributed to the circulated water bath used in the study providing more uniform heat around the feed samples compared to the nonthermal static water bath used in the previous experiments (unpublished data).

"D-value" is defined as the time required to kill 90% of the organism in a specific food system at a heating temperature and the "z-value" is defined as the requiring temperature change for the 90% (1 log) change of D-values (Jay et al., 2005). In this study, the classic Linear and Weibull models calculated D-values and the classic Linear model calculated z-values are smaller than the D-values of previous work with *Salmonella* in poultry feed samples of 6.7 to 24.4 s when heating at 95° to 75°C and the related z-value was 42.1°C (Boltz et al., 2021). Amado et al. (2013) reported that the D-values of *Salmonella* spp. in cattle feed heated at 55° to 65°C were 12.6 to 108 s. The thermal dynamic differences between the current study and Amado et al. (2013) or Boltz et al. (2021) could be due to the higher heating temperatures of 75° to 95°C in a circulated water bath used in the current study compared to the lower heating temperatures of 55° to 65°C in the former study. However, the $D_{85} = 2.9$ s of the current study are very close to $D_{85} = 3.1$ s of *Salmonella* Agona in broiler feed reported by Steghöfer et al. (2021). The z-values for *Salmonella* in thermally processed feed are not widely reported. In a related study, Kim et al. (2012) reported that the z-values of *Salmonella* spp. at 70°, 75°, and 80°C were 49.0° and 30.2°C in fresh chicken litter samples containing 30 and 50% moisture, respectively.

Current feed manufacture practices lack effective methods to control *Salmonella* throughout the pelleting process. Proposed thermal inactivation methods need to be verified in the feed mills, however, biosafety II level foodborne pathogens such as *S.* Typhimurium are not permitted due to potential cross-contamination. Evaluating the behavior of potential surrogate bacteria during the pelleting of broiler feed has become important in recent years. Previous publications have concluded that an ideal non-pathogenic surrogate microorganism should be easy to prepare, behave similarly to the pathogen of interest, and be similar, if not more resistant, to thermal processing (Hu and Gurtler, 2017). *E. faecium* is a gram-positive non-endospore forming facultative cocci that can grow in a wide range of temperatures, pH, and salt concentrations (Fisher and Phillips, 2009). Our previous studies compared *E. faecium* reduction when feed was pelleted using standard pelleting methods (conditioned at 70°C for 15 s without hygieniser use) with more thermally aggressive pelleting (conditioned at 80°C for 30 s and hygieniser retention for 45 s, Boltz et al., 2019). Standard pelleting demonstrated a 3-log reduction while more thermally aggressive pelleting demonstrated a 4-log reduction in *E. faecium* when compared

to unprocessed mash ($P < 0.05$, Boltz et al., 2019). Boney et al. (2018) utilized the same *E. faecium* and found that steam conditioning for 10 and 60 s demonstrated a 3- and 4-log reduction in *E. faecium.*

A side-by-side comparison of thermal resistance between *Salmonella* and *E. faecium* in mash broiler feed was conducted in the current study. Results showed that the D-values and z-value of *E. faecium* in poultry feed samples heated at 75° to 95°C, calculated from classic Linear, Weibull, Linear with Tail, Linear with Shoulder and Tail, and Weibull with Tail models, were significantly greater than those of *Salmonella.* The "Shoulder-time", from Linear with Shoulder and Tail (75°C) and Linear with Shoulder equations (95°C), of *E. faecium* were longer than the *Salmonella.* The "Tail" residual heat resistant subpopulation of *E. faecium* calculated from the Linear with Tail, Linear with Shoulder and Tail, and Weibull with Tail equations were also significantly greater than those of *Salmonella.* These results clearly suggested that *E. faecium* is more resistant to thermal treatment in mash broiler feed compared to the *Salmonella* cells used in this study.

Comparisons between *Salmonella* with *E. faecium* during thermal processing of food products have been well-documented (Bianchini et al., 2014; Ceylan and Bautista 2015; Jiang et al., 2021). Bianchini et al. (2014) reported that reducing 5-log of *E. faecium* in a complex carbohydrate-protein meal required a higher heating temperature (73.7°C) than the *Salmonella* cells (60.6°C). Ceylan and Bautista (2015) found that $D_{76.7C}$ (11.7 min) and $D_{82.2C}$ (4.1 min) values of *E. faecium* in pet food containing 9% of moisture were greater than the 7 tested *Salmonella* strains with $D_{76.7C}$ and $D_{82.2C}$ values equal 6.5 and 2.7 min, respectively. Our previous study found that *E. faecium* was more susceptible to heat than *Salmonella* during double pan-broiling of moisture-enhanced chicken patties. This is due to bacterial reduction being lower after the same cooking time, had longer "Shoulder times", and had greater D-values from Weibull models (Jiang et al., 2021). As reported in previous studies by Martinez et al. (2003), Bianchini et al. (2014), and Ceylan and Bautista (2015), *E. faecium* could be protected during thermal processing by generating a sigma factor mediated adaptation system to direct RNA polymerase to transcribe many genes that can be further translated into heat resistant proteins.

Results from the current study suggest that heating mash broiler feed at 75° to 95°C after 50 to 180 s achieves a 5-log reduction of *Salmonella* and surrogate *E. faecium.* Bacterial thermal inactivation curves fit classic Linear and Weibull equations. Compared to *Salmonella, E. faecium* ATCC 8459 is more resistant to thermal treatment making it a suitable surrogate organism for *Salmonella* during thermal processing of mash broiler feed. Further studies are needed to determine the thermal inactivation kinetics of *E. faecium* in an industrial scale feed manufacture facility.

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