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 (>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 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 feed.
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 physiochemical 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 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

| 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 | 0.25 |
| Total | 100 |

1Metabolizable energy and available phosphorus were based on Agri-stat 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.

2Digestible 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.the poultryfederation.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).

3Supplied the following per kilogram of diet: manganese, 0.02%; zinc, 0.02%; iron, 0.01%; copper, 0.0025%; iodine, 0.0005%; selenium, 0.00005%; folic acid, 0.09 mg; choline, 386 mg; riboflavin, 6.61 mg; biotin, 0.03 mg; vitamin B6, 1.38 mg; niacin, 27.56 mg; pantothentic 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 IU; vitamin A, 7,716 IU.

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 × 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 log10 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 after heating and placed into an ice-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 (log10 CFU/g) with a detection limit of 0.3 log10 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 R2 and root mean square error (RMSE) values (Geeraerd et al., 2005; López-Gámez 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, Kmax1, and Kmax2 values of each treatment were analyzed using JMP mixed model analysis with multiple comparisons. A pairwise t-test was used to compare parameter differences between Salmonella and surrogate Enterococcus. The parameter mean differences were considered...
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 ± 1.2%, respectively. The counts of APCs and total coliforms of feed samples were 4.94 ± 0.61 and 3.44 ± 1.10 log10CFU/g. No generic *E. coli* was detected in the mash broiler feed samples (detection limit is 0.3 log10CFU/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 log10CFU/g to 1.79, < 0.3, and < 0.3 log10CFU/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 log10CFU/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 log10CFU/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 R² and RMSE values of *Salmonella* and *E. faecium* calculated from the 7 bacterial
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, R² = 0.8250−0.9905) and Weibull (RMSE = 0.5464−0.8226, R² = 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 R² 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, R² = 0.9674) and Biphasic Linear (RMSE = 0.7746, R² = 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, R² = 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 D₀ 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 D₀ 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 log₁₀ CFU/g, which were lower (P < 0.05) than those of the E. faecium, ranging from 4.5 to 4.6 log₁₀ 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-, Kmax1 and Kmax2 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-, Kmax1 and Kmax2 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; R² = 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$^{a,A}$ | 18.1 ± 1.8$^{b,B}$ |
| $z$-value | 28.6 ± 6.5$^{a}$ | 34.1 ± 1.8$^{b}$ |
| Log D₀ | 3.7 ± 0.3$^{a}$ | 4.1 ± 0.5$^{a}$ |
| (B) Weibull model (RMSE = 0.246 to 0.978; R² = 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$^{a}$ | 34.4 ± 5.4$^{b}$ |

$^{a-b}$Mean values with different letters within a column differ significantly ($P < 0.05$).

$^{A-B}$Mean values with different capital letters within a row differ significantly ($P < 0.05$).
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 \( \sim 4.5 \log_{10} \text{CFU/g} \). 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 \( \log_{10} \text{CFU/g} \). Hutchison et al. (2007) showed that heating cattle feed to 70°C for 20 or 120 s achieved the reductions of \( E. \text{coli} \) O157:H7 by 1.3 to 2.2 \( \log_{10} \text{CFU/g} \). Our previous study found that heating \( S. \text{Typhimurium} \) in mash broiler feed in a water bath set at 95°, 90°, 85°, 80°, and 75°C achieved more than 7.0 \( \log_{10} \text{CFU/g} \) 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 \( \log_{10} \text{CFU/g} \) 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). 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 \( \log_{10} \text{CFU/g} \) 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 \( \text{K}_{\text{max1}} \) and \( \text{K}_{\text{max2}} \) values (Geeraerd et al., 2005; Li et al., 2018). However, when the heating temperature increased from 75°, 85°, and 95°C, the “Tail”

### 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.

| Model                        | Parameter               | Enterococcus faecium | Salmonella Typhimurium |
|------------------------------|-------------------------|-----------------------|------------------------|
| Linear with Tail             | Tail \( (\log_{10}N_{\text{res}}) \) | 4.4 ± 0.3*            | 2.5 ± 0.7*             |
|                             | D-value (sec)           | 11.4 ± 2.1*           | 6.4 ± 1.1*             |
| Linear with Shoulder and Tail| Shoulder-time (s)       | 26.5 ± 17.6b          | 16.2 ± 9.6a            |
|                             | D-value (s)             | 7.6 ± 3.6b            | 5.7 ± 1.2*             |
|                             | Tail \( (\log_{10}N_{\text{res}}) \) | 4.5 ± 0.2*            | 2.6 ± 0.7*             |
| Weibull with Tail            | Delta                   | 38.1 ± 11.7a          | 26.5 ± 12.4a           |
|                             | Tail \( (\log_{10}N_{\text{res}}) \) | 4.5 ± 0.3*            | 2.5 ± 0.7*             |
| Biphasic linear              | \( f \)                 | 1.00 ± 0.01*          | 1.00 ± 0.01a           |
|                             | \( \text{K}_{\text{max1}} \) | 0.10 ± 0.02a          | 0.16 ± 0.04a           |
|                             | \( \text{K}_{\text{max2}} \) | 0.02 ± 0.01a          | 0.02 ± 0.02a           |

*abMean 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.

| 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                    |
|                  |                             | \( \text{K}_{\text{max1}} \) | 0.14 ± 0.03           | N/A                    |
|                  |                             | \( \text{K}_{\text{max2}} \) | 0.03 ± 0.04           | N/A                    |
| 95               | Linear with Shoulder         | Shoulder-time (sec)     | 12.5 ± 2.0b           | 6.0 ± 1.6a             |
|                  |                             | D-value (s)             | 3.3 ± 0.4b            | 1.4 ± 0.3a             |

*abMean values with different letters within a row differ significantly \((P < 0.05)\). N/A, model is not suitable for this data.
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 $D_{95} = 2.9$ s of the current study are very close to $D_{95} = 3.1$ 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-endosporing facultative cocc i 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 50 s and hygieniser retention for 5 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 $D_{76.7°C}$ (11.7 min) and $D_{82.2°C}$ (4.1 min) values of E. faecium in pet food containing 9% of moisture were greater than the 7 tested Salmonella strains with $D_{76.7°C}$ and $D_{82.2°C}$ 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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REFERENCES

Amado, I. R., J. A. Vázquez, N. P. Guerra, and L. Pastrana. 2013. Thermal resistance of Salmonella enterica, Escherichia coli and Staphylococcus aureus isolated from vegetable feed ingredients. J. Sci. Food Agric. 94:2274–2281.

Aviles, B., C. Klotz, T. Smith, R. Williams, and M. Ponder. 2012. Survival of Salmonella enterica Serotype Tennessee during simulated gastric passage is improved by low water activity and high fat content. J. Food Prot. 76:333–337.

Bianchini, A., J. Stratton, S. Weier, T. Harter, B. Plattner, G. Rokey, G. Hertz, L. Gompa, B. Martinez, and K. M. Eskridge. 2014. Use of Enterococcus faecium as a surrogate for Salmonella enterica during extension of a balanced carbohydrate-protein meal. J. Food Prot. 77:75–82.

Boltz, T. P., J. W. Boney, C. Shen, J. Jaczynski, and J. S. Moritz. 2019. The effect of standard pelleting and more thermally aggressive pelleting utilizing a hygieniser on feed manufacturing and reduction of Enterococcus faecium, a Salmonella Surrogate. J. Appl. Poult. Res. 28:1226–1233.

Boltz, T. P., J. S. Moritz, V. E. Ayres, C. L. Showman, J. Jaczynski, and C. Shen. 2021. Modeling thermal inactivation of Salmonella Typhimurium in mash broiler feed. J. Appl. Poult. Res. 30:100208.

Boney, J. W., J. Jaczynski, J. L. Weidhaas, A. N. Bergeron, and J. S. Moritz. 2018. The effects of steam conditioning and antimicrobial inclusion on feed manufacturing and inactivation of Enterococcus faecium, a Salmonella surrogate. J. Appl. Poult. Res. 27:472–482.

Ceylan, E., and D. A. Bautista. 2015. Evaluating Pediococcus acidilactici and Enterococcus faecium NRRL B-2354 as thermal surrogate microorganisms for Salmonella for in-plant validation studies of low-moisture pet food products. J. Food Prot. 78:934–939.

Cutlip, S. E., J. M. Holt, N. P. Buchanan, A. L. Rack, J. D. Latshaw, and J. S. Moritz. 2008. The effect of steam-conditioning practices on pellet quality and growing broiler nutritional value. J. Appl. Poult. Res. 17:249–261.

Fisher, K., and C. Phillips. 2009. The ecology, epidemiology and virulence of Enterococcus. Microbiology (Reading, England) 155:1749–1757.

Franco, D. A. 2005. A survey of Salmonella serovars and most probable numbers in rendered animal protein meals: Inferences for animal and human health. J. Environ. Health, 67:18–22.

Geeraerd, A. H., C. H. Herremans, and J. F. Van Impe. 2000. Structural model requirements to describe microbial inactivation during a mild heat treatment. Int. J. Food Microbiol. 59:185–209.

Geeraerd, A. H., V. P. Valrandidis, and J. F. Van Impe. 2005. Gima-fit, a freeware tool to assess non-log-linear microbial survivor curves. Int. J. Food Microbiol. 102:95–105.

Hu, M., and J. B. Gurtler. 2017. Selection of surrogate bacteria for use in food safety challenge studies: a review. J. Food Prot. 80:1506–1536.

Huang, L. 2017. IPMP global fit — a one-step direct data analysis tool for predictive microbiology. Int. J. Food Micro. 262:38–48.

Hulebaek, K. L., and W. Schlosser. 2002. Hazard analysis and critical control point (HACCP) history and conceptual overview. Risk Anal. 22:547–552.

Hutchison, M. L., D. J. I. Thomas, and S. M. Avery. 2007. Thermal death of Escherichia coli O157:H7 in cattle feeds. Lett. Appl. Microbiol. 44:357–363.

Jay, J. M., M. J. Loessner, and D. A. Golden. 2005. Modern Food Microbiology. 7th ed. Springer, New York, NY.

Jiang, W., C. Waldman, K. Li, J. Jaczynski, and C. Shen. 2021. Survival of Salmonella and the surrogate Enterococcus faecium in cooking of moisture enhanced reconstituted comminuted chicken patties by double pan-broiling. Poult. Sci. 100:101171.

Jones, F. T. 2011. A review of practical Salmonella control measures in animal feed. J. Appl. Poult. Res. 20:102–113.

Jones, F. T., and K. E. Richardson. 2004. Salmonella in commercially manufactured feeds. Poult. Sci. 83:384–391.

Kim, J., J. Diao, M. W. Shepard, R. Singh, S. D. Heringa, C. Gong, and X. Jiang. 2012. Validating thermal inactivation of Salmonella spp. in fresh and aged chicken litter. Appl. Environ. Microbiol. 78:1302–1307.

Li, K., H. Khouryieh, L. Jones, X. Etienne, and C. Shen. 2018. Assessing farmers market produce vendors’ handling of container and evaluation of survival of Salmonella and Listeria monocytogenes on plastic, pressed-card, and wood container surfaces at refrigerated and room temperature. Food Control. 94:116–122.

Liu, T. S., G. H. Snoeyenbos, and V. L. Carbon. 1969. Thermal resistance of Salmonella senftenberg 77SW in dry animal feeds. Avian Dis. 13:611–631.

López-Gálvez, F. G., D. Posada-Izquierdo, M. V. Selma, F. Perez-Rodriguez, J. Gobet, M. I. Gil, and A. Allende. 2012. Electrochemical disinfection: an efficient treatment to inactivate Escherichia coli O157:H7 in process water washing containing organic matter. Food Microbiol. 30:146–156.

Martinez, S., M. López, and A. Bernardo. 2003. Thermal inactivation of Enterococcus faecium: effect of growth temperature and physiological state of microbial cells. Let. Appl. Microbiol. 37:475–481.

Mellroy, S. G. 1996. How do birds become infected by a Salmonella serotype? Pages 15-17 in World Poultry Special Salmonella Issue. Misset International, Doetinchem, The Netherlands.

Netto Teixeira, M. V., A. Massuquetto, E. L. Krabbe, D. Surek, S. G. Oliveira, and A. Maiorka. 2019. Effect of conditioning temperature on pellet quality, diet digestibility, and broiler performance. J. Appl. Poult. Res. 28:963–973.

Patterson, J. T. 1971. Salmonellae in animal feeding-stuffs. Rec. Agric. Res. 20:27–33.

Shariat, N. W., K. M. Feye, A. K. Richards, B. Booher, Z. Flores, P. M. Rubinelli, and E. G. Olson. 2020. Incidence of Salmonella in feed mills in the United States and serovar identification using CRISPR analysis. J. Appl. Microbiol. 130:2141–2146.

Steghöfer, S., R. Limburn, and E. Margas. 2021. Microbiological assessment of heat treatment of broiler mash at laboratory scale to evaluate Salmonella reduction during feed conditioning. J. Appl. Poult. Res. 30:100122.

U.S. Centers for Disease Control and Prevention (U.S.-CDC). 2020. The Interagency Food Safety Analytics Collaboration. Foodborne illness source attribution estimates for 2018 for Salmonella, Escherichia coli O157, Listeria monocytogenes, and Campylobacter using multi-year outbreak surveillance data, United States GA and D.C. U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, Food and Drug Administration, U.S. Department of Agriculture’s Food Safety and Inspection Service. Accessed May 2022. https://www.cdc.gov/foodsafety/ifsac/pdf/P19-2018-report-TriAgency-508.pdf