FINAL REPORT

Thermal lethality validation for human pathogenic *Salmonella* and the *Salmonella* surrogate *Enterococcus faecium* NRRL B-2354 on chicken feathers and blood

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INDUSTRY SUMMARY

The primary purpose of this research project was to characterize the thermal inactivation (lethality) of human pathogenic *Salmonella enterica* inoculated onto surfaces of poultry animal-derived carcass components (blood, feathers), in order to provide scientific validation of process lethality during the simulated rendering of these carcass components for pathogen control and food safety protection. A secondary purpose of this project was to compare the lethalities achieved for human pathogenic *Salmonella* and the microbe *Enterococcus faecium* NRRL B-2354, a non-pathogenic organism previously used as a pathogen surrogate for in-plant validation study completion. The objectives of the project were to:

i) Generate scientific data describing the inactivation of the pathogen *Salmonella* via heating on chicken feathers and blood as a function of heating temperature and duration.
ii) Generate data allowing the comparison of thermal lethality of *Salmonella* to the non-pathogenic *E. faecium* NRRL B-2354 to determine degree of difference in lethality by application of heat.
iii) Verify the utility of *E. faecium* NRRL B-2354 for thermal inactivation, demonstrating its usefulness for in-plant validation during the rendering of chicken by-products.

The findings from this research demonstrated lethality to both *Salmonella* and *E. faecium* NRRL B-2354 in the rendered products. During the simulated rendering of chicken blood, the pathogen surrogate exhibited a statistically lower $D_{180°F}$ = 0.55 min, differing ($p<0.05$) from that of *Salmonella* in blood ($D_{180°F}$ = 0.99 min). This indicated that at lower rendering temperature conditions the surrogate organism was not useful for process validation. However, at higher temperatures (190, 200 °F), D-values for pathogen and surrogate did not statistically differ from one another for chicken blood. Simulated rendering of chicken feathers resulted in D-values for pathogen and surrogate organisms not differing statistically ($p>0.05$) at all experimental temperatures (190, 200, 210 °F). During simulated rendering of each product, using time/temperature conditions provided by a federally inspected commercial rendering establishment, both the pathogen and surrogate were reduced to non-detectable numbers after cooking at 200°F for 5.0 min (chicken blood) or at 300°F for 18 min (chicken feathers). *E. faecium* NRRL B-2354, compared with *Salmonella*, had similar heat resistance to the pathogen in chicken blood and feathers, demonstrating its usefulness as a suitable surrogate for poultry offal (blood and feathers) in-plant validation during rendering.

Impact of the research will provide both short- and long-term benefits to the poultry rendering industries by the development of data describing the lethality to pathogen and pathogen surrogate by use of high-heat processing on poultry carcass offal (blood, feathers). Additionally, through the evaluation of thermal lethality to *E. faecium* NRRL B-2354, a potential surrogate for *Salmonella* for the validation of lethality during thermal processing of human and animal foods, this research provides data that can be applied to the design and completion of in-plant validation testing for purposes of demonstrating food safety preventive controls are sufficiently effective to protect food safety, and will aid processors in complying with the requirements of the FDA Food Safety Modernization Act (FSMA; 21 CFR§507).

MATERIALS AND METHODS

Microorganisms and inoculum preparation. Isolates belonging to *Salmonella enterica* serovars Senftenberg, Heidelberg, and Typhimurium, recovered from poultry products or U.S. Department of Agriculture Food Safety and Inspection Service (USDA-FSIS)-inspected chicken harvesting environments, were chosen from the Food Microbiology Laboratory culture collection (Department of Animal Science, Texas A&M University, College Station, TX) and revived from -80°C cryo-storage by inoculated each isolate individually into 10 mL sterile brain heart infusion (BHI; Becton, Dickinson and Co., Sparks, MD, USA) broth-containing tubes and then incubating tubes for 24 h at 35°C. Following the initial revival process for preparing bacterial cells for experimental work, cells from each isolate were aseptically transferred to new, sterile BHI-containing tubes and then incubated in identical fashion (35°C, 24 h) in order to activate isolates for subsequent inoculum preparation. Following revival, an inoculum of *Salmonella* isolates (i.e., cocktail) of *S.* Senftenberg, Heidelberg, and Typhimurium was prepared by blending equivalent volumes of cultures incubated at 35°C for 24 h into a sterile 50.0 mL conical tube and then centrifuged (2191 x *g*, 25±2°C, 15 min; Jouan B4i centrifuge, Thermo-Fisher Scientific, Waltham, MA, USA) to pelletize the cells. After centrifuging the cocktail preparation, the resulting supernatant was discarded and then 30.0 mL 0.1% peptone water (Becton, Dickinson and Co.) was added to wash cells. The resulting suspension of cells was centrifuged again under identical settings; the resulting supernatant was poured off and the remaining pellet was hydrated with 3.0 mL of 0.1% peptone water. This was completed to obtain a 9.0-10.0 $\log_{10}$ CFU/mL inoculum in the cocktail. A preliminary experiment was conducted to verify that counts of overnight cultures of individual *Salmonella* isolates utilized for the cocktail do not differ from one another.

Isolate B-2354 of *E. faecium* (Orla-Jensen 1919) Schleifer and Kilpper-Balz 1984 was received from the USDA Agricultural Research Service (ARS) and revived in 10 mL sterile BHI broth for 24 h at 27°C per USDA-ARS guidance. Following the initial revival passage, a second revival passage was completed in identical fashion to activate and prepare the isolate for subsequent inoculum use. Following revival, *E. faecium* NRRL B-2354 (hereafter *E. faecium*) was prepared by blending equivalent volumes of *E. faecium* into a 50.0 mL conical tube and centrifuged (2191 x *g*, 25+2°C, 15 min) to wash the cells, pouring off the supernatant and adding 30.0 mL 0.1% peptone water. This procedure was repeated twice; in the last step, the supernatant was poured off and the remaining pellet was hydrated with 3.0 mL of 0.1% peptone water to obtain a 9.0-10.0 $\log_{10}$ CFU/ml inoculum. A preliminary experiment was conducted to verify that counts of overnight cultures of *E. faecium* NRRL B-2354 isolates utilized for the cocktail do not differ from one another. For the two sample matrices, non-inoculated samples were aseptically collected and analyzed for presence and numbers of background *Salmonella* and *E. faecium* cells in order to guide the determination of final inoculum that would overwhelm naturally occurring numbers of pathogen and surrogate microbes by at least 2 or 3 orders of magnitude.

Sample inoculation with *Salmonella* cocktail or *E. faecium*. Raw chicken blood and chicken feathers were obtained from a commercial rendering establishment located in the southern United States. For blood, inoculation was achieved by pipetting 0.1 mL of the prepared *Salmonella* cocktail into 50.0 mL conical tubes containing 25.0 mL chicken blood and then

vortexed for 1 min to homogenize the cocktail within the sample blood. For inoculation of feathers, 10.0 g of feathers were weighed into a sterile 50.0 mL conical tube, followed by adding 0.1 mL of prepared inoculum. Sample vials were then vortexed/agitated for 1 min in order to distribute the inoculum (*Salmonella* cocktail or *E. faecium*) over the surfaces of the feathers.

Sample Thermality Processing. Metal vessels (1" x 6" galvanized steel by 1" iron screwcap; Southland®, Memphis, TN, USA) were used to simulate the thermal processing equipment material and facilitate simulation of thermal rendering conditions of the commercial rendering establishment with respect to material contacting rendered material during commercial processing. A VWR™ Enviro-Safe® K 50531 thermometer (VWR Int., Radnor, PA, USA) was set inside of an open metallic vessel control filled with 50 mL distilled water to obtain temperature readings within sample vessels and to monitor temperature changes in heated sample material throughout the heating process.

***Salmonella* cocktail and *E. faecium* time/temperature-dependent thermal lethality in chicken blood.** Metal vessels containing were partially submerged in sterile distilled water in a stainless-steel cookpot (11.4 L capacity, TRAMONTINA Inc., Sugar Land, TX, USA) on a Precision™ Induction Cooktop (Figure 1), programmed to produce final cook temperatures in the metal vessels of 82, 87, or 93 °C (180, 190, or 200 °F, respectively). Vessels were heated to one of the three targeted processing temperatures prior to loading in inoculated sample material (blood), so as to heat the vessel and avoid an extended temperature come up period. This was designed to determine the thermal lethality resulting solely from the simulated commercial rendering process (5 min of cooking at one of the three targeted cook temperatures), in an effort to get the most conservative D-value outcomes. Once the metal vessel reached the targeted cook temperature, the inoculated chicken blood samples were poured immediately into metal vessels and immersed in distilled water in a Precision™ induction cooktop heated to the desired cooking temperature. After loading sample vessels into the heating water bath, vessels were removed after 0, 0.5, 1, 2, 3, 4 or 5 min of heat exposure. Once removed from the heating water bath, metal vessels were immediately placed in ice-laden cold water (0°C) to halt further microbial destruction due to heat. Vessels were allowed to cool for at least 1.0 min to ensure complete cooling and allow researcher handling (Figure 2).

Figure 1. Metal vessels immersed in distilled water in a stainless-steel cooktop on a Precision™ induction cooktop.

Sample material (blood) was then subjected to serial dilution in 0.1%

peptone water and enumeration of surviving *Salmonella* on bismuth sulfite (Hi-Media™ L.B.S. Marg, Mumbai, India) agar (BSA) supplemented with 1.0 g/L sodium pyruvate (Sigma-Aldrich Co., St. Louis, MO, USA) to allow for repair and detection of sub-lethally injured salmonellae (Gurtler and Kornacki, 2009). Surviving *E. faecium* cells were enumerated on Kenner Fecal (KF) *Streptococcus* agar (KFSA; Becton, Dickinson and Co.) supplemented with 2,3,5-triphenyltetrazolium Chloride (TTC; 1%) (Sigma-Aldrich Co.) and 1.0 g/L sodium pyruvate to allow the repair and subsequent detection of sub-lethally injured cells on Petri plates, and incubated at 35-37°C for 24-48 h before colony counting (Gurtler and Kornacki, 2009).

Figure 2. Metal vessels in ice-laden cold water (0°C)to halt heat transfer

***Salmonella* cocktail *and E. faecium* time/temperature-dependent thermal lethality on chicken feathers.** Metal vessels were partially submerged in distilled water in a stainless-steel cookpot (11.4 L, TRAMONTINA Inc.) on a Precision™ induction cooktop (Figure 1) and programmed to ensure a heat application inside of 87, 93, or 98 °C (190, 200, or 210 °F, respectively) for the purpose of heating the metal vessels and avoiding having the product experience prolonged "come up" of temperature. Once they reached the target temperature, inoculated chicken feather samples were aseptically placed immediately in the metal vessels using tweezers and immersed in distilled water in a Precision™ induction cooktop for 0, 3, 6, 9, 12, 15, or 18 min, spaced sufficiently in order to not allow any samples to touch one another. The metal vessels were removed at their respective time points and immediately placed in ice-laden cold water (0°C) to halt further microbial destruction due to heat (Figure 2). Following vessel cooling, heat-processed feather samples were aseptically collected and loaded into sterile stomacher bags using flame-sterilized tweezers, and then mixed with 90 mL 0.1% peptone water and placed in a Stomacher 400 blender (Seward Laboratory Systems Inc., Bohemia, NY, USA) for 1 min at 230 rpm. Sample feathers were then subjected to serial dilution in 0.1% peptone buffer. Surviving *Salmonella* were then inoculated onto BSA supplemented with 1.0 g/L sodium pyruvate, and *E. faecium* cells were inoculated onto KFSA supplemented with 1.0% TTC and 1.0 g/L sodium pyruvate. Inoculated plates were then incubated at 35-37°C for 24-48 h before colony counting.

Cumulative thermal lethality for *Salmonella* and *E. faecium* in chicken blood and feathers during simulated commercial rendering. Consulting with the commercial rendering establishment in order to prepare for simulating commercial rendering processes in the Food Microbiology Laboratory, inoculated chicken blood or feathers samples were heated to 93°C (200°F) for 5 min or 149°C (300°F) for 18 min, respectively. In this experiment, total lethality achieved during commercial rendering conditions (total cook time, cook temperature) was determined. For chicken blood, inoculation was achieved by pipetting 0.5 mL of prepared *Salmonella* cocktail or *E. faecium* into a 50.0 mL sterile conical tube containing 50 mL chicken blood. Inoculated sample vials were then vortexed for 1 min for inoculum mixture throughout blood. For feathers inoculation, 10.0 g of feathers were weighed into a sterile 50.0 mL conical tube, followed by adding 0.5 mL of prepared inoculum and then vortexing for 1 min. Samples were then placed in metal vessels that were closed and partially submerged in peanut oil, used for to its high boiling point (441-445°F) in a stainless-steel cookpot (11.4 L, TRAMONTINA Inc.) in a Precision™ Induction Cooktop (Figure 3).

Figure 3. Metal vessels immersed in peanut oil in a stainless-steel cooktop on a Precision™ induction cooktop.

Chicken feathers samples were placed in metallic vessels and immersed in peanut oil in a Precision™ induction cooktop pre-heated to 243°C (470°F) to achieve heating of vessels to 149°C (300°F). The metallic vessels were removed at "come-up" temperature of 149°C (300°F) and inoculated samples were placed in the vessels and immersed again in the heated peanut oil in the cooktop and cooked for 18 min of heating at 149°C (300°F). Following heating, vessels were immediately placed in ice-laden cold water (0°C) to halt further microbial destruction due to heat. Following sample vessel cooling, sample feathers were aseptically transferred to a filter stomacher bag containing 90.0 mL 0.1% peptone buffer and stomached at 230 rpm for 1 min to dilute surviving cells. Following mixing, samples were subjected to serial dilution in 0.1% peptone buffer and surviving cells were inoculated on Petri plates. *E. faecium* cells were plated onto KFSA plates supplemented with 1.0% TTC and 1.0 g/L sodium pyruvate. Surviving *Salmonella* were plated on BSA supplemented with 1.0 g/L sodium pyruvate. Inoculated plates were then incubated at 35-37°C for 24-48 h for before colony counting.

Statistical analysis. Sample vessels were assigned randomly to a cooking period for each of the cooking temperatures for blood or feathers samples, completed all D-value experiments as a full factorial experimental design. For cumulative lethality processes, samples were likewise

randomly assigned to a cooking temperature for the cooking period, for both *Salmonella* and *E. faecium* cells. Plate counts of *Salmonella* and *E. faecium* from time/temperature-dependent D-value, and cumulative lethality, determination lethality experiments were $\log_{10}$-transformed prior to subsequent data analysis. For the determination of D-values for *Salmonella* and *E. faecium*, $\log_{10}$-transformed plate count data were fed into the DM Fit component of ComBase (University of Tasmania/USDA-ARS; https://www.combase.cc/index.php/en/). Once data were uploaded to the modeling program, researchers directed the determination of D-values by the linear regression method and the Baranyi and Roberts modeling method. For both methods, the entire survivor curve was used to determine the D-value for the organism at the targeted cooking temperature. D-values were recorded, along with correlation coefficient (R^2) values; R^2 values range from 0 to 1, with values closer to 1 indicating better model fitting to experimental data and better reliability of calculated D-value.

Statistical analyses of D-values were performed using JMP Pro v12 (SAS Institute, Inc., Cary, NC, USA). To determine whether a replicate-effect existed for D-value experiments on blood and feathers experiments, a Restricted Maximum Likelihood (REML) was completed. D-values were then compared by use of two-way analysis of variance (ANOVA) to determine differences amongst D-values as affected by main effects (organism(s), cooking temperature, and D-value modeling method), and the interactions of these main effects. Statistically significant differences amongst main effects or their interactions ($p<0.05$) were separated using Tukey's Honest Significant Differences (HSD) test. D-value experiments were replicated three times in identical fashion ($N=3$), and cumulative lethality experiments were replicated three times in identical fashion containing duplicate identical samples per replicate ($N=6$).

Following D-values comparison, thermal process constants (z-values) were determined as the negative inverse of the slope of the best-fit linear regression line ($\log_{10}$ D-values). A mean z-value was generated for both *Salmonella* and *E. faecium* in each sample type (blood, feathers).[1] Organism- and temperature-specific D-values for each experimental replicate were first $\log_{10}$-transformed and plotted against their corresponding heating temperatures, after which the best-fit linear regression equation was generated using Microsoft Excel® (Redmond, WA, USA). Mean z-values were then calculated.

[1] While the D-value is defined as the time (min) at constant temperature producing a 1.0 $\log_{10}$ reduction in a microbe's population, the z-value is the temperature change producing a 10-fold change in the D-value of the microbe.

RESULTS AND DISCUSION

***Salmonella* and *E. faecium* time/temperature-dependent D-values on chicken blood.** Completion of REML analysis on D-values of *Salmonella* or *E. faecium* on chicken blood samples indicated that no significant replicate effect existed impacting resulting D-value data (p=0.957; Table 1). Data were then re-analyzed by ANOVA; replication was not included as a main or random effect (Table 2). The ANOVA output for D-values of both organisms (*Salmonella*, *E. faecium*) in chicken blood indicated significant impact on D-values by the experimental main effects (organism, cooking time/temperature) (p=0.0001) (Table 2). The mean thermal process constants (z-values) for *Salmonella* and *E. faecium* NRRL B-2354 in chicken blood was 25.22±3.69 and 125.56±89.02°F, respectively. *E. faecium* NRRL B-2354 produced a much higher temperature difference to reduce the D-value by 10-fold compared to *Salmonella*, indicating an increased resistance to heating, though the excessively high standard deviation does not yield high confidence in the accuracy of the z-value, and is not different from that of *Salmonella*'s z-value. While the z-value of *E. faecium* in chicken blood has not been previously reported in the research literature, the z-value in chicken blood for *Salmonella* agrees with the report by Jones-Ibarra et al. (2017) in chicken offal (raw carcass material) of 21.95±3.87 °F.

Table 1: REML variance component estimates for experimental replicates in chicken blood.

Random Effect	**Variance Ratio**	**Variance Component**	**Standard Error**	**95% Lower CL[a]**	**95% Upper CL**	**Wald *p*-value**	**Variance (%)**
Rep #	0.004	8.4358e-5	0.001	-0.003	0.003	0.957	0.482
Residual		0.017	0.005	0.010	0.034		99.518
Total		0.017	0.005	0.010	0.033		100.000

[a]CL: Confidence limit

Table 2: Two-way analysis of variance: Salmonella *and* E. faecium *D-values for chicken blood.*

Source	**DF**	**Sums of Square**	**Means Squares**	**F-Ratio**	***P*>F**
Model	11	4.577	0.416	23.791	<0.0001
Error	24	0.420	0.017		
C. Total	35	4.997			

The $D_{180°F}$=0.99 min for *Salmonella* in chicken blood was statistically higher than that of the *E. faecium* surrogate ($D_{180°F}$=0.55 min) (p=0.0006; Table 3). In this case, the surrogate produce lower heat resistance, nearly half that of the pathogen. This was not expected and is in fact undesirable, as in other previously completed research, the D-value of *E. faecium* tended to be either non-differing from that of *Salmonella* at a specific cooking temperature, or higher than

that of *Salmonella* (Kopit et al., 2014). Previously published best practices for food safety process validation have directed that candidate pathogen surrogates having similar or greater process resistance than pathogen(s) of concern are desirable as surrogates (CFPVE, 2013). In addition, modeling systems can be included to analyze similar characteristics of the pathogen of concern. Nevertheless, the D-values for the pathogen versus the surrogate organism did not statistically differ from one another at temperatures of 190 and 200 °F. This agrees with the findings of Bianchini et al. (2014), as *E. faecium* demonstrated similar thermal resistance characteristics as *Salmonella* in a balanced carbohydrate-protein meal. Jones-Ibarra et al. (2017), whose study calculated D-values in poultry offal for a cocktail of human and avian-pathogenic *Salmonella* serovars (*S*. Senftenberg, *S*. Enteritidis, and *S*. Gallinarum) at temperatures of 150, 155, or 160°F, reported D-values of 0.254±0.045, 0.172±0.012, and 0.086±0.004 min, respectively. As temperature was increased the D-value also numerically declined initially but then rose again, indicative of some level of imprecision in data collection, or possibly degradation of cooking vessels over replication leading to changes in heat transfer kinetics. Finally, D-values generated in blood for *E. faecium* agree with those reported in previous studies, indicating it can make a suitable surrogate for thermal processing validation in similar types of food or rendered animal carcass components (Goepfert, et al. 1968, Ng. et al., 1969).

Table 3: Least squares means of D-values in chicken blood by the interaction of microorganism x heating temperature.

Organism, Heating Temperature (°F)	**D-value**[a]	***P*>F**
Salmonella, 180	0.99A	0.0006
E. faecium, 180	0.55B	
Salmonella, 190	0.48B	
E. faecium, 190	0.49B	
Salmonella, 200	0.58B	
E. faecium, 200	0.53B	
Pooled Standard Error = 0.053		

[a] Means not sharing a capitalized letter (A, B) differ by Tukey's Honestly Significant Differences (HSD) test at p=0.05. Values depict the means of triplicate identical replicates calculated from like samples (N=3).

Table 4 presents D-values of *Salmonella* and *E. faecium* in chicken blood, as impacted by the interaction of microorganisms x D-value determination model. The statistical analysis indicates a non-statistically significant impact on resulting D-values by this interaction (P=0.7340). The Baranyi and Roberts model has been reported to be more useful for fitting non-linear microbial survival data, in contrast to the linear regression modeling method (Baranyi et al., 1993, Baranyi and Roberts 1994, 1995). The Baranyi and Roberts model offers good predictive capabilities (Grijspeerdt and Vanrolleghem, 1999); it is a dynamic model in that it can accommodate time-varying environmental conditions. In light of previous attention given to quantitative risk analysis of food production, this is a useful tool (McMeekin and Ross, 1996;

Foegeding, 1997). The Baranyi and Roberts model for determining D-values demonstrated a better fit of experimental data to model-fitting parameters, similar to that of previous research studies in the literature (Grijspeerdt and Vanrolleghem, 1999; Fakruddin et al., 2011). As demonstrated in Baranyi et al. (1995), this dynamic inactivation model can describe shoulders and/or tails phenomena, as well as the possible log-linear decrease of a microbial population, in a suitable way. Finally, since the only statistically significant interaction of experimental main effects on resulting D-values was that of microorganism x heating temperature (P=0.0006), experimental data suggest *E. faecium* could be an effective pathogen surrogate for the pathogen *Salmonella* in thermal process validation work. This outcome also agrees with recommendations of Liu and Schaffner (2007), that an ideal surrogate for thermal processing validation be a non-pathogenic organism that provides similar response as the target pathogenic organism when subjected to the same antimicrobial interventions.

Table 4: Least squares means of D-values in chicken blood by the interaction of microorganism x D-value determination method.

Organism, Model	**D-value**[a]	**R^2**	***P*>F**
Salmonella, Linear	0.98	0.69±0.28	0.7340
E. faecium, Linear	0.81	0.74±0.13	
Salmonella, Baranyi and Roberts	0.37	0.91±0.19	
E. faecium, Baranyi and Roberts	0.23	0.94±0.05	
Pooled Standard Error=0.044			

[a] Values depict the means of triplicate identical replicates calculated from like samples (N=3).

***Salmonella* and *E. faecium* time/temperature-dependent D-values on chicken feathers.** To determine if replicates exerted influence over resulting experimental data, a REML analysis was completed. Analysis output indicated that replications did exert a significant effect on resulting data (P=0.0011), but produced 0.0% of total data variation (Table 5). Hence, data were re-analyzed by ANOVA where replication was not identified as a main or random effect (Table 6).

Table 5: REML variance component estimates for experimental replicates in chicken feathers.

Random Effect	**Variance Ratio**	**Variance Component**	**Std Error**	**95% Lower CL**[a]	**95% Upper CL**	**Wald *p*-Value**	**Variance (%)**
Rep #	-0.0818	-0.0256	0.0078	-0.0410	-0.0101	0.0011	0.00
Residual		0.3130	0.0943	0.1872	0.6270		100.00
Total		0.3130	0.0943	0.1872	0.6270		100.00

[a]CL: Confidence limit.

ANOVA output for both *Salmonella* and *E. faecium* D-values in chicken feathers, similar to that for chicken blood experiments, indicated main effects were statistically significant at directing resulting D-values (P=0.0001) (Table 6). Mean z-values for *Salmonella* and *E. faecium* in feathers were 291.64±367.2 and 230.74±213.1°F, respectively, again highly variable and thus likely imprecise. These results may be the result of feathers possessing a substantially reduced water content as compared to blood, increasing the difficulty in gaining efficient heat transfer, as well as the impacts of heavy use of metallic vessels in high heat oil with respect to loss of efficient heat transfer capacity (Paşayev et al., 2017; Reddy and Yang 2007). Chicken feathers moisture content was recently reported as 16.18% on a dry weight basis at 20°C, indicating that during rendering a high degree of heating would be required for pathogen destruction, as is routinely achieved so that feathers can be broken down into useful byproducts (Paşayev et al., 2017).

Table 6: Two-way analysis of variance: Salmonella *and* E. faecium *D-values for chicken feathers.*

Source	**DF**	**Sums of Squares**	**Mean Squares**	**F-Ratio**	***P*>F**
Model	11	40.935	3.7214	12.948	0.0001
Error	24	6.897	0.2874		
C. Total	35	47.833			

In Table 7, for the interaction of microorganism x heating temperature, the D-values for chicken feathers did not statistically differ from one another, regardless of heating/cooking temperature (p=0.4609). Studying the thermal destruction of *S.* Enteritidis in animal feeds, Himathongkham et al. (1996) observed a linear relationship between *Salmonella* reduction and temperature. These researchers, though not working specifically with *E. faecium* and thermal lethality, suggested that heating temperature could be the most important factor on the inactivation of bacterial contaminants in food and feeds (Bianchini et al., 2012). As was discussed above related to the impact of feather moisture on resulting z-value determination, D-values at all temperatures for both organisms were routinely higher than in blood, potentially a function of reduction in feather moisture and the resulting lower heat transfer efficiency.

Table 7: Least squares means for D-values in chicken feathers by the interaction of microorganism x heating temperature.

Organism, Heating Temperature (°F)	**D-value**[a]	**R^2**	***P*>F**
Salmonella, 190°F	1.72	0.84±0.14	0.4609
E. faecium, 190°F	2.26	0.88±0.14	
Salmonella, 200°F	2.04	0.75±0.23	
E. faecium, 200°F	2.02	0.79±0.17	

Salmonella, 210°F	1.96	0.73±0.30
E. faecium, 210°F	2.24	0.78±0.27
Pooled Standard Error=0.218		

[a] Values depict the means of triplicate identical replicates calculated from like samples (*N*=3).

Table 8 describes the interaction of microorganism x model as it relates the determination of D-values for both pathogen and surrogate on chicken feathers. Data analysis indicated that resulting D-values were not influenced by the interaction of these main experimental effects (*P*=0.537). Nevertheless, numerical D-values are not similar to one another, with Baranyi and Roberts models predicting a lower overall D-value as compared to the linear regression model, again likely the result of the multi-variate model being able to accommodate non-linear survival curve data more effectively versus the linear regression. When viewed with the R^2 values generated for each modeling output, researchers suggest the Baranyi and Roberts method to be more reliable for prediction of the D-values for both pathogen and surrogate, given the higher degree of model fitting to the experimental data. Nevertheless, it was surprising to researchers, given the discrepancy in D-values between the two modeling types, that mean D-values were not identified as being statistically differing from one another. This likely arises, to at least a degree, from the variability in experimental data, given the pooled standard error=0.178.

Table 8. Least square means for D-values in chicken feathers by the interaction of microorganism x model.

Organism x D-value Model	**D-value**	**R^2**	***P*>F**
Salmonella, Linear	2.98	0.58±0.16	0.5371
E. faecium, Linear	3.15	0.68±0.20	
Salmonella, Baranyi and Roberts	0.83	0.96±0.05	
E. faecium, Baranyi and Roberts	1.22	0.95±0.03	
Pooled Standard Error=0.178			

Finally, according to Fisher and Phillips (2009), the heat resistance of *E. faecium* is partially the result of its membrane structure and has been related to the lipid and fatty acid content. *E. faecium* has also been shown to be an acceptable pathogen surrogate for the study of thermal inactivation of bacteria in different human food products in past research (Li et al., 1993; Piyasena et al., 2003), though its use in validation of rendering of animal carcass components is not known to exist in the refereed literature by project investigators. Research has indicated *E. faecium* is an adequate surrogate for *Salmonella* for validation of thermal process lethality in almonds and in beef jerky, both of which are low-moisture, low a_w products (Almond Board of California, 2007; Borowski et al., 2009; Jeong et al., 2011).

Cumulative thermal lethality for *Salmonella* and *E. faecium* in chicken blood and feathers. Following consultation with the cooperating commercial rendering establishment, pathogen- or surrogate-inoculated samples were subjected to continuous heating (chicken blood: 200°F, 5.0 min; chicken feathers: 300°F, 18.0 min). Resulting experimental data indicate >7.0 $\log_{10}$-cycles' lethality to *Salmonella* in blood and on feathers (Table 10). Likewise, *E. faecium* lethality was in excess of 7.0 $\log_{10}$-cycles in blood and on feathers (Table 11). These would indicate the production of microbiologically safe products that would comply with other U.S. federal regulatory requirements for production of safe poultry-derived foods (USDA-FSIS, 2017), though no minimum safe *Salmonella* reduction requirement has been proposed for poultry rendering establishments dictating adequate food safety protection by the U.S. FDA.

Table 10: Inactivation of Salmonella *and* Enterococcus faecium *in blood and feathers during heat treatment simulating conditions used in a commercial rendering establishment.*

Salmonella **($\log_{10}$ CFU/g) Pre-Heating**	***Salmonella*** **($\log_{10}$ CFU/mL) Post-Heating**
Blood[a]	
7.4±0.03	ND[c]
Feathers[b]	
8.6±0.02	ND

[a] Values depict means of three replications containing two samples each (N=6) ± one sample standard deviation from mean. Limit of detection for blood = 1 CFU/mL

[b] Values depict means of three identical replications containing two samples each (N=6) ± one sample standard deviation from mean. Limit of detection for feathers = 10 CFU/g.

[c] ND=Non-detectable

Table 11. Inactivation of E. faecium *in blood and feathers during heat treatment simulating conditions used in a commercial rendering establishment.*

E. faecium **($\log_{10}$ CFU/g) Pre-Heating**	***E. faecium*** **($\log_{10}$ CFU/g) Post-Heating**
Blood[a]	
7.8±0.04	ND[c]
Feathers[b]	
8.6±0.07	ND

[a] Values depict means of three replicates containing two samples each (N=6) ± one sample standard deviation from mean. Limit of detection for blood = 1 CFU/mL

[b] Values depict means of three replicates containing two samples each (N=6) ± one sample standard deviation from mean. Limit of detection for feathers = 10 CFU/g.

[c] ND=Non-detectable

Summary of findings. In general, research data and outcomes of data analysis indicate that significant thermal lethality is gained against *Salmonella enterica* serovars likely to cross-contaminate poultry carcasses and their components during the application of rendering under conditions similar to those reported in this research report. As indicated, a >7.0 $\log_{10}$-cycle reduction in *Salmonella* was achieved for both chicken blood and feathers under conditions used that were developed from consultation with a U.S.-located cooperating poultry rendering establishment. Secondly, data indicate that, with one exception, *E. faecium* NRRL B-2354 could be effectively used as a *Salmonella* surrogate for the completion of in-plant lethality validation challenge trials for purposes of scientifically validating food safety preventive controls for use in food safety protection and compliance with the relevant components of the FDA Food Safety Modernization Act. Data analysis did not detect statistical differences in resulting D-values for the pathogen versus the *E. faecium* surrogate in most cases, which would reinforce the argument that the *E. faecium* isolate could be useful for validating pathogen inactivation during in-plant challenge trials of rendering processes. The utilization of the DM Fit-based modeling programs to generate D-values allowed for a straightforward method for analyzing microbiological data. The ComBase system is free for users, and provides simple guidance in using the models. The data generated in this project is suitable for sharing with the ComBase system in order to further the usefulness of ComBase to the foods industry, and will be shared with the ComBase database upon final publication of these data in a refereed journal article. The Baranyi and Roberts predictive model appeared to be a better choice as compared to the linear regression model for determining D-values from microbiological survival data, as indicated by higher R^2 values as compared to R^2 values obtained from linear regression-based survivor curve analyses. Ultimately, *Salmonella* were inactivated to a significant extent (>7.0 $\log_{10}$-cycles) by use of a thermal process designed to simulate a commercial rendering process for both chicken blood and feathers, and would thus indicate good control of microbiological safety of these products.

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LIST OF PRESENTATIONS AND PUBLICATIONS

Presentations

Taylor, T.M. 2019. Validating *Salmonella* lethality during rendering of poultry by-products: update. U.S. Poultry and Egg Association – Poultry Protein and Fat Educational Seminar, Nashville, TN. October 3-4.

Publications

Wong de la Rosa, C., T. M. Taylor. Thermal lethality to *Salmonella* and the pathogen surrogate *Enterococcus faecium* during simulated commercial rendering of poultry carcass by-products. *J. Food Prot.* In preparation for submission.

Wong de la Rosa, C. 2019. Thermal lethality for human pathogenic *Salmonella* and the *Salmonella* surrogate *Enterococcus faecium* on chicken feathers and blood. M.S. Thesis: Animal Science. Texas A&M University, College Station, TX.