Final Report to American Meat Institute Foundation

Project Title:

Development and Validation of Thermal Surrogate Microorganisms in Ground Beef for In-Plant Critical Control Point Validation Studies

Principal Investigator: Michael P. Doyle

Position/Title: Regents Professor and Center Director

Mail address: Center for Food Safety
University of Georgia
Griffin, Georgia 30223

Phone No. 770-228-7284

Fax No. 770-229-3216

E-mail: mdoyle@uga.edu

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Collaborators: Jeffrey Kornacki
Li Ma
Chia-Min Lin

Project Summary

Thermal processing of meat and poultry is among the most common processing treatments used to assure microbiological safety and is often a critical control point in HACCP programs. However, it is not feasible in factory settings to validate thermal processes using pathogenic bacteria. Hence, a suitable non-pathogenic (surrogate) microorganism is needed for process validation. We compared thermal inactivation relationships of *Enterococcus faecium* to those of *Listeria monocytogenes* and *Salmonella* in ground beef.

Several trials were done to determine the relationship of thermal destruction of *E. faecium* 2354 to *L. monocytogenes* 101M and *Salmonella* Senftenberg 775W at 58, 62, 65, and 68°C in ground beef containing 4% and 12% fat. In lean (4% fat) ground beef, *L. monocytogenes* was more sensitive to thermal inactivation at 58 and 62°C than was *S.* Senftenberg, but slightly more resistant at temperatures above 62°C. However, in ground beef containing 12% fat, *L. monocytogenes* was consistently more heat sensitive than *S.* Senftenberg at all four temperatures tested.

D values for *E. faecium* in lean and 12% fat ground beef were 4.4 to 17.7 and 3.6 to 14.6 times greater, respectively, than those for *L. monocytogenes* or *S.* Senftenberg 775W at all temperatures tested, with greatest difference in *D* values occurring at 58 and 62°C. This indicates that thermal treatments that kill *E. faecium* 2354 in ground beef would also kill *L. monocytogenes* and *Salmonella*. Depending on the margin of safety desired, processors could use this strain of *E. faecium* as a surrogate for validation studies of thermal processes for lean and 12% fat ground beef at 58 and 68°C.

In a search for a less heat resistant surrogate than *E. faecium* 2354, the heat resistance of *Pediococcus parvulus* HP and *Pediococcus acidilactici* LP, which are used as commercial meat starter cultures, was compared with that of *L. monocytogenes* 101M, *S.* Senftenberg 775W, and *E. faecium* 2354 in broth at 62°C. *D* values of *P. parvulus* HP and *P. acidilactici* LP were lower than those of *E. faecium* 2354, but 4.1 and 2.5 times greater, respectively, than those of *S.* Senftenberg 775W, the most resistant pathogen. These two *Pediococcus* strains may be alternate surrogates for validation studies when a less heat resistant surrogate is desired; however, studies at additional temperatures are needed with these strains for validation of a temperature range of 58 to 68°C.

MATERIALS AND METHODS

Bacterial strains and preparation of test cultures. Three bacterial strains, *Salmonella enterica* serotype Senftenberg 775W, *Listeria monocytogenes* 101M, and *Enterococcus* sp. B2354 (formerly *Pediococcus* sp. MRRL B-2354 and *Micrococcus freudenteihii*), were used for ground beef and broth studies. *Pediococcus parvulus* HP and *Pediococcus acidilactici* LP, commercial meat starter cultures (Chr. Hansen Inc., Milwaukee, WI) and identified by 16S rRNA gene sequencing (MIDI Labs, Newark, DE), were used in broth studies only. Each strain was incubated in 10 ml of tryptic soy broth with 0.6% yeast extract (TSB-YE) at 37 °C for three successive 24-h intervals. After incubation, each culture was harvested by centrifugation at 5000 x g for 10 min, then washed twice and reususpended in 0.25 ml of sterile Bufferfield's phosphate buffer (BPB, pH 7.2) for ground beef studies and in 3 ml of sterile BPB for broth studies.

Ground beef. Lean (4% fat content) and 12% fat ground beef were purchased from a local store. Total moisture and fat contents of ground beef were determined based on AOAC methods and pH was measured by a surface pH electrode (Sensorex, Garden Grove, CA).

Inoculation of ground beef. Ground beef (75 g) was weighed on sterile foil, spread into a thin layer, and 0.25 ml of each bacterial suspension (a total of 0.75 ml) was dripped onto the meat. The inoculated ground beef was kneaded aseptically by hand for 2 min to distribute the inocula. One gram of the ground beef was placed into a Whirl-Pak bag. The meat was flattened to as thin as possible by rolling a bottle over the surface of the bag. Two bags were initially samples to enumerate of inoculated bacteria. BPB (9 ml) was added to each bag which was then hand massaged for one min. The beef suspension

was serially diluted (1:10) in 0.1% peptone water and 0.1-ml portions of appropriate dilutions were plated onto duplicate plates of modified Oxford (MOX), xylose lysine thinosulfate (XLT4), and modified phenol red agar base (mPRAB) for enumeration of *L. monocytogenes* 101M, *S. enterica* serotype Senftenberg 775W, and *Enterococcus* sp. B2354, respectively. The plates were incubated at 37 °C for 24 h before enumeration. Thermal couples (12-channel thermo couple scanner, Barnant, Barrington, IL) were placed into three bags for temperature determinations during heat treatment and the remaining bags were vacuum-sealed and placed on ice until heat treatment.

Heat treatment of ground beef. The vacuum-sealed Whirl-Pak bags were submersed into a circulating water bath (Fisher Scientific) and adjusted to 58, 62, 65, or 68°C and three bags with thermal couples were placed in the middle and at the two sides of the water bath to obtain interior temperatures of the bags during heat treatment. When the interior temperature of the three bags reached the desired temperature, three Whirl-Pak bags were removed from the water bath and placed into ice water, and inoculated bacteria were enumerated for time 0 determinations. Three bags were removed subsequently at each sampling time. At 58 °C, the sampling time was every 2 min for up to 14 min for *L. monocytogenes* 101M and *S. enterica* serotype Senftenberg 775W, and at 15, 30, 60, 90, 120, and 150 min for enumeration of *Enterococcus* sp. B2354. At 62 °C, sampling was every 30 sec up to 5 min for *L. monocytogenes* 101M and *S. enterica* serotype Senftenberg 775W, and at 5, 10, 15, 25, 35, 45, and 55 min for *Enterococcus* sp. B2354. Sampling at 65 °C was every 10 sec for up to 60 sec for *L. monocytogenes* 101M and *S. enterica* serotype Senftenberg 775W, and every min for up to 6 min for *Enterococcus* sp. B2354. Sampling at 68 °C was every 5 sec for up to 30 sec for *L. monocytogenes* 101M

and *S. enterica* serotype Senftenberg 775W, and every 10 sec for up to 70 sec for *Enterococcus* sp. B2354.

Enumeration procedures of inoculated bacteria in ground beef. Three selective media, MOX, XLT4, and mPRAB, were used for *L. monocytogenes* 101M, *S. enterica* serotype Senftenberg 775W, and *Enterococcus* sp. B2354, respectively. Tryptic soy agar (TSA) (12 ml) was overlaid on selective medium plates on the day of the experiment to enable recovery of heat-injured cells. Nine ml of BPB was added to each Whirl-Pak bag, followed by hand massaging for one min. The beef suspension was serially diluted (1:10) with 0.1 % peptone water and 0.1-ml portions of appropriate dilutions were plated onto duplicate overlaid plates, then incubated at 37 °C for 24 h. In addition, the original cell suspension was enumerated by plating 1-ml onto four TSA-YE plates (0.25 ml each) for low cell numbers. All treatments were repeated at least three times.

Inoculation, heat treatment and enumeration of bacteria in broth. Only one temperature, 62 °C, was tested for all five strains in broth. For each strain, one ml of resuspended cells was transferred into 49 ml of pre-warmed (ca. 64 °C) TSB-YE broth and the tube was briefly vortexed before putting into a water bath at 62 °C. When the temperature of the broth reached 62 °C as monitored by thermal couples, 1 ml was removed from the test tube and placed into a sterile tube in ice water for 0-time bacterial counts. Thereafter, 1 ml of bacterial suspension was removed at each sampling time. The cell suspension was serially diluted (1:10) with 0.1 % peptone water and 0.1-ml portions of appropriate dilutions were plated onto duplicate TSA-YE plates. At later sampling times, the entire 1-ml sample was plated (four 0.25-ml portions) onto four TSA-YE plates to enable enumeration of small numbers of cells. The treatment was repeated three times.

Calculation of D- and z-values. D-values (time to inactivate 90% of the cells), expressed in seconds, were determined from the straight line portion of the survival curves by plotting the log number of survivors against time for each heating temperature using Microsoft Excel software (Microsoft Corp. 2000). Only survival curves with more than five values in the straight-line portion, and descending through more than 3 to 5 log cycles were used. Linear regression lines were fitted to experimental data points and D-values were calculated by taking the absolute value of the inverse slope. z-values were determined by computing the linear regression of mean log D-values vs. their corresponding heating temperatures using Microsoft Excel software. The z-values were the absolute value of the inverse slope.

RESULTS

Chemical characteristics of ground beef.

Moisture content, fat content, pH and water activity (a_w) of the ground beef were 72.6 %, 3.7 %, 5.5, and 0.990, respectively, for lean ground beef, and 73.7%, 12.4%, 5.5, and 0.989, respectively for 12% fat ground beef. Variations of different batches of ground beef were within 1% for moisture and fat contents and 0.1 pH value.

Bacterial strain identification and name change of *Pediococcus sp*. NRRL B-2354

We obtained two strains of bacteria from commercial meat starter cultures as additional potential surrogates and identified them by 16S rRNA gene sequencing (MIDI Labs). 16S rRNA gene alignment profiles and phylogenetic analysis identified one isolate as *Pediococcus parvulus* and the other as *Pediococcus acidilactici. Pediococcus* sp. NRRL B-2354, the potential surrogate we were using for thermal inactivation studies also was

identified by 16S rRNA gene sequencing and was determined to be an *Enterococcus* sp. It appeared to be either *E. faecium*, *E. hirae*, or *E. durans* but the species could not be determined by sequence data.

Thermal inactivation of bacteria in ground beef

D-values of *Salmonella* Senftenberg 775W, *Listeria monocytogenes* 101M and *Enterococcus sp*. B2354 in lean and 12% fat ground beef at 58, 62, 65, and 68 ºC in both matrixes are shown in Tables 1 and 2. In lean ground beef, *L. monocytogenes* was more sensitive to thermal inactivation than *S.* Senftenberg at 58 ºC and 62 ºC but slightly more resistant at temperatures above 62 ºC. This pattern was not observed in 12% fat ground beef as *L. monocytogenes* was consistently more heat sensitive than *S.* Senftenberg at all temperature points tested. D-values for *Enterococcus* sp. B2354 in lean and 12% fat ground beef were 4.4 to 17.7 and 3.6 to 14.6 times greater, respectively, than those for the most resistant pathogenic strain (*L. monocytogenes* or *S.* Senftenberg 775W) at all temperatures tested. Heat treatments of ground beef at 58 to 68ºC sufficient to kill *Enterococcus* sp. B2354 would also inactivate *Salmonella* and *L. monocytogenes*. Hence, depending on the margin of safety desired, processors could use *Enterococcus* sp. B2354 as a surrogate for validation studies of thermal processes in lean and 12% fat ground beef at 58 to 68ºC. The dynamics of thermal inactivation in both matrixes at all four temperatures tested are shown in Figures 1 and 2 and z values are presented in Tables 3 and 4.

Influence of heating menstruum on rates of inactivation of test bacteria

The composition of food matrices can have an effect on rates of thermal inactivation of bacteria. The fat content of ground beef did influence the rates of thermal inactivation of

all three test bacteria, with reduced rates of inactivation (higher D values) in beef with the higher fat content (Figure 3). However, this protective effect was greatly diminished at 68ºC.

Thermal inactivation of bacteria in broth

In search of a less heat-resistant surrogate than *Enterococcus* sp. B2354, the heat resistance of two other potential surrogate microorganisms, *Pediococcus parvulus* HP and *Pediococcus acidilactici* LP, isolated from commercial meat starter cultures, were determined and compared to those of *L. monocytogenes* 101M, *S.* Senftenberg 775W, and *Enterococcus* sp. B2354 in broth at 62 ºC. D-values for each under these conditions are shown in Table 5. D-values of *P. parvulus* HP and *P. acidilactici* LP were less than that of *E. faecium* 2354 but 4.1 and 2.5 times greater, respectively, than those of the most resistant pathogenic strain (*S.* Senftenberg 775W). Hence, these two *Pediococcus* strains may be alternate surrogates for validation studies when a less heat resistant surrogate is desired; however, studies at additional temperatures are needed with these strains for validation of the entire range of 58 to 68°C.

Table 1. D values (sec) of *S. senftenberg* 775 W, *L. monocytogenes* 101M, and *Enterococcus* sp. B2354 in lean ground beef at 58, 62, 65, and 68°C.

Bacterial strains/temperatures	58°C	62°C	65°C	68°C
L. monocytogenes 101M	152 ± 39	31.2 ± 2.2	9.17 ± 0.71	4.27 ± 0.27
S. Senftenberg 775W	154 ± 3	40.2 ± 2.8	8.66 ± 1.36	3.31 ± 0.51
Enterococcus sp. B2354	2724 ± 226	476 ± 48	70.2 ± 11.0	18.6 ± 0.4
	(17.7)*	(11.8)	(7.7)	(4.4)

*Ratios between D values of *Enterococcus* sp. B2354 and those of most resistant pathogen at the temperature tested

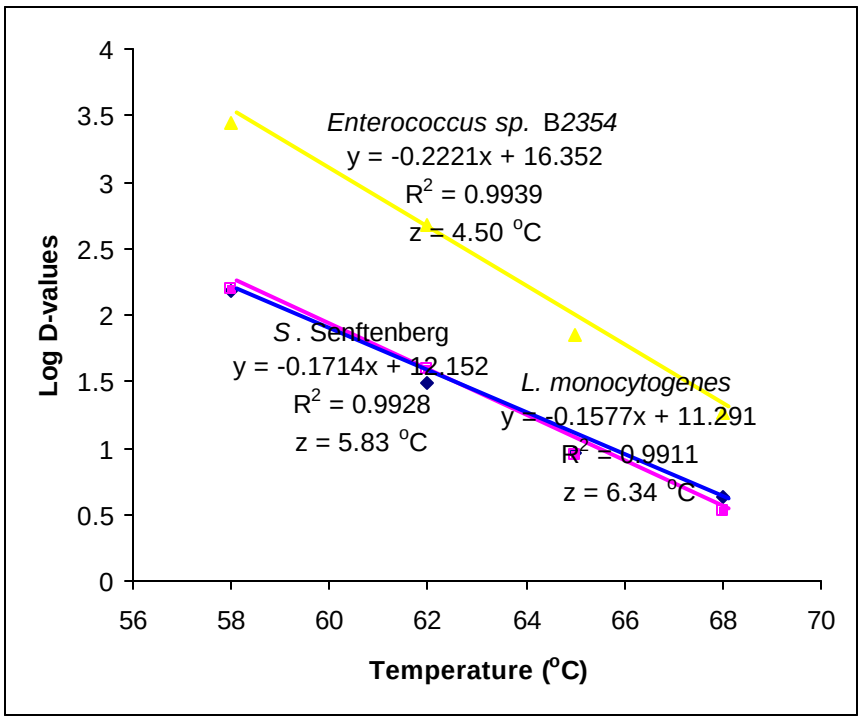

Figure 1. Dynamics of thermal inactivation for *S.* Senftenberg 775 W, *L. monocytogenes* 101M, and *Enterococcus* sp. B2354 in lean ground beef between 58 and 68 ^{0}C

Table 2. D values (sec) of *S. senftenberg* 775 W, *L. monocytogenes* 101M, and *Enterococcus* sp. B2354 in 12% fat ground beef at 58, 62, 65, and 68°C.

Bacterial strains/temperatures	58°C	62°C	65°C	68°C
L. monocytogenes 101M	264 ± 28	36.2 ± 1.3	13.6 ± 0.7	4.21± 0.41
S. Senftenberg 775W	269 ± 11	50.3 ± 2.3	14.6 ± 2.7	4.53 ± 0.43
Enterococcus sp. B2354	3902 ± 193	736 ± 110	118 ± 22	16.5 ± 3.3
	(14.5)*	(14.6)	(8.1)	(3.6)

*Ratios between D values of *Enterococcus* sp. B2354 and those of most resistant pathogen at the temperature tested

Table 3. z values (°C) of *S. senftenberg* 775 W, *L. monocytogenes* 101M, and *Enterococcus* sp. B2354 in lean ground beef.

Bacteria	z value (°C)
L. monocytogenes 101M	6.34
S. senftenberg 775 W	5.83
Enterococcus sp. B2354	4.50

Table 4. z values (°C) of *S. senftenberg* 775 W, *L. monocytogenes* 101M, and *Enterococcus* sp. B2354 in 12% fat ground beef.

Bacteria	z value (°C)
L. monocytogenes 101M	5.63
S. senftenberg 775 W	5.64
Enterococcus sp. B2354	4.21

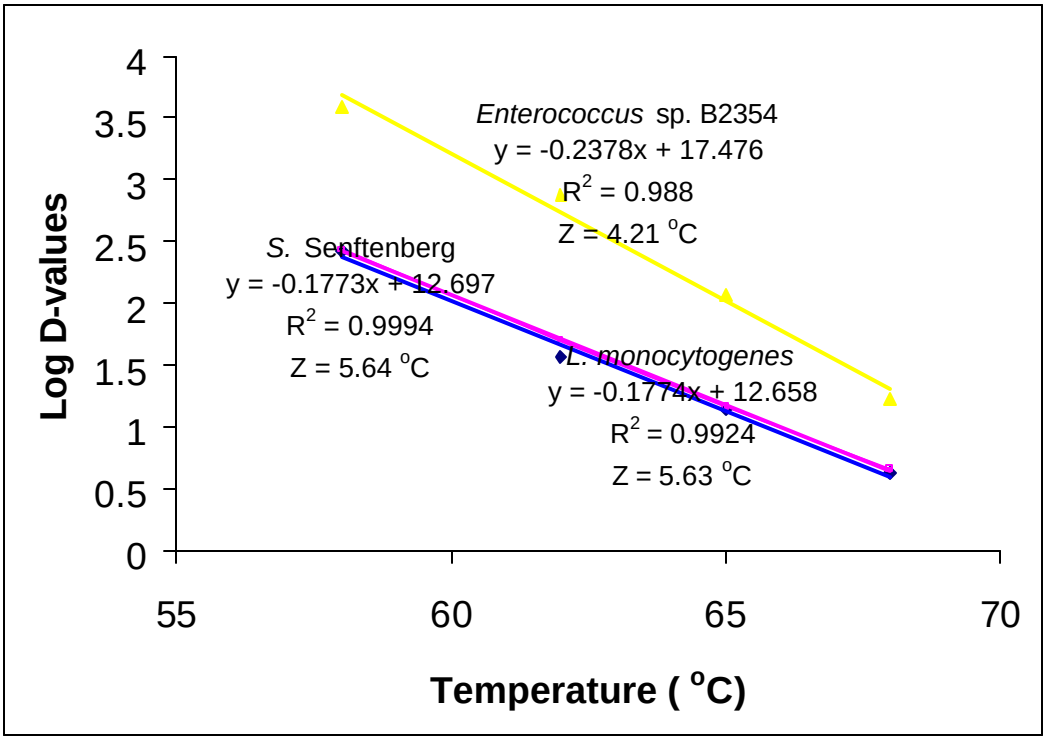

Figure 2. Dynamics of thermal inactivation for *S. senftenberg* 775 W, *L. monocytogenes* 101M, and *Enterococcus* sp. B2354 in 12% fat ground beef between 58 and 68 ^{0}C.

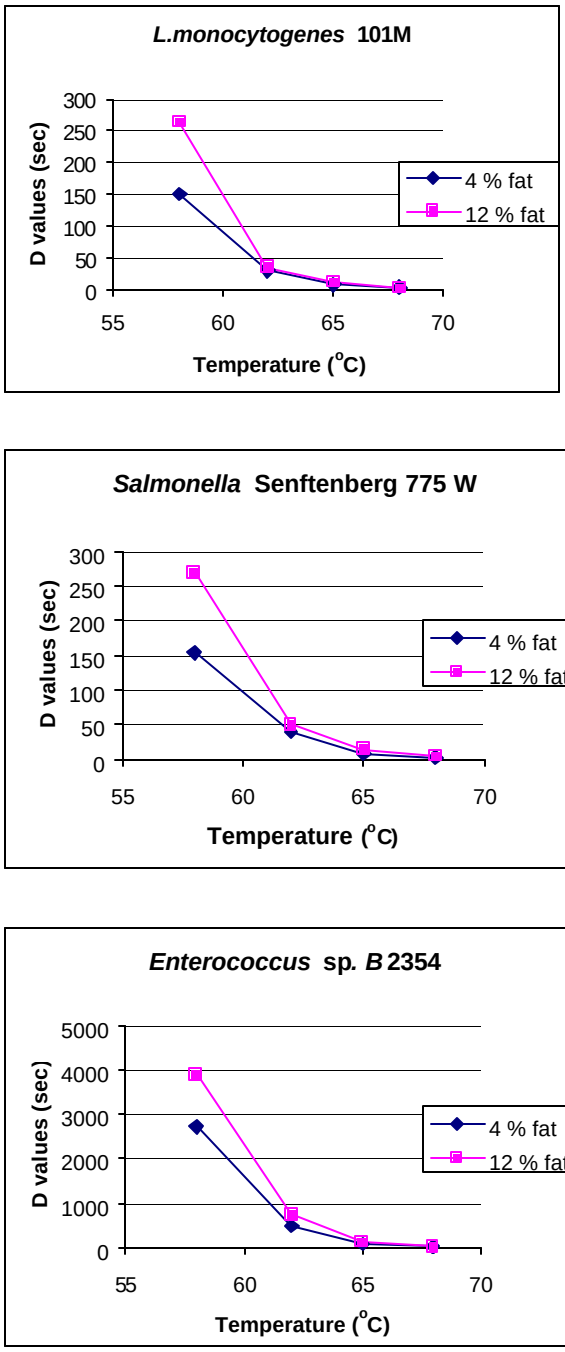

Figure 3. Comparison of D-values of test strains at each test temperature in lean ground beef (blue) and in 12% fat ground beef (pink).

Table 5. D values (min) of *S. senftenberg* 775 W, *L. monocytogenes* 101M, *Enterococcus* sp. B2354, *P. parvulus* HP, and *P. acidilactici* LP in TSB-YE broth at 62°C.

Bacterial strains	D values (min)
L. monocytogenes 101M	1.71 ± 0.02
S. Senftenberg 775W	1.86 ± 0.03
Enterococcus sp. B2354	11.7 ± 0.4
P. parvulus HP	7.65 ± 0.30
P. acidilactici LP	4.68 ± 0.04