CENTER for PRODUCE SAFETY

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FINAL PROJECT REPORT

Project Title
Validating a physically heat-treated process for poultry litter in industry settings using the avirulent *Salmonella* surrogates or indicator microorganisms

Project Period
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Principal Investigator
Xiuping Jiang
Clemson University
Department of Food, Nutrition, and Packaging Sciences
228A Life Science Facility
Clemson, SC 29634
T: 864-656-6932
E: xiuping@clemson.edu

Co-Principal Investigator
Annel Greene
Clemson University
Department of Animal and Veterinary Sciences
247 Poole Agricultural Center
Clemson, SC 29634
T: 864-656-3123
E: agreene@clemson.edu

Objectives
1. *Select avirulent Salmonella surrogates and indicator microorganisms for process validation of physically heat-treated poultry litter.*
2. *Validate thermal processes for physically heat-treated poultry litter in industrial settings (turkey litter and chicken litter processing plants) using Salmonella surrogate and indicator microorganisms.*

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FINAL REPORT

Abstract

Poultry litter, commonly used as soil amendment or organic fertilizer, may contain human pathogens such as *Salmonella*. In order to reduce the microbiological risks associated with the use of raw poultry litter, physical heat treatments are recommended to inactivate pathogenic microorganisms. However, there are no validation studies or guidelines available to the litter processing industry. In this project, we proposed to select avirulent *Salmonella* surrogates and indicator microorganisms by comparing thermal resistance with *Salmonella* in poultry litter under various conditions (**Obj. 1**), and to use the indicator and surrogate microorganisms to validate processes for physically heat-treated poultry litter in industry settings (**Obj. 2**).

As microorganisms may be injured during thermal exposure, first we evaluated several recovery media for allowing heat-injured cells to resuscitate. Then, both the aged broiler litter and partially composted turkey litter with 20, 30, 40, and 50% moisture content were inoculated with desiccation-adapted *Salmonella* Senftenberg 775/W and *Enterococcus faecium* NRRL B-2354 as surrogate microorganisms for *Salmonella*, separately, followed by heat treatment at 75, 85 or 150°C. The correlation analysis was performed on thermal inactivation data for *S*. Senftenberg, *E. faecium* NRRL B-2354*,* presumptive indigenous enterococci, and total aerobic bacteria in poultry litter. Our results revealed that the indigenous enterococci counts predict the survival behavior of *Salmonella* much better than total aerobic bacterial counts, under most treatment conditions. Among the three temperatures (75, 85, and 150°C) tested, the desiccation-adapted *E. faecium* NRRL B-2354 was more heat resistant ($P < 0.05$) than desiccation-adapted *S.* Senftenberg 775/W. Based on thermal inactivation data, we can conclude that reductions of 1.2 to 2.7 log or more of *E. faecium* NRRL B-2354 may predict >5-log reductions of *Salmonella* in poultry litter, depending on heating temperature, moisture content and types of poultry litter. Therefore, our results confirmed that *E. faecium* NRRL B-2354 can be used as a surrogate for *Salmonella* and presumptive indigenous enterococci as indicator microorganisms to provide sufficient safety margin when validating the thermal processing of poultry litter (Obj. 1).

For thermal process validation, we teamed up with three poultry litter processors—one turkey litter processor (plant A) and two chicken litter processors (plant B and plant C)—to conduct 7 pilot trials throughout the project. A multiple component sampler was custom-designed in this project to carry the inoculated samples through the industrial dryers. This sample was capable of withstanding the harsh conditions in the dryers, and at the same time measure the residence time more accurately than using magnetically retrievable MicroTracers. For each pilot trial, the poultry litter at target moisture levels was inoculated with *E. faecium* NRRL B-2354 at an initial population of ca.10^7 cfu/g and placed into the customized sampler, which was shipped overnight to plant A and plant B, added into the inlet of the dryer, and collected at the exit end. For the validation study conducted in plant C, ca. 2.5 kg of laying hen litter compost inoculated with *E. faecium* NRRL B-2354 was mixed with the bulk of laying hen litter (680 kg) on site at initial population of ca. 10^5 cfu/g. All the heat-treated samples collected were shipped back to the lab overnight and analyzed immediately for the presence or population of the inoculated *E. faecium* NRRL B-2354*,* presumptive indigenous enterococci and total aerobic bacteria (Obj. 2). The thermal process in the three plants yielded 2.93–7.45, >1.72–3.74, and 1.49–2.61 log CFU/g reductions of *E. faecium* NRRL B-2354, presumptive indigenous enterococci, and total aerobic bacteria, respectively. Statistical analysis of validation data in plant A did not show a significant effect ($P > 0.05$) of either seasonality or moisture content of turkey litter compost on reductions of *E. faecium* NRRL B-2354 prior to the dryer. Based on thermal inactivation comparison data collected in Obj. 1, the processing conditions in these plants were adequate to achieve at least

5-log reductions of *Salmonella*, even though the processing conditions varied among trials. Additionally, the microbial community profiles varied significantly among different types of poultry litter, and the thermal process reduced 95 to 99.9% of total aerobic bacterial populations in poultry litter/compost. Metagenomic sequencing analysis revealed a significant decrease in the most abundant communities (i.e., *Bacillaceae*) and species evenness became more distinct in the turkey litter compost after heat treatment, indicating that heat treatment had different effects on the microbial community. Results generated from this study have validated current processes for physically heat-treated poultry litter in industry settings, and provide tools (surrogate and/or indicator microorganism for *Salmonella,* and custom-designed sampler) and guidelines (time/temperature and moisture level) for litter processors to modify their existing process parameters to produce *Salmonella*-free physically heat-treated poultry litter, which can be used by the produce industry to grow microbiologically safe products.

Background

According to the U.S. Department of Agriculture (USDA) 2012 Census of Agriculture, broilers and turkeys in the U.S. produced ~6.8 and 2 million tons of litter, respectively. Poultry litter, rich in nutrients and organic matter, has great value as a soil amendment and organic fertilizer for produce production. Poultry litter can be used in organic farming under certain conditions; however, under the Organic Materials Review Institute (OMRI)/USDA National Organic Program (NOP) rules and the California Leafy Green Marketing Agreement, the use of raw manure on fresh produce that is intended for human consumption is discouraged due to the possible presence of human pathogens such as *Salmonella* spp. (California Leafy Green Handler Marketing Board 2013; NOP 2006; Timmenga & Associates Inc. 2003). Physically heat-treated poultry litter is used as a biological soil amendment or organic fertilizer by both organic and conventional farms (Kim *et al.* 2012). To produce heat-treated poultry litter products, heat treatments, such as pelletization, pasteurization, and dry heat at higher temperatures (>65°C for 1 h or more), are recommended to reduce or eliminate potential pathogenic microorganisms in poultry litter while lowering the moisture content of raw materials to below 10 to 12% (Cox *et al.* 1986; López-Mosquera *et al.* 2008). However, research on the microbiological safety of physically heat-treated poultry litter, especially on litter processing plant validation, is limited.

Our lab has carried out a series of studies on evaluating whether certain recommended heat treatment conditions with specific time-temperature combinations are sufficient to produce finished products free from foodborne pathogens (Chen *et al.* 2013, 2015a, 2015b; Kim *et al.* 2012). Desiccation adaptation of *S. enterica* during aged broiler litter stockpiling was studied to determine the impact on heat resistance during subsequent exposure to heat treatments (Chen *et al.* 2015b). We found ca. >3-, >4-, 4 to 10-, and >6-fold increases in the exposure times required for reducing desiccation-adapted *Salmonella* cells by 5 log at 70, 75, 80, and 85°C, respectively, as compared to non-adapted cells. Therefore, to validate thermal processing of poultry litter, *Salmonella* cells subjected to desiccation stress during storage should be used.

Due to biosafety concerns, it is not feasible to validate thermal processing with pathogenic bacteria in poultry litter processing plants. To understand pathogen behavior in processing environments, suitable surrogates or indicator microorganisms with similar characteristics to pathogens are needed (Harris *et al.* 2013). Our study revealed that *S.* Senftenberg was the most heat-resistant serotype among four *Salmonella* serotypes used for validating thermal inactivation of *Salmonella* in physically heat-treated chicken litter (Chen et al. 2013). Since *S.* Senftenberg is pathogenic, a nonpathogenic surrogate with similar thermal resistance is needed for validating *Salmonella* thermal inactivation in poultry litter in an actual processing plant. *Enterococcus faecium* NRRL B-2354 has been used as a surrogate for *S. enterica* for validating

thermal processing of almonds and carbohydrate-protein meal (Bianchini *et al.* 2014; Jeong *et al.* 2011). The Almond Board of California (2014) also recommends this strain as a surrogate for *S.* Enteritidis PT30 during moist-air heating of almonds. Therefore, *E. faecium* NRRL B-2354 may be used as surrogate to validate poultry litter thermal processing for *Salmonella* inactivation, but this needs to be confirmed by the lab and plant trials.

Another approach for predicting pathogen survival during thermal processing is to monitor the behavior of indigenous microflora as indicator microorganisms. However, limited information exists on the comparison of survival behavior of foodborne pathogens and indigenous microflora in foods or animal wastes during heat treatment. A similar thermal death rate of indigenous microflora in ground beef versus those of *Listeria monocytogenes*, *Escherichia coli* O157:H7, and *S. enterica* has been reported (Juneja 2003; Juneja *et al.* 1997). It has been recognized that enterococci can be resistant to various environmental stresses such as high temperature and desiccation (Fernández *et al.* 2009; Flahaut *et al.* 1996). For biosolids, the enterococci indicator is a more representative surrogate of both bacterial and viral pathogen inactivation than fecal coliforms (Viau and Peccia 2009). Graham *et al.* (2009) sampled three conventional broiler chicken farms and reported enterococci populations ranging from 3.00 to 7.50 log cfu/g in chicken litter during 120 d of stockpiling. In our previous study, mesophiles (incubated at 35°C) in broiler litter ranged from 6.55 to 7.80 log cfu/g during a 9-month storage period (Chen *et al.* 2015b). Therefore, the populations of these indigenous microflora in poultry litter are sufficiently high for indicating the survival behavior of *S. enterica*, suggesting that they can be considered as a potential choice to validate the effectiveness of thermal processing.

There is a lack of knowledge on microbial community changes after heat processing of poultry litter. The identification of indigenous microbial species in poultry litter surviving the physical heat treatment may lead to future studies on biological control of pathogens in biological soil amendments. High-throughput metagenomic sequencing has become a powerful tool for characterizing and profiling complex microbial communities. Genomic techniques based on polymerase chain reaction (PCR) amplification of the conserved and variable regions of the microbial genome (16S rRNA) allows direct sequencing of these PCR amplicons using high-throughput next generation sequencing. In this approach, each group of PCR amplicons that share a similar variable region is considered an “operational taxonomic unit” (OTU). Based on the sequencing similarity, the phylogenetic diversity of the microbial community can be categorized by the analysis of OTUs (Vida *et al*. 2016). The 16S rRNA sequencing has been used to characterize the microbial community structure in poultry litter (Smith et al., 2014) and in agricultural soil amended with composted almond shells, responsible for suppressing the avocado soil-borne phytopathogen, *Phytophthora cinnamomi* (Vida *et al*. 2016).

Research Methods and Results

Objective 1*: Select avirulent* Salmonella *surrogates and indicator microorganisms for process validation of physically heat-treated poultry litter.*

Compost preparation: Aged broiler litter was collected from an organic farm in California. To prepare for the broiler litter, the litter inside the chicken house was removed annually, followed by a partial windrow composting for 7–10 days. After composting, the litter was then screened to remove rice hulls. Composted turkey litter was obtained from plant A. All compost samples were dried under a fume hood until moisture content was reduced to <20%, and then screened to particle size of <3 mm using a sieve (sieve pore size, 3 by 3 mm). Sufficient samples were collected for the entire study and stored in a sealed container at 4°C until use. Moisture content was measured with a moisture analyzer (Model IR-35, Denver Instrument, Denver, CO). Water activity (a_w) was measured with a dew-point a_w meter (Aqualab Series 3TE, Decagon Devices,

Pullman, WA). Ammonia content and pH value were measured based on the methods described by Weatherburn (1967) and the U.S. Composting Council (2002), respectively.

Culture preparation: S. enterica Senftenberg ATCC 43845 (775/W), as *S.* Senftenberg 775/W, was identified as the most heat resistant among 4 *Salmonella* serotypes tested during thermal processing of aged broiler litter in our previous study (Chen *et al*. 2013). *Enterococcus faecium* NRRL B-2354 (ATCC 8459) was evaluated as a potential *Salmonella* surrogate for thermal process validation of poultry litter. Both strains were induced to rifampin resistance (100 µg/ml) using the gradient plate method. Each strain was grown overnight at 35°C in tryptic soy broth (TSB; Becton, Dickinson and Company, Sparks, MD) containing 100 µg of rifampin/ml (TSB-R). The overnight cultures were then washed three times and resuspended with sterile 0.85% saline to desired cell concentrations by measuring the optical density at 600 nm. Enumeration of *S*. Senftenberg 775/W and *E. faecium* NRRL B-2354 was performed on Xylose-Lysine-Tergitol 4 agar (XLT-4; Becton, Dickinson and Co.) and enterococcosel agar (EA), respectively, both supplemented with 100 µg rifampin/ml (XLT4-R and EA-R).

Compost inoculation and desiccation adaptation of inocula: Poultry litter compost with adjusted moisture content was inoculated with the above *S.* Senftenberg 775/W and *E. faecium* NRRL B-2354 cultures (1:100, v/w), separately, using a sterile spray nozzle and then thoroughly mixed to a desired concentration. Afterwards, the inoculated poultry litter compost was kept in a container covered loosely by sterile aluminum foil to allow the bacterial cells to acclimatize at room temperature. After 24-h desiccation adaptation, 100 g of poultry litter inoculated with desiccation-adapted cells were further mixed (1:100, w/w) with 500 g of poultry litter with the same moisture content using a sterile blender for subsequent heat treatment. For enumeration of indigenous enterococci and total aerobic bacteria during thermal treatment, poultry litter (100 g) without added *S.* Senftenberg 775/W was prepared the same way as above.

Thermal inactivation: The initial populations of *S.* Senftenberg 775/W, *E. faecium* NRRL B-2354, indigenous enterococci, and total aerobic bacteria were determined right before thermal inactivation. Twenty grams of samples in duplicate were distributed evenly inside an aluminum pan (I.D. 10 cm). The temperature of a controlled convection oven (Binder Inc., Bohemia, NY) was initially set 5°C higher than the target temperature to reduce the come-up time. When the oven temperature reached the set temperature, the aluminum pans with inoculated samples were quickly placed at two different locations on the oven shelf and then exposed to the experimental temperature. Temperature was monitored using T-type thermocouples (DCC Corp., Pennsauken, NJ), with one cord kept inside the oven chamber and others inserted into litter samples at three different locations. When the interior temperature of litter sample reached the target temperature (0 h), the temperature setting was readjusted to the designated temperature. For heat treatments at 75 and 85°C, litter samples in duplicate were withdrawn quickly from the oven at 0 h and every 0.5 h during come-up time and holding time (up to 3 h) to determine microbial populations. For heat treatment at 150°C, duplicate samples were withdrawn every 15 min up to 60 min. Samples were transferred into sterile Whirl-Pak bags already in an ice-water bath to cool down the samples and stop further cell death.

Optimizing the culturing methods for recovering heat-injured E. faecium *NRRL B-2354 and indigenous enterococci:* Composted turkey litter with moisture adjusted to 30% (a_w 0.925) was inoculated with overnight-grown *E. faecium* NRRL B-2354 cells. Approximately 50 g of turkey litter compost, with and without desiccation-adapted cells, was distributed into Tyvek pouches, separately, and exposed to 75°C in an oven, as described above. Samples were taken out at 0, 0.5 and 1 h. Recovery media for heat-injured indigenous enterococci were bile esculin agar (BEA) or enterococcosel agar (EA), and the modified two-step overlay (OV) method (OV/BEA and OV/EA) and modified thin agar layer (TAL) method (TAL/BEA and TAL/EA) were used. To recover heat-injured *E. faecium* NRRL B-2354, BEA and EA recovery media supplemented with

rifampin (100 µg/ml) were used as above. These experiments were performed in two separate trials.

Bacterial enumeration: The surviving *Salmonella* and *E. faecium* NRRL B-2354 cells were enumerated using a modified two-step overlay method to allow heat-injured cells to resuscitate, with XLT-4 and EA supplemented with 100 µg rifampin/ml as the selective media, respectively, and tryptic soy agar (TSA; Becton, Dickinson and Co.) as the nonselective media. The samples negative for *S.* Senftenberg 775/W by the direct plating method (detection limit: 1.30 log cfu/g) were pre-enriched in universal pre-enrichment broth followed by a secondary enrichment in Rappaport-Vassiliadis (RV) broth supplemented with 100 µg rifampin/ml. After 24-h incubation at 42°C, enriched samples were then plated onto XLT-4-R. Samples negative for *E. faecium* NRRL B-2354 by the direct plating method were pre-enriched in brain heart infusion (BHI) broth supplemented with 100 µg rifampin/ml at 37°C for 24 h, followed by plating onto EA-R.

Thermal inactivation kinetics and linear regression analysis: All experiments were conducted in two separate trials. Plate count data were converted to log cfu/g in dry weight. Analysis of variance (ANOVA), followed by the least significant differences (LSD) test, was carried out to determine whether significant differences ($P < 0.05$) existed among different treatments and different microorganisms using SigmaPlot 12.3 (Systat Software Inc., San Jose, CA). Linear regression analysis was performed to calculate the correlation between mean log reductions of desiccation-adapted *S*. Senftenberg 775/W and presumptive indigenous enterococci or total aerobic bacteria. An adjusted regression coefficient R^2 was used to evaluate the correlation. For the thermal inactivation kinetics of *E. faecium* NRRL B-2354, mathematical models, including Weibull, modified Gompertz, and log-logistic models, were applied to fit the thermal inactivation curves. An adjusted regression coefficient R^2 was used to evaluate and compare the goodness-of-fit of the proposed models.

All original compost samples were tested free from rifampin-resistant enterococci or *Salmonella*. To determine the best medium for recovering heat-injured surrogate or indicator microorganisms, *E. faecium* NRRL B-2354 was inoculated at 8.21 log cfu/g turkey litter compost that contained presumptive indigenous enterococci at 5.45 log cfu/g. Among six recovery methods, OV/EA-R plates yielded the highest populations ($P < 0.05$) of *E. faecium* NRRL B-2354 (5.09±0.33 log cfu/g; Table 1A – see Appendix A). For heat-treated turkey litter compost without inoculation, background microorganisms growing on OV/EA, OV/BEA, TAL/EA and TAL/BEA caused interference with the enumeration of indigenous enterococci. Therefore, **BEA and EA were selected to recover the heat-injured presumptive indigenous enterococci, and OV/EA-R was selected for recovering heat-injured *E. faecium* NRRL B-2354.** These media and methods were used for all following thermal inactivation experiments.

Selection of indicator microorganisms for validating desiccation-adapted Salmonella *reduction in physically heat-treated poultry litter:* Aged broiler and turkey litter with 20, 30, 40, and 50% moisture content (MC) were inoculated with desiccation-adapted *S.* Senftenberg 775/W (ca. 10^7 log cfu/g) and then heat-treated at 75 or 85°C for 3 h. Table 2A shows the chemical composition of aged broiler litter and composted turkey litter. Organic matter, C, N, S, and Na contents, C/N, pH, EC, and ammonia content in broiler litter were much higher than those in turkey litter ($P < 0.05$), while P, K, Ca, Mg, and heavy metal (Zn, Cu, Mn, Fe, and Al) contents, in turkey litter were much higher than those in broiler litter ($P < 0.05$). The a_w values of broiler litter with 20, 30, 40, and 50% MC were 0.808, 0.916, 0.952, and 0.974, respectively, whereas the a_w values of turkey litter were 0.839, 0.917, 0.953, and 0.972, respectively. Come-up times for heating broiler and turkey litter with different MCs at 75 and 85°C ranged from 0.82 to 3.00 h (Table 3A). Poultry litter with higher moisture content required longer come-up times. The initial populations of inoculated *S.* Senftenberg 775/W, presumptive indigenous enterococci, and total aerobic bacteria in broiler litter and turkey litter were 7.00–7.20, 3.90–4.10, and 7.70–7.90 log

cfu/g, respectively. There were reductions in microbial populations in all poultry litter samples during heat treatment at 75 and 85°C (Figure 1A–4A). Overall, microbial cells were inactivated much faster when treatment temperature and moisture content of poultry litter increased (P < 0.05). Additionally, microbial cells in broiler litter were more heat-sensitive as compared to those in turkey litter (P < 0.05).

Table I (below) lists the regression equations between the mean log reductions of desiccation-adapted *S.* Senftenberg 775/W and those of indigenous microflora with different treatments, except for poultry litter with 50% MC exposed to 85°C. There were better correlations between mean log reductions of *S.* Senftenberg 775/W and presumptive indigenous enterococci in broiler litter samples with 20, 30, 40, and 50% MC at 75°C (R^2 >0.91), and 20, 30, and 40% MC at 85°C (R^2 >0.87). The mean log reductions of *S.* Senftenberg 775/W were better correlated with those of indigenous enterococci in turkey litter samples with 20, 30, 40, and 50% MC at 75°C (R^2 >0.88), 20 and 30% MC at 85°C (R^2 =0.83) than those of total aerobic bacteria, which had a better correlation in turkey litter sample with 40% MC (R^2 =0.98) at 85°C (Table 4A). **Our results clearly demonstrated that presumptive indigenous enterococci may be used to validate the thermal processing of poultry litter, as it predicts the survival behavior of *Salmonella* under most treatment conditions**.

Table I. Regression equations between mean log reductions of desiccation-adapted *S.* Senftenberg 775/W and presumptive indigenous enterococci or total aerobic bacteria in aged broiler litter and composted turkey litter with 20, 30, 40, and 50% MC at 75 and 85°C

Moisture content (%)	Temperature (°C)	Regression equation with poultry litter	
		Aged broiler litter	Composted turkey litter
20	75	S*=2.63E-1.68	S=2.71E+0.05
	85	S=1.04E+0.31	S=2.38E
30	75	S=1.18E-1.04	S=2.74E-0.29
	85	S=0.77E+0.35	S=1.34E+0.73
40	75	S=2.08E+0.98	S=1.30E-0.29
	85	S=2.89E+1.41†	S=1.20B[a]+4.58†
50	75	S=0.06E-0.06	S=0.31E+0.02
	85	N.A.‡	N.A.

*S, *S.* Senftenberg 775/W; E, enterococci; B, total aerobic bacteria.
†Population data during come-up time was used. ‡N.A., not applicable.

Evaluation of E. faecium *NRRL B-2354 as a surrogate for validating desiccation-adapted* Salmonella *reduction in physically heat-treated poultry litter:* Both desiccation-adapted *S.* Senftenberg 775/W and *E. faecium* NRRL B-2354 were inoculated into aged broiler litter and turkey litter compost with 20, 30, and 40% MC at a final concentration of ca.10^5 cfu/g. The inoculated broiler litter was exposed to 75, 85, or 150°C, while turkey litter compost was exposed to 75 or 150°C. The thermal inactivation experiments were performed as described above. The Weibull, modified Gompertz, and log-logistic models were fitted into the survival curves for modeling the thermal inactivation of *S.* Senftenberg and *E. faecium* NRRL B-2354.

Come-up times for heating poultry litter with different moisture contents at 75 and 85°C ranged from 86 to 150 min (data not shown). During come-up time at 75 and 85°C, the populations of desiccation-adapted *S.* Senftenberg 775/W and *E. faecium* NRRL B-2354 in aged broiler litter decreased in all samples exposed to heat treatment. However, *E. faecium* NRRL B-2354 yielded significantly (P < 0.05) lower average log reductions (24 of the 24 data points) compared with *S*. Senftenberg 775/W, indicating that *S*. Senftenberg 775/W was more heat-sensitive than *E. faecium* NRRL B-2354 under the same treatment condition (P < 0.05) (Figure 5A).

Figure 6A shows the linear regression results between the mean log reductions of *S*. Senftenberg 775/W and *E. faecium* NRRL B-2354 in broiler litter with various moisture contents at 75°C. The log reductions for *E. faecium* NRRL B-2354 were below those for *S*. Senftenberg 775/W, indicating more thermal resistance in *E. faecium* NRRL B-2354 than *Salmonella*. Specifically, during come-up time at 75°C, we observed 4–5.5 log reductions of *Salmonella*, whereas reductions of *E. faecium* NRRL B-2354 were 2.50, 2.75, and 2.96 log cfu/g for 20, 30, and 40% MC, respectively (Figure 7A). At 85°C, regardless of MC, >5-log reductions of *S.* Senftenberg 775/W were observed compared with 1.5–3.75-log reductions of *E. faecium* NRRL B-2354 (Figure 8A).

Significantly more heat resistance of desiccation-adapted *E. faecium* NRRL B-2354 than desiccation-adapted *S.* Senftenberg 775/W was also observed in turkey litter compost with 20, 30, and 40% MC at 75°C. *S.* Senftenberg 775/W was not detectable by enrichment (>5-log reduction) during holding time at 75°C, whereas *E. faecium* NRRL B-2354 could still be detected by direct plating throughout the entire heat treatment (Figure 9).

At 150°C, *E. faecium* NRRL B-2354 survived for up to 15 min in aged broiler litter with 20 and 30% MC, and in turkey litter compost with 20% MC, whereas it was not detectable either in aged broiler litter with 40% MC nor in turkey litter compost with 30 and 40% MC. *S.* Senftenberg could not be detected under all experimental conditions throughout heat treatment (90 min).

Overall, both species were inactivated much faster when temperature and MC of poultry litter were increased (Figure 7A–9A). For example, for the entire heat treatment at 75 and 85°C, the log reductions of desiccation-adapted *E. faecium* NRRL B-2354 in aged broiler litter with 30% MC were 2.89 and 4.02 log cfu/g, respectively, as compared with 3.37 and 4.12 log cfu/g with 40% MC, respectively. After come-up time, the population of *E. faecium* NRRL B-2354 was stabilized around 1.5–2.5 log cfu/g at both 75 and 85°C, indicating no further reduction for all treatments during the extended heat treatment. A similar trend was observed for *E. faecium* NRRL B-2354 survival in turkey litter compost when exposed to 75°C.

Due to the lack of sufficient data points for modeling, inactivation curves of desiccation-adapted *S.* Senftenberg were not fitted to mathematical models. The Gompertz model produced the best description of *E. faecium* NRRL B-2354 inactivation, with mean adjusted R^2 values of 0.970 and 0.991 at 75 and 85°C, respectively (Table 5A). The "*B*" values in the Gompertz model were lower at 75°C than at 85°C in aged broiler litter at the same MC, which suggested a slower population decrease at a lower temperature (Table 6A). The "*C*" values became higher as moisture content increased from 20 to 40%, except at 75°C when no difference was found for "*C*" values between broiler litter with 20 and 30% MC, indicating a higher population reduction at higher moisture content. Clearly, thermal inactivation of the surrogate microorganism can be best predicted by the modified Gompertz model.

Overall, the desiccation-adapted *E. faecium* NRRL B-2354 in both types of poultry litter was more heat resistant at 75, 85, and 150°C as compared to desiccation-adapted *S.* Senftenberg. By summarizing all thermal inactivation data under different combinations of temperature, moisture, and compost types, our results clearly demonstrated that >1.2–2.7 log reductions of *E. faecium* NRRL B-2354 can predict >5 log reductions of *Salmonella*, depending on heating temperature and compost moisture combinations (Table II [broiler litter], Table 7A [turkey litter]). Therefore, ***E. faecium* NRRL B-2354 can be used as a surrogate for *Salmonella* to provide a sufficient safety margin when validating the thermal processing of poultry litter compost.**

Table II. Relationship between *Salmonella* and surrogate reductions during heat treatment at 75 and 85°C in aged broiler litter

Temperature (°C)	MC (%)	*Salmonella* reduction (log CFU/g)	*E. faecium* NRRL B-2354 reduction (log CFU/g)
75	20	5.55	2.65
	30	5.27	2.74
	40	5.44	1.96
85	20	5.62	1.23
	30	5.79	1.27
	40	5.71	1.18

Objective 2: *Validating processes for physically heat-treated poultry litter in industry settings*

For this objective, we teamed up with a turkey litter processor (plant A), broiler litter processor (plant B), and laying hen litter processor (plant C) to validate their heat-treatment processes.

Specifications of industrial dryers and residence time determination: Plant A has a dryer with diameter of 12 ft and length of 50 ft, which had an inlet temperature of ~593°C and an outlet temperature of 65–82°C (Table 1B – see Appendix B). Plant B used a similar dryer, with an inlet temperature of 218–232°C and an outlet temperature of ~74°C. The dryer in plant C has a diameter of 36 inches and length of ~90 inches, with a capacity of 680 kg; the temperature ranged from 65–104°C. All dryers were operated at 13–22 rpm rotation rate.

To determine the residence time for the poultry litter going through the dryer, magnetically retrievable MicroTracer F-Tracers (Micro-Tracers, Inc.) were evaluated. Based on testing results of red, blue, violet, and yellow color-coded F-Tracers in the lab, both red and blue MicroTracers were used to determine the residence time of the dryer in plant A (Figure 1B). Magnetic F-Tracers (1 kg) were mixed well with about 10 kg of turkey compost, and then applied at the inlet of the dryer at once and collected at the exit end at different time intervals for up to 30 min. A total of 3 trials were conducted, using red F-Tracers for trials 1 and 3 and blue F-Tracers for trial 2 in order to differentiate among these three trials. As shown in Figure 2B, the amount of F-Tracers detected in collected samples increased rapidly within 10 min after being added to the dryer and decreased thereafter, suggesting that the average residence time of F-tracers inside the dryer was around 10 min. Due to the small particle sizes (0.1–0.3 mm) of F-tracers as compared with the majority of turkey compost pellets (2 mm), the residence time measured by F-tracers may be significant shorter than the actual residence time for turkey litter compost.

Sampler design: Based on our previous compost study and heat tolerance test, we decided to use a Tyvek® bag to hold the poultry litter samples without leaking out the contents. However, our preliminary study in a rendering processing plant showed the destruction of the bag after going through the industrial dryer. Therefore, sampler systems that can protect the Tyvek bag were designed. Initially, three sampler prototypes (tea infuser, mesh cylinder, and suet wire basket) were tested in plant A. All prototypes were retrieved from the knockout unit (for trapping the stones) immediately after the dryer. Both the tea infuser and mesh cylinder were torn apart, whereas the suet wire basket remained intact. By adding stainless steel mesh as the liner in the basket, the Tyvek bag remained intact after passing through the dryer for at least two transits. The same sampler system was tested and used in plant B. For plant C, instead of using a sampler, a small amount of poultry litter inoculated with desiccation-adapted *E. faecium* NRRL B-2354 was mixed with the bulk of poultry litter in the dryer prior to heat treatment.

Compost inoculation and shipping: One week prior to each plant trial, poultry litter samples were obtained from each processor. Poultry litter samples were prepared and analyzed in duplicate for physical, chemical and microbiological characteristics, as described in Obj.1 (Table 2B). The

rifampin-resistant *E. faecium* NRRL B-2354 was used for the plant validation study. For plant trials conducted at plants A and B, the washed overnight culture was added (1:100, vol/wt) into either partially composted turkey litter (plant A) or broiler litter (plant B), both adjusted with desired moisture levels (within the moisture range for incoming raw litter material), to a final concentration of ca. 10^7 cfu/g. About 50 g of inoculated poultry litter was packed into each Tyvek pouch (5 x 5 in), all sides reinforced with heat-tolerant duct tape. Two Tyvek pouches, one inoculated and one uninoculated, were placed into the customized stainless-steel mesh inside the sampler basket (Figure 3B). For plant trials conducted at plant C, *E. faecium* NRRL B-2354 was added (1:100, vol/wt) into ca. 2,460 g of laying hen litter with ca. 15% MC in the lab to a final concentration of ca.10^9 cfu/g, followed by mixing with sterilized kitchen cloth swatches (ca. 80 pieces for each trial, 1 x 1 in, with ca. 20% MC; Figure 4B) as an indicator for the mixing process. Afterwards, all the prepared samplers (for plants A and B) and inoculated laying hen litter (for plant C) were packaged and shipped overnight to the processing plant at room temperature, allowing the bacterial cells to be desiccation-adapted in the litter. Tyvek bags containing poultry litter with and without inoculation of *E. faecium* NRRL B-2354, in duplicate, served as shipment controls for each trial.

Sample collection and analysis: For plant trials in plants A and B, after sample baskets were dropped into the entry of the dryer, each was retrieved at the exit end of the dryer and the residence time for each sampler was recorded. Compost samples before and after heat treatment were also collected from the processing line to serve as process controls. For the trial in plant C, process control samples were taken from the dryer prior to the addition of inoculated samples. After each pilot run, the distribution of the kitchen cloth swatches from the catch bin was observed to determine if the inoculated sample was sufficiently mixed. Meanwhile, 12 samples from representative locations of the catch bin were collected for sample analysis. Two separate pilot runs were conducted sequentially for each trial. All collected samples were shipped overnight with cold packs to Clemson University, and analyzed immediately, as described in Obj.1. The flow chart for conducting plant trials is shown in Figure 5B.

Validation study conducted at plant A: A total of 4 trials in three different seasons were performed with two separate pilot runs per trial (Table 3B). The residence times of the turkey litter compost samplers inside the dryer for the winter trial (44% MC), spring trial (36% MC), and summer trials (36 and 44% MC) were 28–198, 23–72, 26–60, and 25–59 min, respectively, with the median time as 51, 45, 43, and 35 min, respectively. During the second pilot run of the summer trial (36% MC), there was an 8-min shutdown due to a power outage.

Among all 4 trials, an average >7-log reduction of *E. faecium* NRRL B-2354 in composted turkey litter was achieved in the dryer (Table 3B). However, for the winter trial with 44% MC and summer trial with 36% MC, there were two samples positive for *E. faecium* NRRL B-2354 after enrichment. Those two positive samples were obtained from the samplers exiting from the dryer with the shortest residence times of each trial, i.e., 36 and 28 min, respectively.

The >3-log reductions of presumptive indigenous enterococci were found in both uninoculated samples contained in Tyvek bags and in the finished products collected from the processing line (Table 3B). After heat treatment, in the processing control samples, the average population reductions of indigenous mesophilic counts were 2.22, 2.49, 1.74, and 2.21 log cfu/g, whereas the average population reductions of indigenous thermophiles were 1.08, 1.51, 0.38, and 1.08 for winter trial (44% MC), spring trial (36% MC), and summer trials (36 and 44% MC), respectively (data not shown). Similar population reductions in indigenous microorganisms were found in the samples contained in Tyvek bags. However, the MC reduction was much higher in process controls than in samples contained in Tyvek bags. Additionally, no significant difference ($P > 0.05$) in *E. faecium* NRRL B-2354 counts in turkey litter compost was found among samples analyzed on the initial day, just before dryer and shipment control (4 days).

To best simulate actual conditions in a commercial processing plant, 4 plant trials were conducted in plant A at different seasons, with two moisture levels for the compost samples in each trial. Based on the ANOVA analysis, the moisture content of turkey litter compost (prior to the dryer) and seasonality had no significant effect ($P > 0.05$) on reductions of *E. faecium* NRRL B-2354, but had a significant effect ($P < 0.05$) on presumptive indigenous enterococci, implying indigenous enterococci are inactivated faster in turkey litter compost at lower moisture content than at higher moisture content (Table 4B). In addition to small differences between the two moisture levels tested, this discrepancy between surrogate and indicator microorganisms may be partially explained by variation of bacterial species enumerated as presumptive indigenous enterococci from trial to trial. Based on the data obtained from Obj. 1 (Table 7A), **regardless of the weather or moisture content of turkey litter compost, the validation trials using *E. faecium* NRRL B-2354 and presumptive indigenous enterococci demonstrated that the processing conditions in plant A were adequate to reduce *Salmonella* levels by >5 log.**

Validation study conducted at plant B: Broiler litter with moisture contents of 37 and 42% (two trials with two pilot runs for each) was inoculated with *E. faecium* NRRL B-2354, and the sampler baskets (n = 24) were prepared and shipped as described above. Due to the shortage of available workers in the plant B, only one pilot run for the first trial (37% MC) was conducted as planned, whereas the second pilot run for trial #1 and duplicate runs for trial #2 were left at room temperature for two weeks before thermal treatment. As a result, the chicken litter MC level of 37% in pilot run #2, 42% in pilot run #1, and 42% in pilot run #2 dropped to 18.71, 22.84, and 24.95%, respectively. Therefore, the data of 37% MC litter were presented by separate trial. Data of 42% MC were presented by the mean of two separate runs. For 37% MC trial pilot run #1 (Table 5B), the initial MC level of the chicken litter was 36.31%. *E. faecium* NRRL B-2354 counts decreased by ca. 0.56 log cfu/g during 48-h desiccation adaptation at room temperature.

The residence times of samplers containing chicken litter inside the dryer of 37% MC pilot run #1 were 50–55 min. After heat processing, the average population reductions of *E. faecium* NRRL B-2354 cells in chicken litter with 37% MC pilot run #1, 37% MC pilot run #2, and 42% MC were 2.93, 1.87, and 1.9 log cfu/g, as compared with the average population reductions of presumptive indigenous enterococci of 1.87, 1.73, and 1.25 log cfu/g, respectively, and average population reductions of mesophiles of 1.81, 1.28, and 1.53 log cfu/g, respectively. As for the process control (collected only with 37% MC, pilot run #1), the average population reductions of presumptive indigenous enterococci and mesophiles in the chicken litter were 1.31 and 1.81 log CFU/g, respectively. Although, some unexpected conditions occurred during the plant trials, **our results demonstrated that the processing conditions in plant B were adequate to eliminate *Salmonella* levels by 5 log.**

Validation study conducted at plant C: The initial moisture level of the laying hen litter was 16.98%, and *E. faecium* NRRL B-2354 counts decreased by ca. 2 log cfu/g during 48-h desiccation adaptation at room temperature (Table 6B). As the total weight of the compost in each batch was ca. 680 kg, the expected population of desiccation-adapted *E. faecium* NRRL B-2354 in poultry litter prior to heat treatment was 4.47 log cfu/g. The heat treatment condition in the dryer was ca. 99.44°C for 7 min. The cloth swatches (mixing indicators) were well-distributed, indicating a homogeneous litter mixture during the drying process.

After heat processing, *E. faecium* NRRL B-2354 was detected by enrichment from 14 out of 20 samples. As the detection limit of directly plating was 1.32 log cfu/g, our data showed that an average >3.4-log reduction in surrogate microorganism population was achieved after the physical heat-treatment process in the pilot runs (Table 7B).

For the processing control samples, after heat treatment, the reductions of presumptive indigenous enterococci, mesophiles (total aerobic bacteria), and thermophiles were >1.69, 2.58,

and 2.53 log, respectively (Table 8B). According to our lab results for laying hen litter with ca. 15% MC, a 1.45-log reduction of *E. faecium* NRRL B-2354 at 75°C can predict a >5-log reduction of *Salmonella* (Figure 6B).

Table III summarizes the average reductions of *E. faecium* NRRL B-2354, presumptive indigenous enterococci, and total aerobic bacteria during thermal processing at the three plants. As the processing conditions (temperature, residence time, poultry litter types and moisture, dryer type) varied significantly among the three processing plants, the reductions of the above microorganisms were also quite different. Apparently, plant A's thermal process was more severe than the other two plants. Regardless, **all three plants reduced the surrogate *E. faecium* NRRL B-2354 by >2.9 log, suggesting a >5-log reduction of *Salmonella* if ever present in poultry litter**.

Table III. Reductions of *E. faecium* NRRL B-2354, presumptive indigenous enterococci, and total aerobic bacteria during trials in three poultry litter processing plants

Plant	Trial	Pilot run	log reductions of		
			E. faecium NRRL B-2354	Presumptive indigenous enterococci	Total aerobic bacteria
Plant A	4	8	7.45±0.17	3.74±0.45	1.49±0.34
Plant B*	1	1	2.93	1.87	1.6
Plant C	1	2	3.40±0.34	>1.72±0.40	2.61±0.16

*Samples from three pilot runs were left at room temperature for 2 weeks prior to trials at plant B, therefore the microbial reduction data were not used for the calculation of averages.

Isolation and identification of microbial species from turkey litter compost enumerated on EA or BEA: Both BEA and EA are agars recommended for enumerating enterococci species, but we were interested in learning which species of enterococci and other species can grow on these two selective agars. Figure 7B presents typical colony morphologies on BEA and EA isolated from turkey litter compost. All the isolates picked were purified and characterized by Gram-staining. BHI broth supplemented with 6.5% sodium chloride and bromcresol purple (as a pH indicator) was used for identification of presumptive indigenous enterococci. DNA was extracted from those gram-positive and salt-tolerant cocci using a DNA isolation kit, followed by PCR amplification using enterococcus specific primers Ent1/Ent2 (Table 10B). PCR-positive and some randomly selected interfering isolates from these two agar plates were prepared for the subsequent Sanger Sequencing. Figure 7B illustrates the steps used for identifying presumptive indigenous enterococci.

A total of 479 isolates (260 from EA plates, and 219 from BEA plates) were isolated from the last two highest dilution plates of turkey litter compost (427 isolates from turkey litter compost and 52 from heat-treated turkey litter compost); all isolates were gram-positive. From those 427 isolates, 185 (43%) were able to grow at 45°C in BHI broth containing 6.5% NaCl with color change, and 78 (18%) were further confirmed as Ent1/Ent2 positive by PCR. From those 52 isolates obtained from heat-treated samples, all were characterized as *Bacillus* by Gram-staining. Compared with BEA, a high percentage (56%) of isolates from EA was considered as presumptive enterococci (Table 11B). Overall, 39% of isolates grown on BEA and EA are presumptive enterococci based on microscopic and growth observations, while only 16% were confirmed by PCR assay.

Those PCR-positive isolates and 24 randomly selected interfering isolates were further identified to species levels by 16S rRNA sequencing. The preliminary results are presented in

Table 12B. In the turkey litter prior to heat treatment, 29 isolates (37.18% from the total sequenced isolates) were identified as enterococci, including *E. gallinarum*, *E. hirae*, and *E. faecalis*. *Aerococcus viridans* was previously found as the most abundant, dominant species in poultry litter. Similar to enterococci, *Aerococcus* is also bile-esculin positive when grown on BEA and EA. Clearly, the presumptive indigenous enterococci counts enumerated on EA or BEA include multiple species of enterococci and other species as well. As poultry litter compost is a complex matrix, the difficulty in differentiating interfering microorganisms from enterococcal isolates phenotypically was not unexpected. Furthermore, in the turkey litter after heat treatment, the spore-forming *Bacillus* spp. was found to survive the heat treatment, suggesting a further study of investigating its capability to serve as a competitive exclusion strain against plant or human pathogens. Overall**, all enterococci isolates were obtained by using EA, indicating EA is a better selective agar as compared to BEA for poultry litter sample analysis.** However, EA has limitations in not being able to exclude other interfering species.

Application of denaturing gradient gel electrophoresis (DGGE) to the analysis of microbial communities of poultry litter compost: Genomic DNA samples were extracted from partially composted turkey litter (plant A), raw chicken litter (plant B), and heat-treated partially composted turkey litter (plant A) using a DNA extraction kit. The nested PCR procedure to amplify bacterial 16s rRNA genes was used to obtain a highly specific PCR product. The primer sets used were 27F and 907R for the first PCR, and 341F and 517R for the second PCR (Table 10B). DGGE analysis of the amplified bacterial 16S rRNA gene was performed on the DCode Universal Mutation Detection System (Bio-Rad, USA). Polyacrylamide gels (7% w/v) containing a linear formamide/urea gradient ranging from 30% to 60% denaturant were used. The gels were run for 6 h at 60V and 60°C.

As shown in Figure 8B, the DGGE patterns of the two different poultry litter composts were distinctly different, with only a few common bands. As compared to the microbial community in the heat-treated turkey litter compost profiling by DGGE, there were more bands and complexity in the turkey litter compost without heat treatment. Our results showed a higher resolution when using nested PCR protocols. Microbial community profiling by DGGE was drastically different among poultry litter samples. In agreement with results from the plate counting method, the reduced bands in the heat-treated turkey litter confirmed the reduction of microbial species and population level in compost due to heat exposure.

Microbial community profile of turkey litter compost using 16S rRNA and 18S rRNA sequencing: Turkey litter compost samples prior to and after dryer treatment were sourced from plant A. Before genomic DNA extraction, DNA from the dead cells (or no longer intact cells) was removed by the photo-reactive viability PCR indicator propidium monoazide (PMA), followed immediately by DNA extraction using Qiagen PowerFecal extraction kit. The extracted DNA was checked by Nanodrop 2000 and Qubit. The universal primers were used for PCR amplification of specific variable regions of the prokaryotic 16S rDNA and eukaryotic 18S rDNA (Table 10B). Amplicons were barcoded and processed through an Illumina library preparation protocol and sequenced on an Illumina HiSeq (2500) sequencer using a 2x250bp sequencing chemistry.

Figure 9B presents the PCR amplification of specific variable regions of the prokaryotic 16S rDNA and eukaryotic 18S rDNA, which showed the good quality of the DNA. Based on metagenomic sequencing analysis, the top-10 dominant bacterial and fungal genera found in turkey litter compost prior to and after heat treatment are listed in Table 13B. The most abundant communities (i.e., *Bacillaceae*) decreased and species evenness became more distinct in the turkey litter after heat treatment, indicating the different effects of heat treatment on the microbial community. Also, the changes in the community abundance, as shown in Figure 10B, indicated that DNA from dead cells was removed by application of PMA treatment.

Outcomes and Accomplishments

Objective 1: *Select avirulent* Salmonella *surrogates and indicator microorganisms for process validation of physically heat-treated poultry litter.*

The thermal resistance of desiccation-adapted *S.* Senftenberg 775/W was compared with those of desiccation-adapted *E. faecium* NRRL B-2354 as a surrogate for *Salmonella*, and with presumptive indigenous enterococci and total aerobic bacteria in poultry litter with 20, 30, 40 and 50% MC when exposed to 75, 85, or 150°C. As compared to total aerobic bacteria, there were better correlations between mean log reductions of desiccation-adapted *S.* Senftenberg and presumptive indigenous enterococci in both broiler and turkey litter samples with different moisture contents at most treatment conditions. The desiccation-adapted *E. faecium* NRRL B-2354 was more heat resistant than desiccation-adapted *S.* Senftenberg 775/W ($P < 0.05$) in the poultry litter with 20, 30, and 40% MC, at all tested temperatures. Overall, the data generated from modeling clearly revealed that microbial cells were inactivated much faster when the temperature and moisture content of poultry litter were increased. Based on thermal inactivation data, we can conclude that 1.2–2.7 log or more reductions of *E. faecium* NRRL B-2354 may predict >5-log reductions of *Salmonella* in poultry litter, depending on heating temperature, moisture content, and type of poultry litter.

Objective 2: *Validate thermal processes for physically heat-treated poultry litter in industrial settings (turkey litter and chicken litter processing plants) using* Salmonella *surrogate and indicator microorganisms.*

We designed a multiple component sampler that can carry the inoculated samples to pass through the industrial dryers and withstand the harsh conditions inside, and at the same time measure the residence time more accurately than using magnetically retrievable MicroTracers. As compared with bile esculin agar, enterococcosel agar was better for enumeration of presumptive indigenous enterococci from poultry litter samples due to less growth of interfering species; however, some bacterial species besides enterococci could grow on enterococcal selective agar plates. By working with our industry collaborators, we conducted 7 plant trials throughout the project to validate the thermal process in one turkey litter compost processing plant and two chicken litter processing plants using indicator microorganisms and litter inoculated with *E. faecium* NRRL B-2354 as the *Salmonella* surrogate. The thermal processes in all three plants yielded 2.93–7.45, >1.72–3.74, and 1.49–2.61 log CFU/g reductions of *E. faecium* NRRL B-2354, presumptive indigenous enterococci, and total aerobic bacteria, respectively. For plant A, both seasonality and moisture content of turkey litter compost prior to the dryer had no significant effect ($P > 0.05$) on the reduction of *E. faecium* NRRL B-2354 but did have a significant effect ($P < 0.05$) on the reduction of presumptive indigenous enterococci, probably due to the variation of presumptive indigenous enterococci species in compost from trial to trial. Based on thermal inactivation comparison data collected in the Obj. 1 experiments, the processing conditions in these plants are adequate to reduce *Salmonella* levels by at least 5 log, even though the processing conditions varied among trials. Additionally, the microbial community profiles varied significantly among different types of poultry litter, and the thermal process reduced 95 to 99.9% of total aerobic bacterial populations in poultry litter/composts. The metagenomic sequencing analysis revealed a significant decrease of the most abundant communities (i.e., *Bacillaceae*) and that species evenness became more distinct in the turkey litter after heat treatment, indicating that heat treatment had different effects on the microbial community.

Summary of Findings and Recommendations

Objective 1: *Select avirulent* Salmonella *surrogates and indicator microorganisms for process validation of physically heat-treated poultry litter.*

Our results clearly demonstrated that presumptive indigenous enterococci and desiccation-adapted *E. faecium* NRRL B-2354 were more heat resistant at 75, 85, and 150°C as compared to desiccation-adapted *S.* Senftenberg 775/W in both turkey and chicken litter/compost, suggesting the use of these indicator and surrogate microorganisms can provide sufficient safety margin when validating the thermal processing of poultry litter. However, the thermal resistance of these microorganisms is affected by heating temperature, moisture content of poultry litter, and other factors. As these thermal inactivation data were collected under controlled experimental conditions in the lab, the effectiveness of using these as surrogate and indicator microorganisms should be validated in a commercial thermal processing plant of poultry litter under various processing conditions.

Objective 2: *Validate thermal processes for physically heat-treated poultry litter in industrial settings (turkey litter and chicken litter processing plants) using* Salmonella *surrogate and indicator microorganisms.*

We have used *E. faecium* NRRL B-2354 and presumptive indigenous enterococci in successfully validating the thermal processes of three poultry litter plants under different seasons. Even though the processing conditions in these plants varied greatly, the validation results showed that *Salmonella* levels could be reduced by at least 5 log, based on the reductions of surrogate and indicator microorganisms. The custom-designed sampler basket was capable of withstanding the harsh environment (high temperature, strong physical forces) inside the industrial dryers, and provided a carrier for the inoculated poultry litter samples to be exposed to the actual thermal process inside the dryer. Therefore, both the indicator and surrogate microorganisms along with the custom-designed sampler can be practical tools for poultry litter processors to routinely monitor or validate their thermal processes without introducing pathogens into the industrial environment. In consideration of the low cost to enumerate indigenous enterococci, poultry litter processors could do routine monitoring of their thermal process by using indicator microorganisms if minor changes occur to their processing parameters. Due to the fluctuation of indigenous enterococci populations in animal waste–based soil amendments, the use of spiking with *E. faecium* NRRL B-2354 is preferable for a validation study. Additionally, the microbial community analysis of poultry litter products will expand our knowledge on microbial community structure and functions, and identify potential beneficial microorganisms for agricultural applications. Overall, our findings from this study will assist poultry litter processors in optimizing their existing process parameters to produce *Salmonella*-free physically heat-treated poultry litter, which can be used by the produce industry to grow microbiologically safe products.

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APPENDICES

Publications and Presentations

Publications:

1. Chen, Z. and X. Jiang. 2017. Microbiological safety of animal wastes processed by physical heat treatment: An alternative to eliminate human pathogens in biological soil amendments as recommended by the food safety modernization act. J. Food Prot. 80:392-405.
2. Chen, Z. and X. Jiang. 2017. Selection of indigenous indicator microorganisms for validating desiccation-adapted *Salmonella* reduction in physically heat-treated poultry litter. J. Appl. Microbiol. 122(6):1558-1569. doi: 10.1111/jam.13464
3. Chen, Z. and X. Jiang. 2017. Thermal resistance and gene expression of both desiccation-adapted and rehydrated *Salmonella enterica* Typhimurium in aged broiler litter. Appl. Environ. Microbiol. 83(12):e00367-17. doi: 10.1128/AEM.00367-17
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5. Chen, Z., J. Kim, and X. Jiang. 2018. Survival of *Escherichia coli* O157:H7 and *Salmonella enterica* in animal wastes-based composts as influenced by compost type, storage condition, and inoculum level. J. Appl. Microbiol. (online 6 February) doi: 10.1111/jam.13719
6. Wang, H., Z. Chen, M. Li., X. Jiang, and J. Wang. Selection of surrogate microorganism for validating desiccation-adapted *Salmonella* reduction in physically heat-treated broiler litter. J. Food Prot. (in submission)

Presentations:

1. Jiang, X. and A. Greene. 2016. Validating a physically heat-treated process for poultry litter in industry settings using the avirulent *Salmonella* surrogates or indicator microorganisms. 7th Annual CPS Produce Research Symposium, June 28-29, Seattle, WA.
2. Wang, H., Z. Chen, C. Gong and X. Jiang. 2016. Thermal inactivation of avian virus surrogates in aged chicken litter. IAFP Annual Meeting, St. Louis, MO.
3. Chen, Z. and X. Jiang. 2016. Selection of indigenous indicator microorganisms for validation of desiccation-adapted *Salmonella* reduction in physically heat-treated poultry litter. IAFP Annual Meeting, St. Louis, MO.
4. Jiang, X. and A. Greene. 2017. Validating a physically heat-treated process for poultry litter in industry settings using the avirulent *Salmonella* surrogates or indicator microorganisms. 8th Annual CPS Produce Research Symposium, June 20-21, Denver, CO.
5. Wang, H., Z. Chen, and X. Jiang. 2017. Optimizing methods for recovering heat-injured *Enterococcus faecium* and indigenous enterococci in turkey litter compost. IAFP Annual Meeting, Tampa, FL, 7/9-12.
6. Wang, H., Z. Chen, M. Dharmasena, M. Li, A. Greene, B.M. Gardener, B. Holden, J. Wang and X. Jiang. 2018. Thermal inactivation of *Salmonella* surrogate and indicator microorganisms in turkey litter compost during physical heat-treatment process - A plant validation study. IAFP Annual Meeting, Salt Lake City, UT, 7/8-11 (submitted).

Budget Summary

The funds provided by CPS were adequate for us to carry out the project. The approximate breakdown of the grant funds spent by category is:

Grad	$66,792
Wages	$15,459
Fringe	$8,725
GAD	$28,686
Travel	$12,198
Other	$52,883
Indirect	$4,549
Total	$189,292

Travel funds remaining ($3,307) will be used to attend the 2018 CPS Research Symposium.

Suggestions to CPS

We enjoyed the close contact with CPS, and all those activities such as attending research symposiums and making industry contacts, which helps us to refine our research approaches in order to develop effective solutions for the produce industry.

Tables and Figures

Please see **Appendix A** & **B** in following pages:

APPENDIX A: Table 1A–7A and Figures 1A–9A

Table 1A. Comparison of recovery media for heat-injured *E. faecium* NRRL B-2354 in composted turkey litter

Recovery media	Population of *E. faecium* NRRL B-2354 (log cfu/g) after exposure to 75°C		
	0 h	0.5 h	1 h
EA-R	8.21±0.13a [a]	3.97±0.34c	<2
BEA-R	7.34±0.79b	3.27±0.20b	<2
OV/EA-R	**-**	**5.09±0.33a**	**<2**
OV/BEA-R	-	4.74±0.49b	<2
TAL/EA-R	-	4.75±0.24b	<2
TAL/BEA-R	-	4.16±0.83c	<2

[a] Data are expressed as mean±SD of three trials. Means with different letters in the same column are significantly different ($P < 0.05$).

Table 2A. Chemical characteristics of aged broiler litter and composted turkey litter*

Poultry litter	Nutrient (%)									
	OM†	C	N	C/N	P	K	Ca	Mg	S	Na
Broiler litter	67.10± 1.41a	34.26± 0.36a	3.75± 0.01a	9.13± 0.13a	2.77± 0.13b	2.89± 0.14b	2.56± 0.10b	0.56± 0.02b	1.06± 0.04a	0.91± 0.04a
Turkey litter	41.40± 2.69b	25.37± 0.46b	2.97± 0.05b	8.56± 0.01b	5.43± 0.18a	4.06± 0.19a	5.31± 0.29a	1.10± 0.07a	0.85± 0.03b	0.68± 0.03b

	Heavy metal (μg g^{-1})					pH	EC‡ (ms cm^{-1})	NH_4-N (μg g^{-1})
	Zn	Cu	Mn	Fe	Al			
Broiler litter	439.00± 21.21b	250.50± 14.85b	472.00± 21.21b	3103.00± 104.65b	2710.00± 11.31b	8.70± 0.00a	14.84± 0.22a	820.35± 67.37a
Turkey litter	488.50± 23.33a	626.00± 35.36a	592.00± 22.63a	6880.00± 429.21a	4526.50± 246.78a	8.00± 0.00b	13.31± 0.06b	591.26± 25.61b

*Data are expressed as mean±SD of two samples. Means with different letters in the same column are significantly different ($P < 0.05$). The values of nutrients and metals are all calculated based on dry weight.

†OM, organic matter.

‡Electrical conductivity.

Table 3A. Come-up times for aged broiler litter and composted turkey litter at 75 and 85°C

Moisture content (%)	Temperature (°C)	Come-up time (h) with poultry litter	
		Aged broiler litter	Composted turkey litter
20	75	0.95	0.85
	85	0.88	0.82
30	75	1.38	1.52
	85	1.27	1.35
40	75	2.05	2.07
	85	1.70	1.77
50	75	2.48	3.12
	85	2.00	3.00

Table 4A. Regression correlations between mean log reductions of desiccation-adapted *S.* Senftenberg 775/W and indigenous enterococci or total aerobic bacteria in aged broiler litter and composted turkey litter with 20, 30, 40, and 50% MC at 75 and 85°C

Moisture content (%)	Temperature (°C)	Microorganism	Regression correlation (R^2) with poultry litter	
			Aged broiler litter	Composted turkey litter
20	75	Enterococci	0.91±0.03a*	0.89±0.02a
		Total aerobic bacteria	0.84±0.02b	0.81±0.03b
	85	Enterococci	0.98±0.02a	0.94±0.05a
		Total aerobic bacteria	0.91±0.03b	0.63±0.07b
30	75	Enterococci	0.91±0.04a	0.89±0.02a
		Total aerobic bacteria	0.75±0.05b	0.87±0.02a
	85	Enterococci	0.87±0.04a	0.83±0.06a
		Total aerobic bacteria	0.80±0.01b	0.45±0.05b
40	75	Enterococci	0.93±0.06a	0.88±0.02a
		Total aerobic bacteria	0.62±0.05b	0.83±0.02b
	85	Enterococci	0.98±0.03a†	0.88±0.03b†
		Total aerobic bacteria	0.91±0.03b†	0.98±0.04a†
50	75	Enterococci	0.94±0.02a	0.92±0.02a
		Total aerobic bacteria	0.86±0.03b	0.90±0.01a
	85	Enterococci	N.A.‡	N.A.
		Total aerobic bacteria	N.A.	N.A.

*Data are expressed as mean±SD of two trials. For each temperature, means with different letters in the same column are significantly different ($P < 0.05$).
†Population data during come-up time was used.
‡N.A., not applicable.

Table 5A. Goodness-of-fit of the Weibull, modified Gompertz, and log-logistic models for the survival curves of desiccation-adapted *E. faecium* NRRL B-2354 in aged broiler litter with 20, 30, and 40% MC at 75 and 85°C

Temperature (°C)	Moisture content (%)	Adjusted R^2 with following models.		
		Weibull	Modified Gompertz	Log-logistic
75	20	0.911	0.992	0.991
	30	0.911	0.964	0.960
	40	0.924	0.954	0.948
85	20	0.931	0.983	0.872
	30	0.870	0.997	0.998
	40	0.928	0.992	0.987

Table 6A. Parameters of the Weibull, modified Gompertz, and log-logistic models for the survival curves of desiccation-adapted *E. faecium* NRRL B-2354 in aged broiler litter with 20, 30, and 40% MC at 75 and 85°C

Temperature (°C)	Moisture content (%)	Parameters		
		Weibull	Modified Gompertz	Log-logistic
75	20	b=0.501, n=0.326	M=28.358, B=0.035, C=3.009	A=2.887, σ=-3.739, τ=1.615
	30	b=0.246, n=0.439	M=39.471, B=0.024, C=3.005	A=2.947, σ=-3.245, τ=1.792
	40	b=0.333, n=0.396	M=-27.8891, B=0.015, C=6.375	A=3.404, σ=-2.768, τ=1.809
85	20	b=0.646, n=0.299	M=23.127, B=0.037, C=3.465	A=6.955, σ=-1.453, τ=2.672
	30	b=0.929, n=0.275	M=32.035, B=0.054, C=3.977	A=4.009, σ=-7.044, τ=1.584
	40	b=0.479, n=0.385	M=26.223, B=0.021, C=4.954	A=4.337, σ=-4.352, τ=1.773

Table 7A. Relationship between *Salmonella* and surrogate reductions during heat treatment at 75°C in turkey litter compost

Temperature (°C)	MC (%)	*Salmonella* reduction (log CFU/g)	*E. faecium* NRRL B-2354 reduction (log CFU/g)
75	20	4.28	1.32
	30	5.20	2.20
	40	5.54	1.83

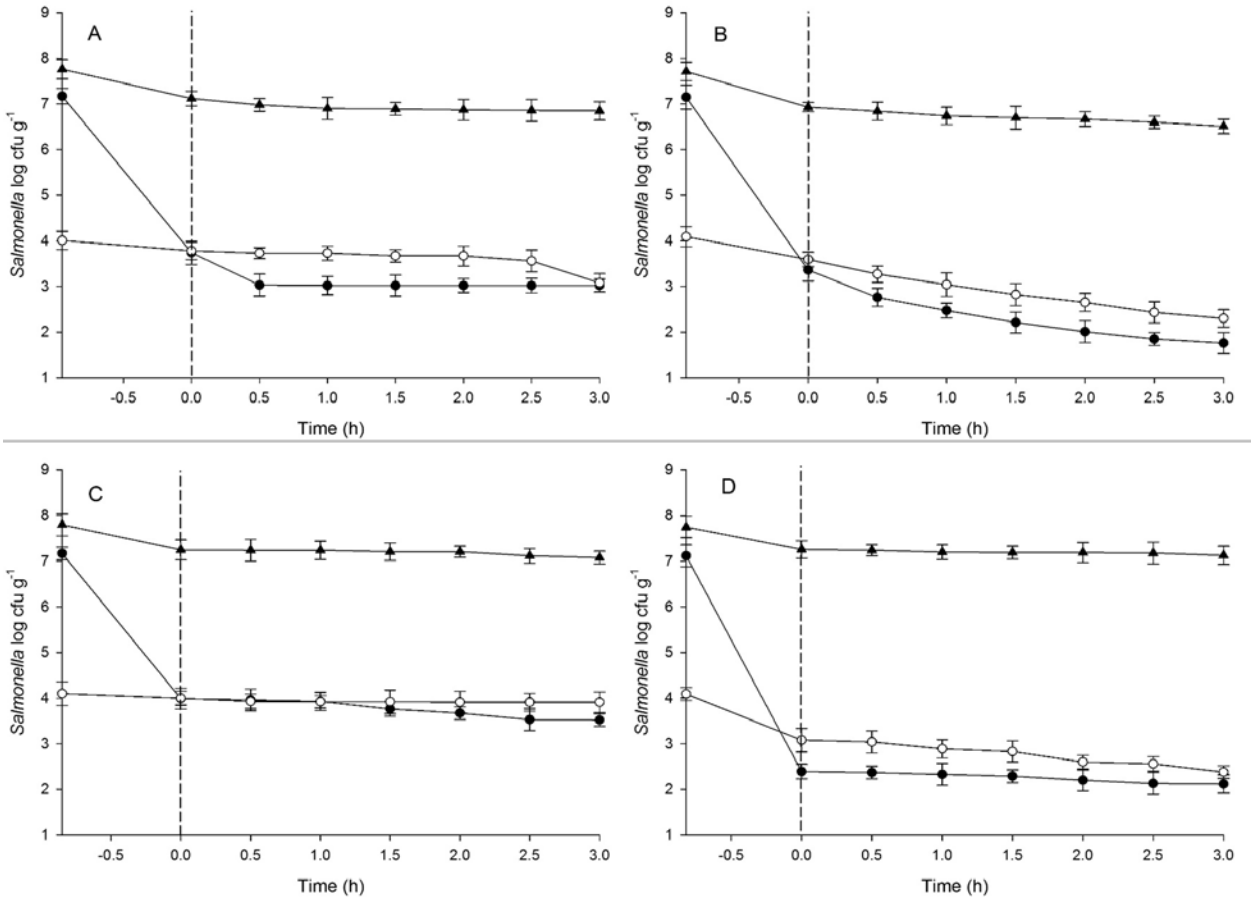


Figure 1A. Survival of desiccation-adapted *S.* Senftenberg 775/W (solid circle), indigenous enterococci (hollow circle), and total aerobic bacteria (solid triangle) in aged broiler litter and composted turkey litter with 20% moisture content at 75 and 85°C. A: aged broiler litter at 75°C; B: aged broiler litter at 85°C; C: composted turkey litter at 75°C; D: composted turkey litter at 85°C. Inactivation curves during come-up times (on the left of the vertical dashed line) and during holding times (on the right of the vertical dashed line) are shown.

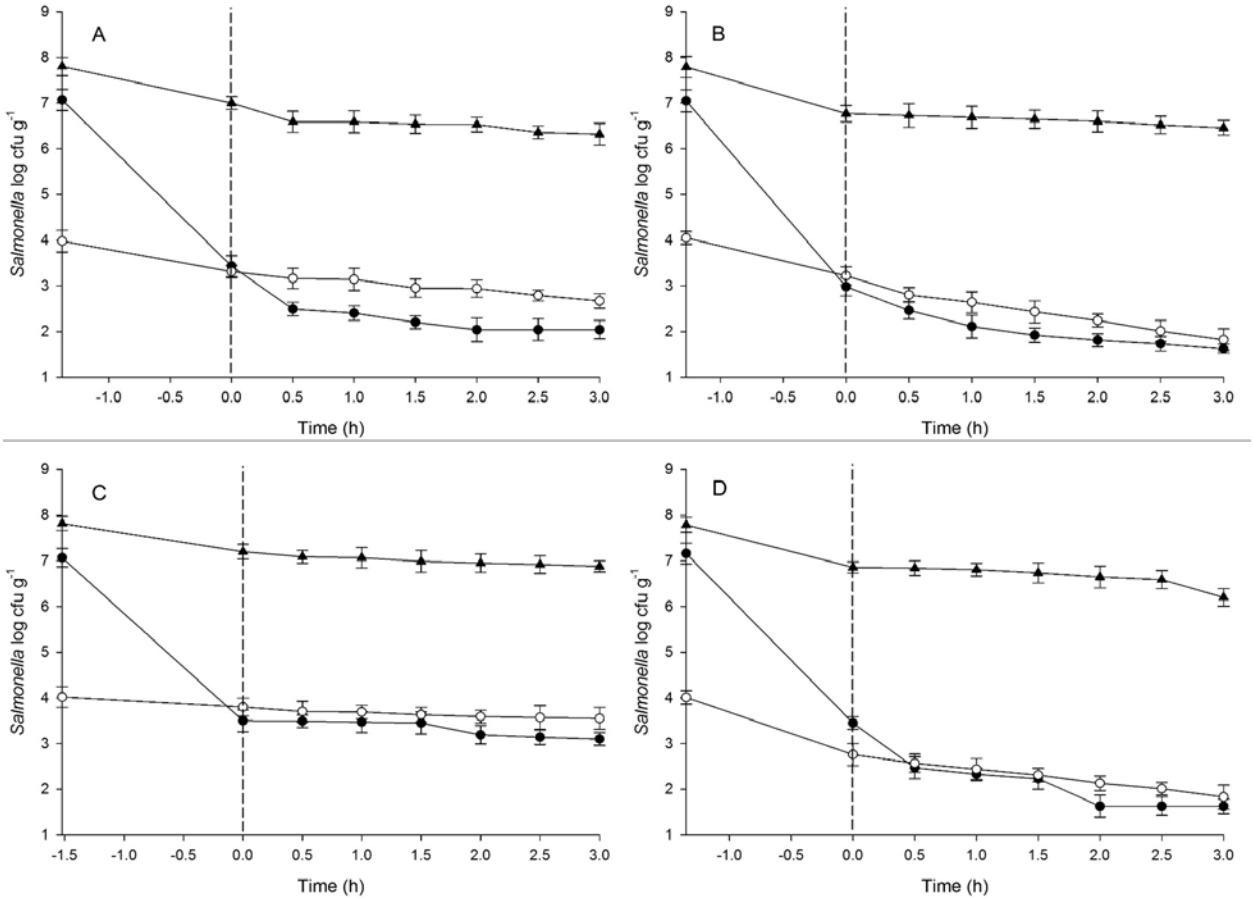


Figure 2A. Survival of desiccation-adapted *S.* Senftenberg 775/W (solid circle), indigenous enterococci (hollow circle), and total aerobic bacteria (solid triangle) in aged broiler litter and composted turkey litter with 30% moisture content at 75 and 85°C. A: aged broiler litter at 75°C; B: aged broiler litter at 85°C; C: composted turkey litter at 75°C; D: composted turkey litter at 85°C. Inactivation curves during come-up times (on the left of the vertical dashed line) and during holding times (on the right of the vertical dashed line) are shown.

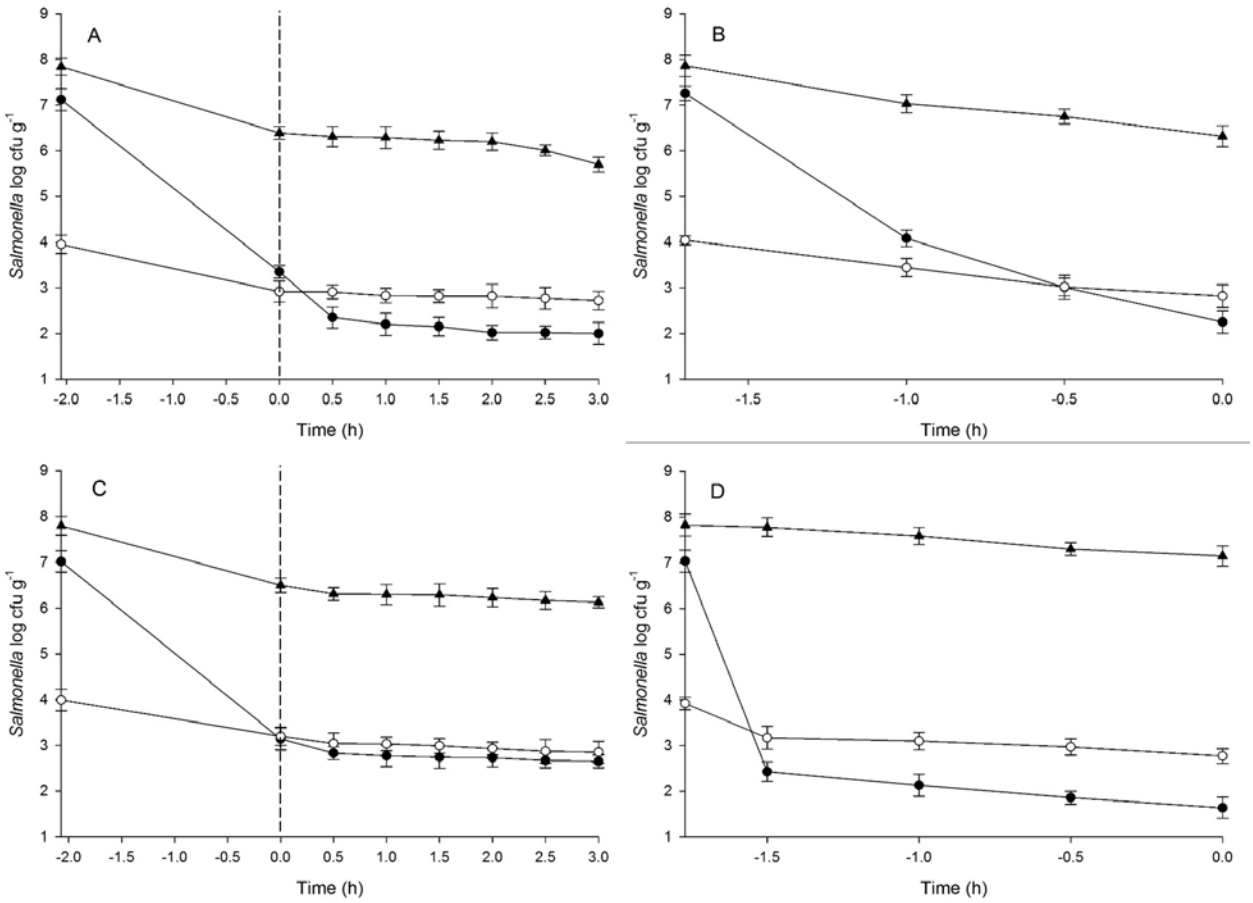


Figure 3A. Survival of desiccation-adapted *S.* Senftenberg 775/W (solid circle), indigenous enterococci (hollow circle), and total aerobic bacteria (solid triangle) in aged broiler litter and composted turkey litter with 40% moisture content at 75 and 85°C. A: aged broiler litter at 75°C; B: aged broiler litter at 85°C; C: composted turkey litter at 75°C; D: composted turkey litter at 85°C. Inactivation curves during come-up times (on the left of the vertical dashed line) and during holding times (on the right of the vertical dashed line) are shown.

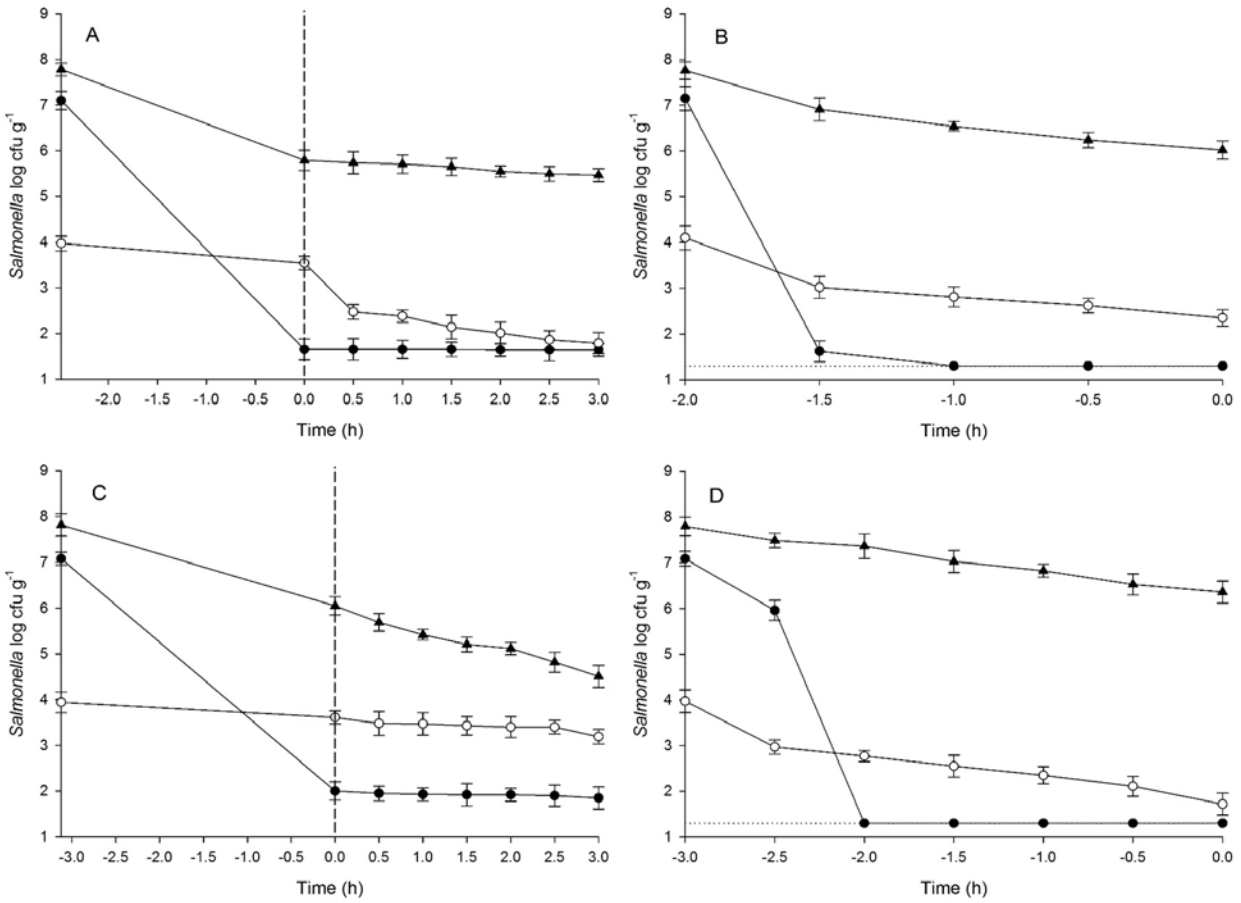


Figure 4A. Survival of desiccation-adapted *S.* Senftenberg 775/W (solid circle), indigenous enterococci (hollow circle), and total aerobic bacteria (solid triangle) in aged broiler litter and composted turkey litter with 50% moisture content at 75 and 85°C. A: aged broiler litter at 75°C; B: aged broiler litter at 85°C; C: composted turkey litter at 75°C; D: composted turkey litter at 85°C. Inactivation curves during come-up times (on the left of the vertical dashed line) and during holding times (on the right of the vertical dashed line) are shown. The horizontal dotted line indicates that Salmonella was detectable only by enrichment (detection limit by direct plating: 1.30 log cfu/g).

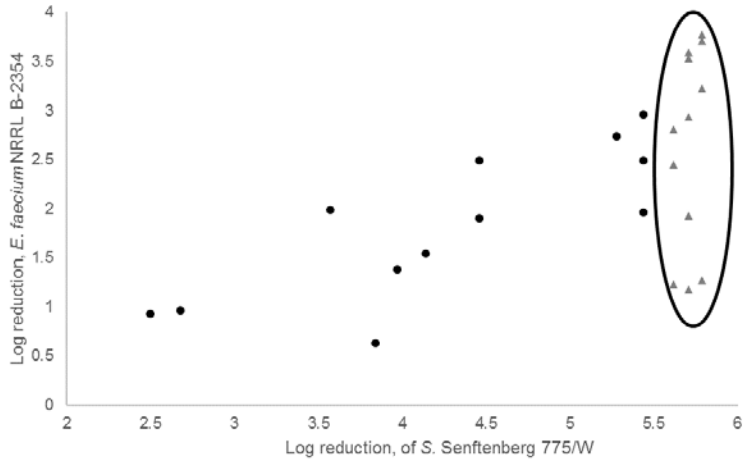


Figure 5A. Direct comparison between the mean log reductions for desiccation-adapted *S.* Senftenberg 775/W and *E. faecium* NRRL B-2354 in aged broiler litter with 20, 30, and 40% MC at 75°C (dotted data points outside the circle) and 85°C (triangle data points in the circle) (n=24). Each treatment was conducted with two separate trials.

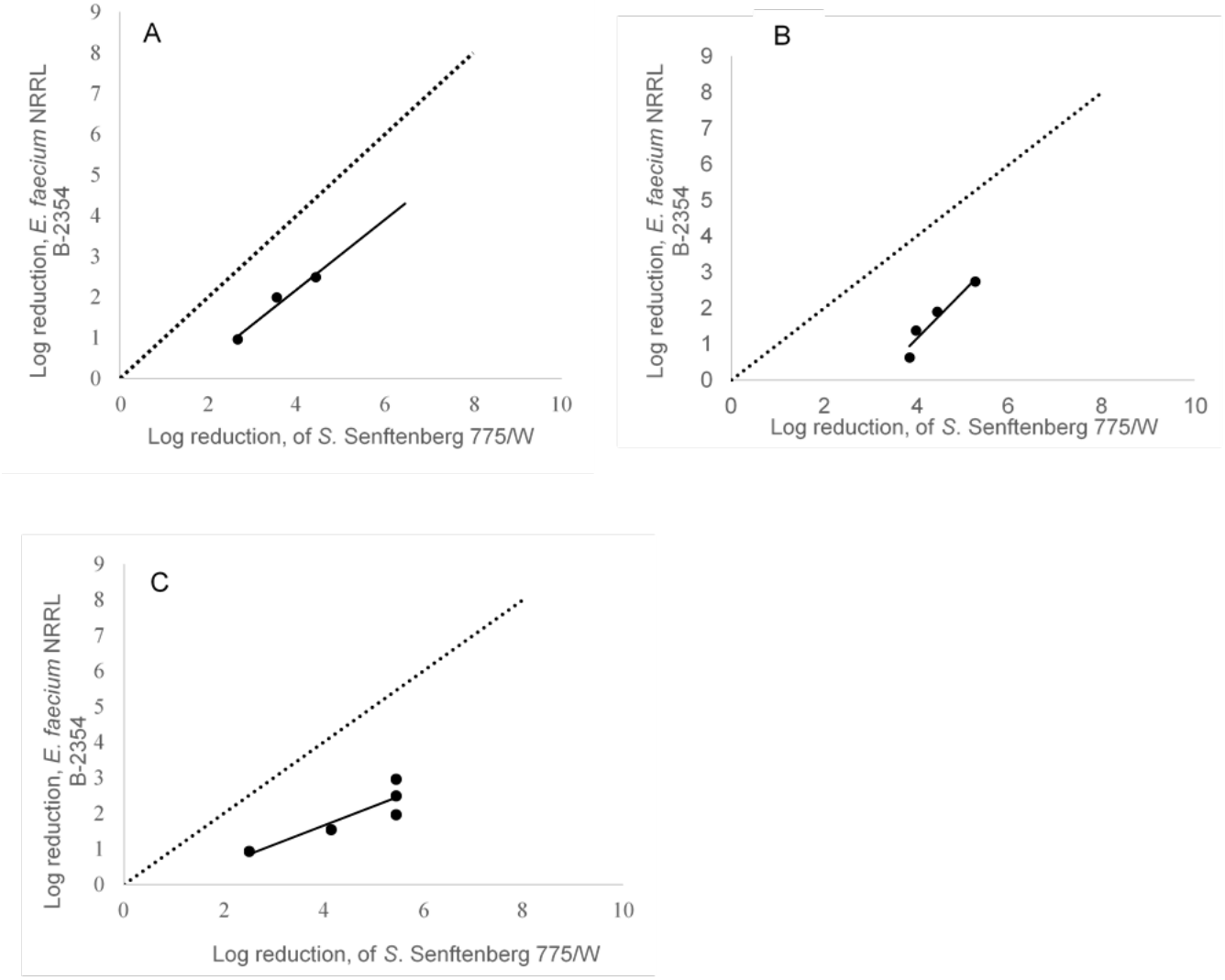


Figure 6A. Linear regression results between the mean log reductions of desiccation-adapted *S.* Senftenberg 775/W and *E. faecium* NRRL B-2354 in aged broiler litter with 20% (A), 30% (B), and 40% (C) MC at 75°C. The dotted line showed the 1:1 standard line, and the line connecting data points indicates the linear regression between the surrogate microorganism and the target pathogen.

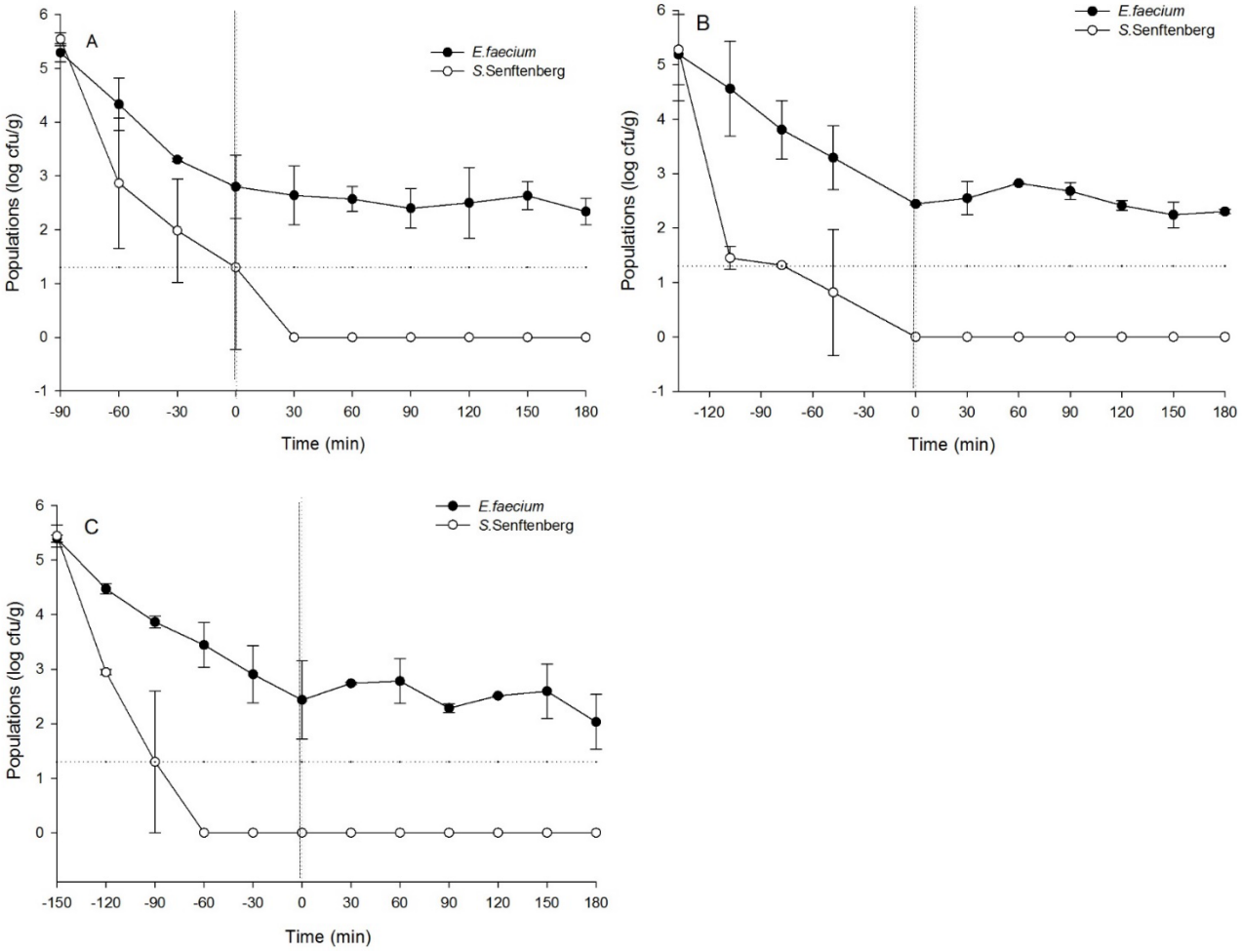


Figure 7A. Survival of desiccation-adapted *S*. Senftenberg 775/W and *E. faecium* NRRL B-2354 in aged broiler litter with 20% (A), 30% (B), and 40% (C) MC at 75°C. Inactivation curves during come-up times (on the left of the vertical dotted line) and during holding times (on the right of the vertical dotted line) are shown. The horizontal dotted line indicates that Salmonella was detectable only by enrichment (detection limit by direct plating: 1.30 log cfu/g).

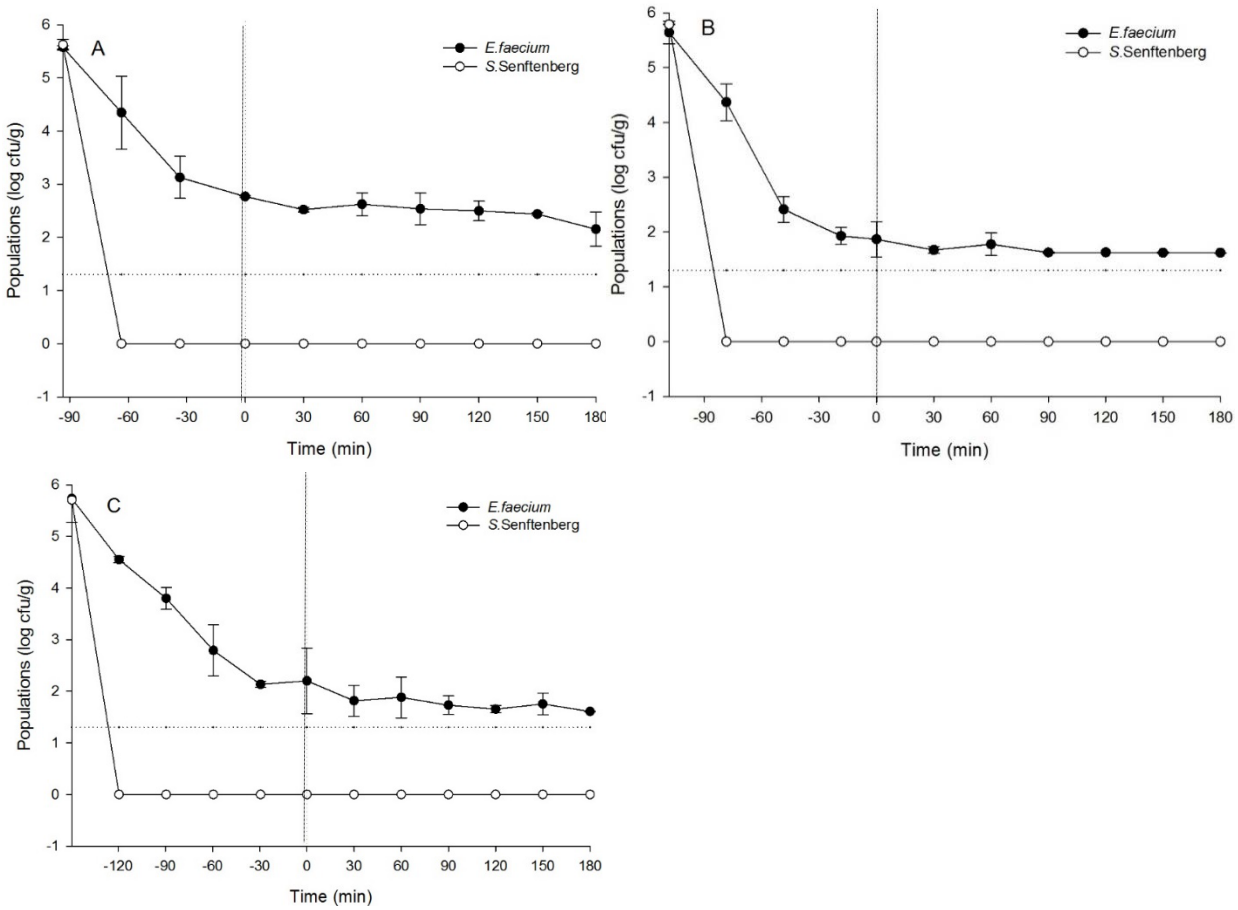


Figure 8A. Survival of desiccation-adapted *S.* Senftenberg 775/W and *E. faecium* NRRL B-2354 in aged broiler litter with 20% (A), 30% (B), and 40% (C) MC at 85°C. Inactivation curves during come-up times (on the left of the vertical dotted line) and during holding times (on the right of the vertical dotted line) are shown. The horizontal dotted line indicates that *Salmonella* was detectable only by enrichment (detection limit by direct plating: 1.30 log cfu/g).

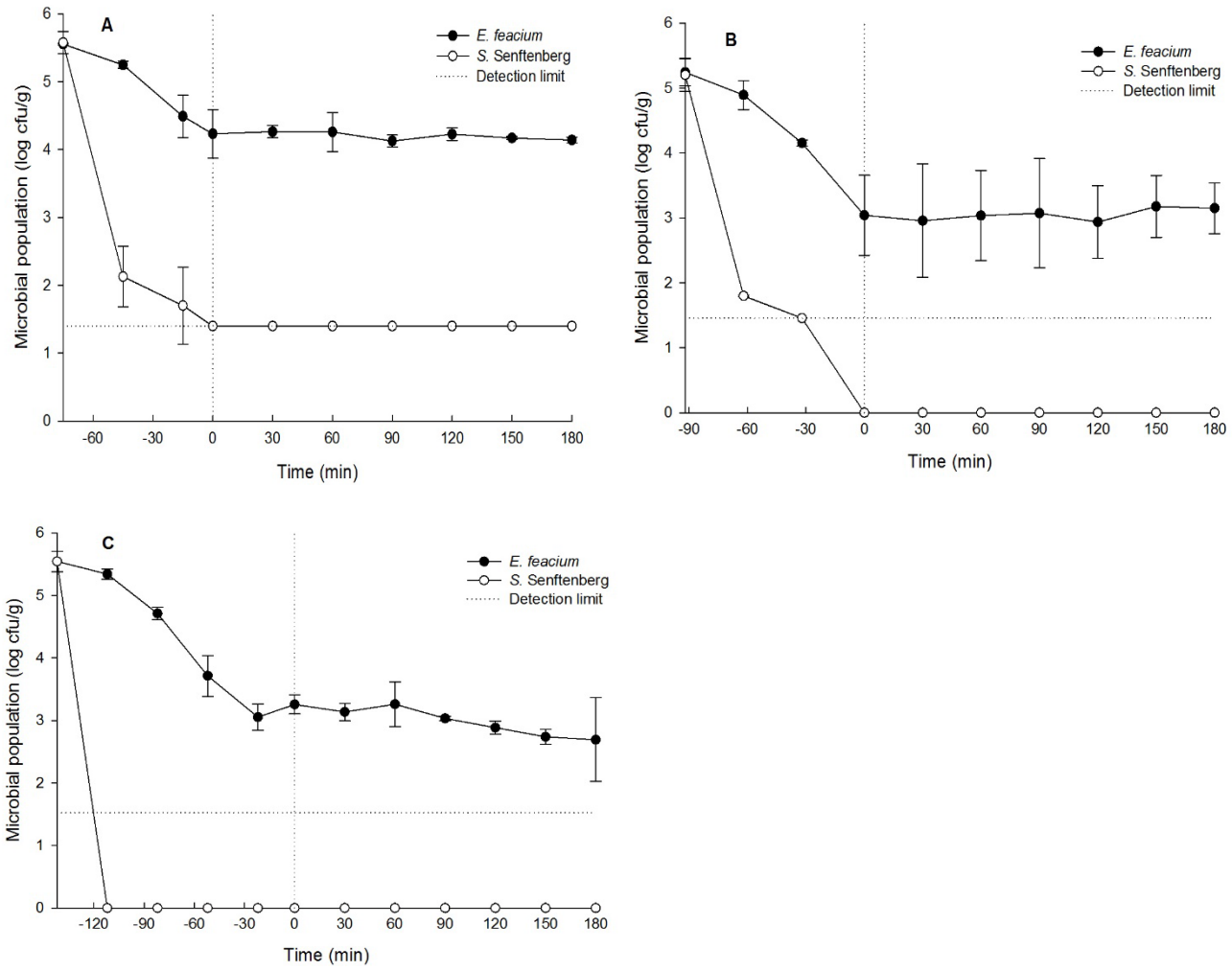


Figure 9A. Survival of desiccation-adapted *S.* Senftenberg 775/W and *E. faecium* NRRL B-2354 in turkey litter compost with 20% (A), 30% (B), and 40% (C) MC at 75°C. Inactivation curves during come-up times (on the left of the vertical dotted line) and during holding times (on the right of the vertical dotted line) are shown. The horizontal dotted line indicates that *Salmonella* was detectable only by enrichment (detection limit by direct plating: 1.30 log cfu/g).

Appendix B: Table 1B–13B and Figure 1B–10B

Table 1B. Detailed information for validation trials at Plant A, B, and C

Plant	Dryer conditions			Target moisture content (%)	Date Performed	Total samples analyzed after heat treatment	Processing controls collected
	Size	Temperature	Process capacity				
Plant A	12 ft diameter 50 ft length	Inlet: 593ºC Outlet: 65–82ºC	4.5 tons/h	44	November 16, 2016	22	4
					July 19 and Aug 8, 2017	24	4
				36	April 28, 2017	30	4
					June 29, 2017	24	4
Plant B	12 ft, 50 ft length	Inlet: 232ºC Outlet: 74ºC	300 tons/day	37	March 9 and 27, 2017	20	1
				42	March 27, 2017	20	N.C.[a]
Plant C	36 inch diameter, ca. 90 inch length	65–104ºC	16–17 tons/day	15	December 13, 2017	24	2

[a] N.C, not collected; process control was not collected by the industry collaborator.

Table 2B. Chemical characteristics of poultry litter received before plant studies

Sample	Nutrient (%)									
	OM[†]	C	N	C/N	P	K	Ca	Mg	S	Na
Plant A (11/16)	58.00± 0.28	30.42± 0.39	3.24± 0.10	9.40± 0.18	4.59± 0.21	4.19± 0.05	4.76± 0.01	1.04± 0.06	0.98± 0.01	5588.50± 92.63
Plant A (4/17)	53.95 ±0.64	29.11 ±0.28	2.95 ±0.00	9.86 ±0.09	5.64 ±0.16	4.54 ±0.11	5.99 ±0.07	1.13 ±0.00	1.08 ±0.02	6320.50 ±169.00
Plant A (6,7,8/17)	43.67 ±4.51	22.55 ±0.10	2.61 ±0.06	8.65 ±0.16	6.98 ±0.57	3.24 ±0.08	5.45 ±0.61	1.10 ±0.10	0.73 ±0.01	5948.50 ±95.46
Plant B (3/17)	59.80± 0.21	32.20± 0.75	4.00± 0.01	8.1± 0.20	5.50± 0.33	4.40± 0.11	4.30± 0.16	1.00± 0.03	1.6± 0.04	14036.00± 537.40
Plant C (12/17)	49.70 ±6.93	29.39 ±2.49	7.12 ±0.22	4.21 ±0.11	6.07 ±0.23	2.35 ±0.21	8.31 ±0.02	0.94 ±0.05	0.72 ±0.05	4768.50 ±177.48

Sample	Heavy metal (μg g^{-1})					pH	EC[‡] (ms cm^{-1})	NH_4-N (μg g^{-1})
	Zn	Cu	Mn	Fe	Al			
Plant A (11/16)	528.50± 9.19	589.50± 13.43	625.00± 46.67	7483.50± 788.42	2726.00± 444.06	8.50± 0.00	29.70± 0.9	599.50± 31.82
Plant A (4/17)	551.50 ±10.61	638.50 ±20.51	693.50 ±20.51	8316.50 ±1232.49	3124.50 ±289.21	8.75 ±0.07	30.35 ±0.21	769.50 ±89.80
Plant A (6,7,8/17)	562.00 ±21.21	712.50 ±19.09	644.00 ±48.08	5717.00 ±364.87	5226.50 ±74.25	7.90± 0.14	9.91 ±0.51	456.50 ±56.65
Plant B (3/17)	871.00± 52.33	585.50± 36.06	796.50± 27.58	1976.00± 82.02	1856.50± 23.34	8.20± 0.07	41.1± 0.28	949.00 ±29.70
Plant C (12/17)	431.00 ±29.70	50.50 ±4.95	397.50 ±30.41	2179.00± 214.96	2157.00 ±453.96	6.15 ±0.07	12.23 ±0.33	215.52± 3.35

[a] Data are expressed as mean±SD of two samples. The values of nutrients and metals are all calculated based on dry-weight.
[b] OM, organic matter.
[c] Electrical conductivity.

Table 3B. Inactivation of *E. faecium* NRRL B-2354 and indigenous enterococci in turkey litter compost after passing through industrial dryer (plant A)

Moisture content tested and date	n [b]	Before dryer			After dryer		
		MC (%)	*E. faecium* (log cfu/g)	Indigenous enterococci [d] (log cfu/g)	MC (%)	*E. faecium* (log cfu/g)	Indigenous enterococci (log cfu/g)
44% MC [a] (Winter, 11/16)	11	43.84±0.57	7.35±0.05	5.45±0.15	32.34±12.12	+/- (1/11) [e]	2.17±0.05
Process control		51.17±2.32	N.A. [c]	6.01±0.10	3.11±0.63	N.A.	2.33±0.06
36% MC (Spring, 4/17)	10	36.35±0.91	7.46±0.06	5.36±0.01	14.76±2.65	- [f]	1.89±0.29
Process control		28.64±3.95	N.A.	4.79±0.17	2.84±0.09	N.A.	1.76±0.21
36% MC (Summer, 6/17)	12	35.15±1.13	7.66±0.03	5.73±0.01	20.48±0.62	+/- (1/12)	1.83±0.03
Process control		29.91±6.16	N.A.	5.16±0.42	4.80±2.04	N.A.	1.79±0.01
44% MC (Summer, 7,8/17)	12	41.76±1.42	7.53±0.02	6.03±0.14	21.88±0.42	-	1.72±0.00
Process control		36.07±3.63	N.A.	4.82±0.86	2.29±0.88	N.A.	1.61±0.00

[a] Two trials were conducted, and the data were expressed as mean±SD.
[b] Number of sampler collected from the dryer.
[c] Not applicable.
[d] Enterococci counts enumerated on EA plates.
[e] +, Detected by enrichment. The detection limit of directly plating is 1.49 log CFU/g by dry weight.
[f] -, not detected by enrichment.

Table 4B. Moisture content and seasonal effect on the reduction of microorganisms in plant A*

Effect source	On the log reduction of	Prob >F	Outcome
Moisture content	Surrogate (*E. faecium*)	0.1293	Not Significant effect
	Indicator (Indigenous enterococci)	0.0064	Significant effect
	Total aerobic bacteria	0.1520	Not Significant effect
	Thermophiles	0.4054	Not Significant effect
Season	Surrogate (*E. faecium*)	0.2681	Not Significant effect
	Indicator (Indigenous enterococci)	0.0249	Significant effect
	Total aerobic bacteria	0.0208	Significant effect
	Thermophiles	0.6176	Not Significant effect

* Analysis of variance (ANOVA), followed by Fisher's Protected Least Significant Differences (LSD) test, was carried out to determine whether effects were significant, and P-value less than 0. 05 was considered evidence of a significant effect.

Table 5B. Inactivation of *E. faecium* NRRL B-2354 and indigenous enterococci in broiler chicken litter compost after passing through industrial dryer (plant B)

Moisture tested and date	n [d]	Before dryer			After dryer		
		MC (%)	*E. faecium* (log cfu/g)	Indigenous enterococci [g] (log cfu/g)	MC (%)	*E. faecium* (log cfu/g)	Indigenous enterococci (log cfu/g)
37% MC trial 1; 3/17[a]	5	36.31±0.47	7.46±0.05	4.24±0.04	33.32±0.00	4.53±0.64	2.37±0.76
Process control		31.53±0.22	N.A.[f]	5.63±0.10	10.61±0.30	N.A.	4.32±0.38
37% MC trial 2; 3/17[b]	5	18.71±0.56	5.83±0.06	4.01±0.01	16.91±1.35	3.96±0.25	2.28±0.79
Process control		N.C.[e]					
42% MC ; 3/17[c]	10	35.15±1.13	5.51±0.16	4.12±0.00	16.21±1.48	3.61±0.42	3.03±0.17
Process control		N.C.	N.A.	5.16±0.42	4.80±2.04	N.A.	1.79±0.01

[a,b] Data were expressed as mean±SD of 5 replicates.
[c] Two trials were conducted, and the data were expressed as mean±SD.
[b,c] Delayed, and the inoculated sample were kept at room temperature for two weeks.
[d] Number of sampler collected from the dryer.
[e] N.C, not collected; process control was not collected by the industry collaborator.
[f] Not applicable.
[g] Enterococci counts enumerated on EA plates.

Table 6B. Population change of *E. faecium* NRRL B2354 in laying hen litter during shipping to plant C

	MC (%)	*E. faecium* (log cfu/g)
Initial (Day 0) [a]	16.89±0.23 [b]	8.94±0.01
After desiccation adaptation	15.69±0.08	6.94±0.02
Expected initial prior to dryer	N.A.	4.47±0.02
Shipment control	10.44±0.47	6.52±0.03

[a] Initial moisture content was ca. 15%
[b] Data are expressed as mean±SD of two trials.

Table 7B. Inactivation of *E. faecium* NRRL B2354 as surrogate microorganism for *Salmonella* in laying hen litter after passing through industrial dryer (plant C)

	n	Trial/Replicate	MC (%)	pH	*E. faecium* (log cfu/g)
Before dryer	4		N.A	6.08±0.01	4.47±0.02[c]
After dryer	20[a]	T1R9 [b]	4.56	5.92	1.78±0.15
		T2R1	4.37	5.94	1.92±0.30
		T2R6	4.24	5.97	1.77±0.15
		Others	4.78±0.47	5.96±0.04	+/- (11/17)[d]

[a] The number of sample collected from two trials after heat treatment.
[b] Detected by direct plating from the following heat treated samples: T1R9, T2R1, and T2R6.
[c] Expected initial surrogate population prior to dryer.
[d] +, detected by enrichment. The detection limit of directly plating is 1.32 log CFU/g by dry weight.

Table 8B. Inactivation of indigenous microorganisms in poultry litter after going through industrial dryer (plant C)

Sample ID	Before dryer					After dryer				
	MC (%)	pH	Indigenous enterococci (log cfu/g)	Mesophiles (log cfu/g)	Thermophiles (log cfu/g)	MC (%)	pH	Indigenous enterococci (log cfu/g)	Mesophiles (log cfu/g)	Thermophiles (log cfu/g)
Process control I [a]	6.26±0.08	6.08±0.14	3.33±0.28	5.80±0.19	5.46±0.04	4.46±0.19 [b]	5.98±0.21	<1.61[d]	3.22±0.06	2.93±0.02
Process control II[c]	N.A.	N.A.	N.A.	N.A.	N.A.	2.17±0.00	6.02±0.11	<1.61	2.90±0.22	2.23±0.13

[a] Sample collected before mixing with the inoculum.
[b] The randomly selected heat treated sample.
[c] Heat treated sample collected from a separated trial, not the validation trials.
[d] Indigenous enterococcus was not detected by directly plating after heat treatment, the detection limit of direct plating was 1.62 log cfu/g in dry weight.

Table 9B. Reductions of *E. faecium* NRRL-B2354, presumptive indigenous enterococci, and total aerobic bacteria during pilot trials in three poultry litter processing plants

Plant	Trial	Run	log reductions of		
			E. faecium NRRL B-2354	Presumptive indigenous enterococci	Total aerobic bacteria
Plant A	1 (11/2016)	1	7.39	3.45	1.58
		2	7.10	3.11	1.59
	2 (4/2017)	1	7.51	3.27	1.54
		2	7.42	3.69	1.95
	3 (6/2017)	1	7.42	3.87	1.00
		2	7.68	3.93	0.96
	4 (7 & 8/2017)	1	7.54	4.21	1.67
		2[+]	7.51	4.41	1.62
Plant B	1 (6/2017)	1	2.93	1.87	1.6
		2*	1.87	1.73	1.28
	2 (3/2017)	1*	1.49	1.2	1.58
		2*	2.31	0.88	1.61
Plant C	1 (12/2017)	1	3.64	>2.00	2.72
		2	3.16	>1.44	2.50

[+]Performed in Aug. 2017.

*The samples were left at room temperature for 2 weeks prior to validation study at plant B.

Table 10B. Primers used in the compost microbial community analysis

Primers		Sequences
Enterococcus specific primers	Ent1	5'-TACTGACAAACCATTCATGATG- 3'
	Ent2	5'-AACTTCGTCACCAACGCGAAC- 3'
Sanger sequencing primers	8F	5'-AGAG TTTGATCCTGGCTCAG-3'
	787R	5'-CGACTACCAGGGTATCT AAT-3'
Nested PCR 1 for DGGE	27F	5'-AGAGTTTGATCMTGGCTCAG-3'
	907R	5'-CCGTCAATTCMTTTRAGTTT-3'
Nested PCR 2 for DGGE	341F-GC	5'-CGCCCGCCGCGCGCG GCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAG GCAGCAG-3'
	517R	5'-ATTACCGCG GCTGCTGG-3'
Universal primers for prokaryotic 16S rDNA[a]	515F	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGYCAGCMGCCGCGGT-3'
	806R	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACNVGGGTWTCTAA-3'
Universal primers for eukaryotic 18S rDNA[b]	1441F	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCAGGTCTGTGATGCCC-3'
	1641R	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGGCGGTGTGTACAARGRG-3'

[a, b] The sections in color are the region specific sequences. The rest are adapter sequences.

Table 11B. Comparison of EA and BEA plates used for compost sample analysis

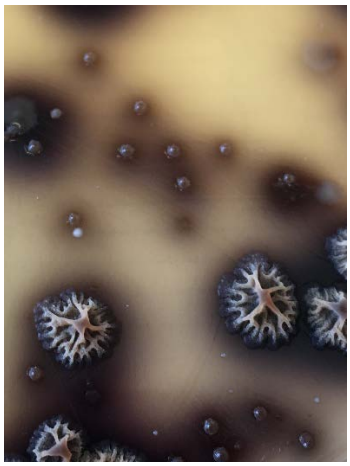
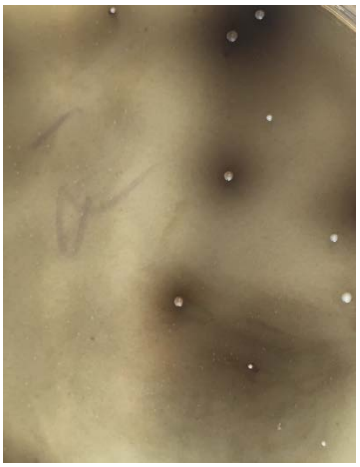
	Bile esculin agar (BEA)	Enterococcosel agar (EA) [Bile esculin azide agar]
Colony morphology	The colonies grown on BEA showed different size, with smooth surface	Most colonies grown on EA have the similar pinpoint size
Halo around colonies	Colonies with dark brown or black halos	With black halos (sometimes dark brown)
Incubation period	Normally 24–48 h, some fast growing colonies can grow after 24-h incubation. Most colonies need more than 24 h. The best count time is less than 48 h.	Need ~24–48 h incubation
Recovery from the litter sample	For the indigenous enterococci in the raw poultry litter, the higher counts were obtained on EA plates (ca. 1 log more) For the indigenous enterococci in the heat-treated poultry litter, the higher counts were obtained on BEA plates (ca. 0.5 log more)	
Recovery of desiccation-adapted *E. faecium*	EA-R is better to enumerate *E. faecium* (surrogate) after desiccation adaptation.	
Growth inhibition by interfering colonies		

Table 12B. Preliminary Sanger sequencing results of isolates from EA and BEA plates

	Sanger sequencing identification	Number of isolates	Isolates from heat-treated compost	EA or BEA
Ent1/Ent2 PCR-positive isolates	*Aerococcus viridans*	37	-	34 from EA and 3 from BEA
	Enterococcus gallinarum	18	-	EA
	Enterococcus hirae	9	-	EA
	Staphylococcus cohnii	6	-	EA
	Staphylococcus devriesei	4	-	EA
	Enterococcus faecalis	2	-	EA
	Pisciglobus halotolerans	2	-	EA
Randomly selected interfering isolates	*Bacillus ruris*	5	3	4 from EA and 1 from BEA
	Bacillus lentus	4	1	2 from EA and 2 from BEA
	Oceanobacillus	7	-	BEA
	Atopostipes suicloacalis	4	-	EA
	Staphylococcus hominis	1	-	BEA
	Staphylococcus stepanovicii	1	-	EA
	Staphylococcus lentus	1	-	EA
	Virgibacillus kekensis	1	-	BEA

Table 13B. Top-10 dominant bacteria and fungi found in turkey litter compost prior to and after heat treatment based on reads

Prior to dryer		After dryer	
Bacteria	Fungi	Bacteria	Fungi
Bacillaceae g-	*Cryptosporidium*	*Trueperaceae B-42*	*Fungi-other*
Psychrobacter	*Fungi-other*	*Bacillaceae g-*	*Arachnida*
Trueperaceae B-42	*Arachnida*	*Aequorivita*	*Thelebolaceae other*
Bacillaceae Other	*Cercomonadidae other*	*Flavobacteriaceae g Zhouia*	*Cercomonadidae other*
Moraxellaceae g-	*Heteromita*	*Bacillales g-*	*Cryptosporidium*
Sporosarcina	*Verticillium*	*Marinimicrobium*	*Verticillium*
Bacillaceae g-	*Uncultured eukaryote*	*Saprospiraceae g*	*Ascobolaceae other*
Alphaproteobacteria o-f-g-	*Diplofasterida*	*Bacillaceae; Other*	*Paramicrosporidium*
Halomonas	*Ambiguous taxa*	*Pirellulaceae_*	*Uncultured soil fungus*
Clostridia o-f-g-	*Letomyxida*	*Acidimicrobiales F-G-*	*Letomyxida*

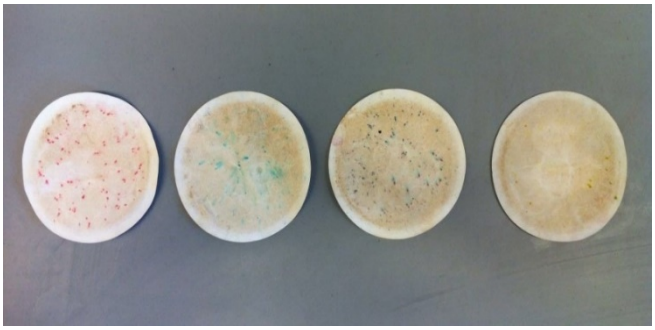


Figure 1B. Developed color on filter papers using MicroTracers with different colors (from left to right: Red, Blue, Violet, Yellow).

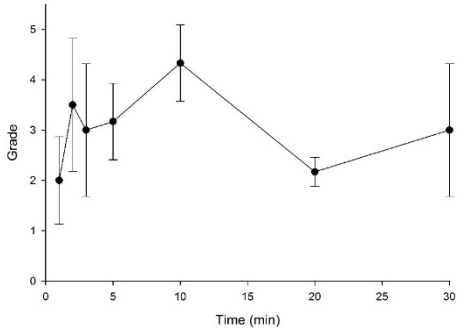


Figure 2B. Grades of developed colors of F-Tracers on filter papers at different residence time intervals.

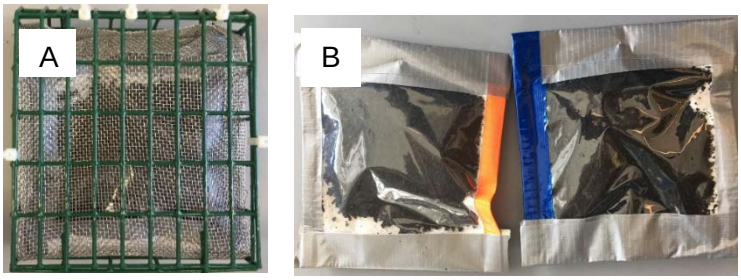


Figure 3B. Customized stainless-steel mesh sampler (A) and color-coded Tyvek pouch containing poultry litter sample (B).

Figure 4B. Inoculated laying hen litter mixed with kitchen cloth swatches as mixing indicators.

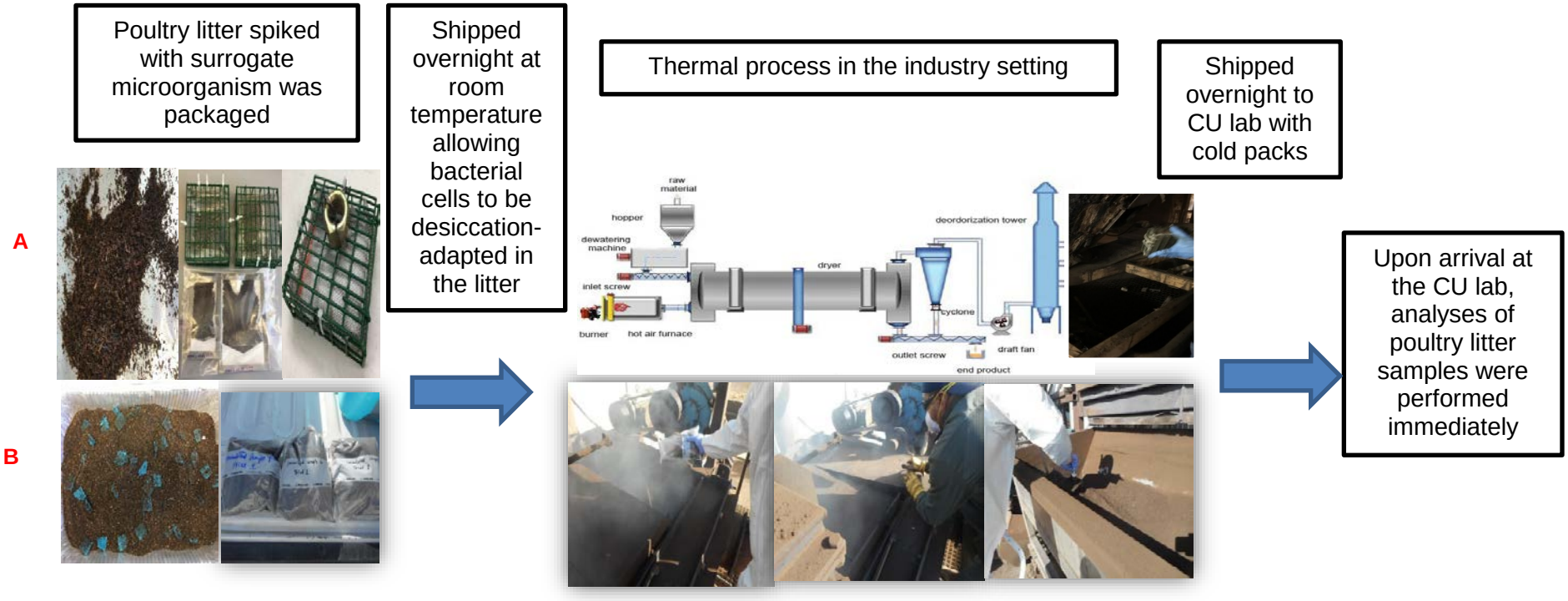


Figure 5B. General flow chart for plant validation study in plant A and B (A), plant C (B).

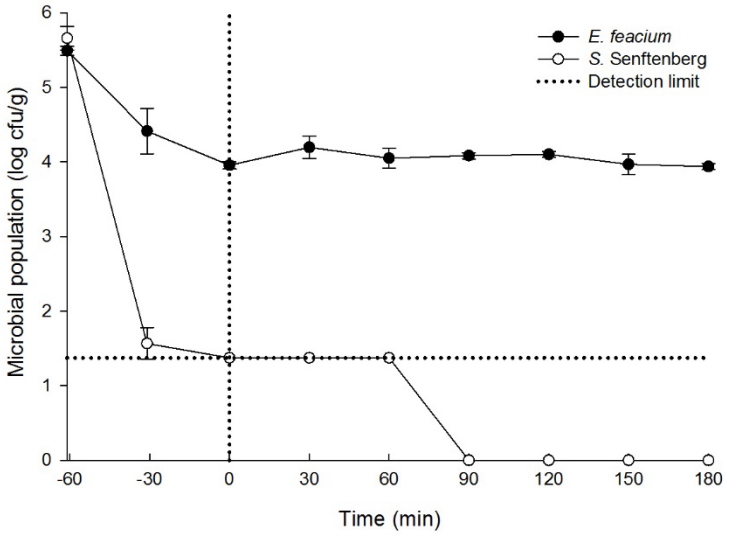


Figure 6B. Survival of desiccation-adapted *S.* Senftenberg 775/W and *E. faecium* NRRL B-2354 in chicken litter compost with 15% MC at 75°C. Inactivation curves during come-up times (on the left of the vertical dotted line) and during holding times (on the right of the vertical dotted line) are shown. The horizontal dotted line indicates that *Salmonella* was detectable only by enrichment (detection limit by direct plating: 1.37 log cfu/g).

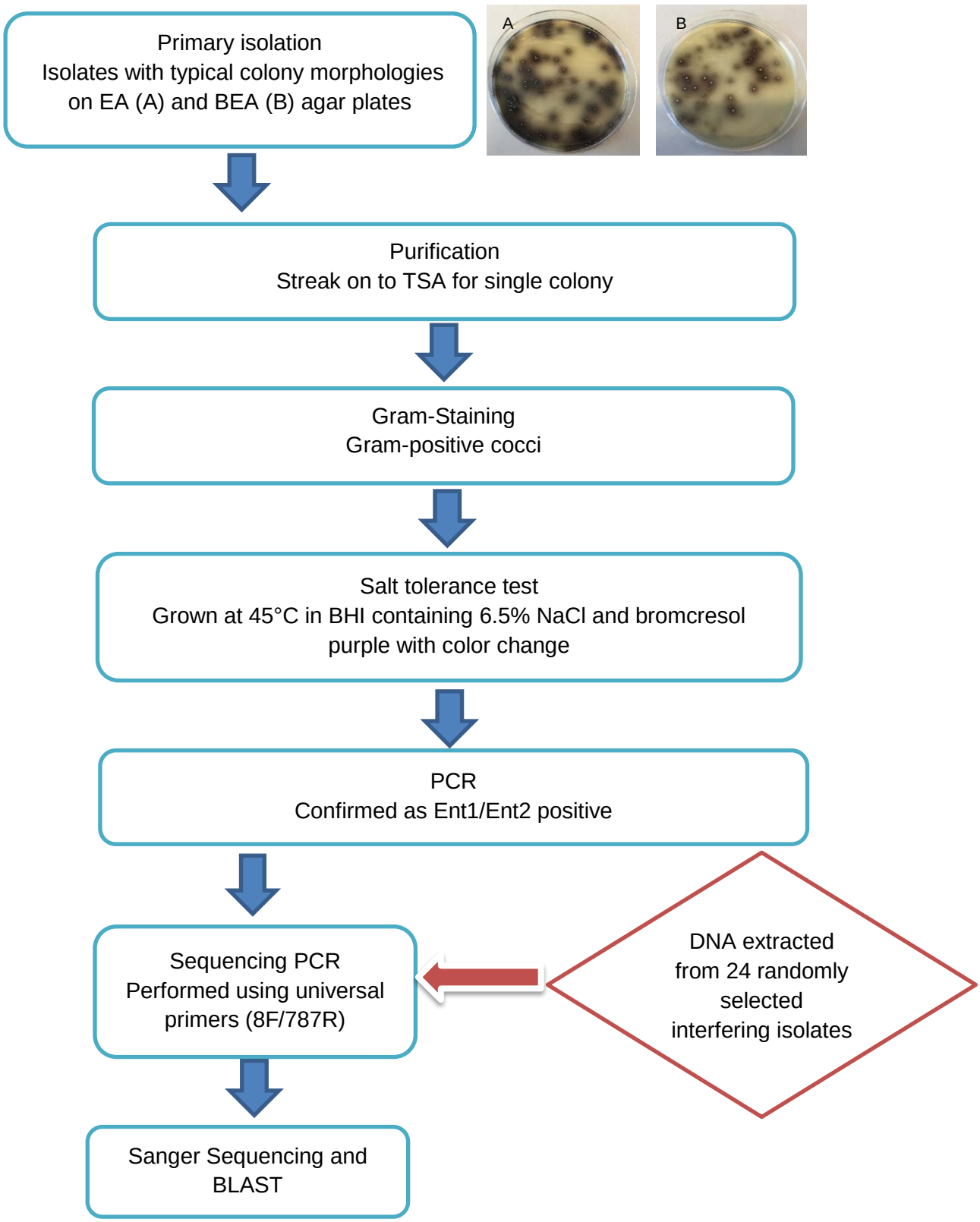


Figure 7B. Isolation and identification of presumptive indigenous enterococci isolated from turkey litter compost.

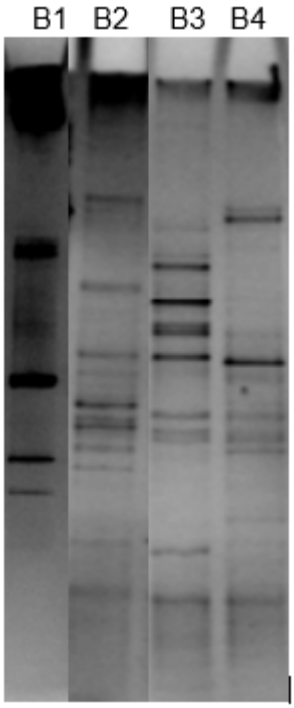


Figure 8B. DGGE profiles of PCR-amplified 16S rDNA fragments from poultry litter compost. Labels on top of the gel indicate the sample identity: B1, 1 Kb ladder, B2, raw chicken litter; B3, partially composted turkey litter; B4, heat-treated partially composted turkey litter.

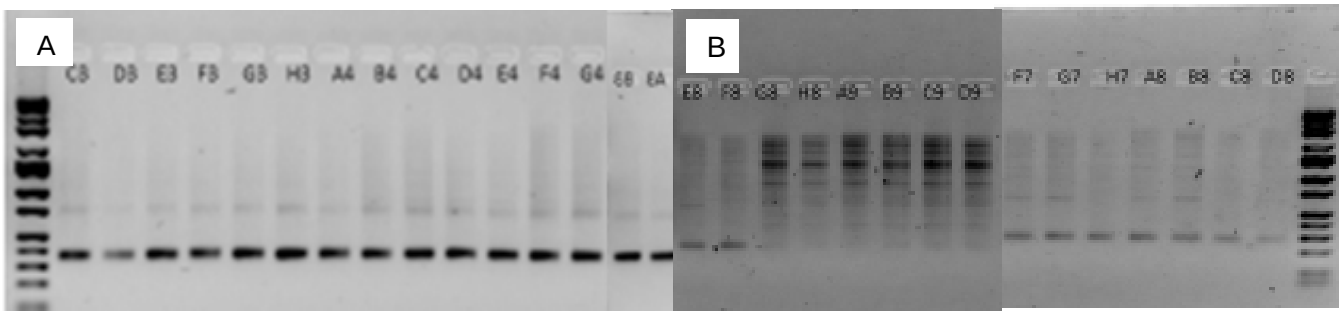


Figure 9B. PCR amplification of specific variable regions of the prokaryotic 16S rDNA (A) and eukaryotic 18S rDNA (B).

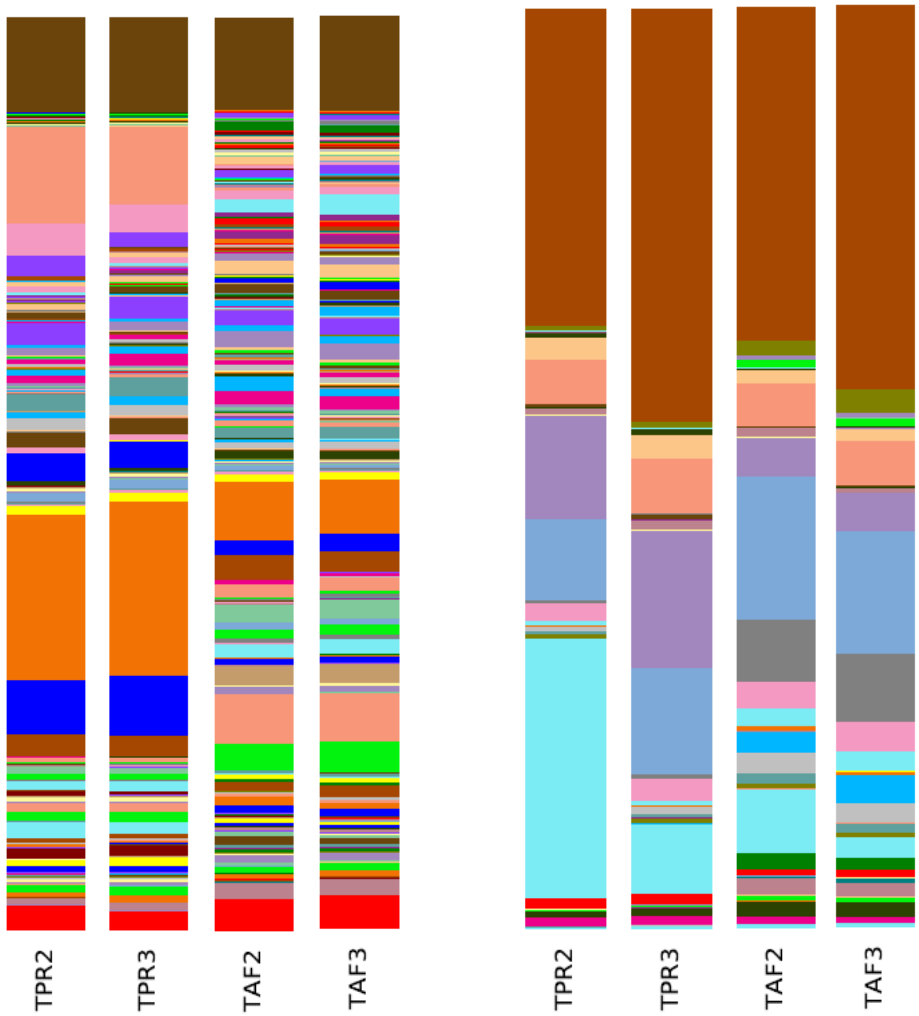


Figure 10B. Prokaryotic (A) and eukaryotic (B) community abundance (at the genus level) in the turkey litter compost prior to and after industry dryer. TPR2 and TPR3: Turkey litter compost prior to dryer; TAF2 and TAF3: Turkey litter compost after dryer.