Effects of Chicken Litter Storage Time and Ammonia Content on Thermal Resistance of Desiccation-Adapted *Salmonella* spp.

Zhao Chen,^a Hongye Wang,^b Claudia Ionita,^b Feng Luo,^c Xiuping Jiang^b

Department of Biological Sciences,^a Department of Food, Nutrition, and Packaging Sciences,^b and School of Computing,^c Clemson University, Clemson, South Carolina, USA

Broiler chicken litter was kept as a stacked heap on a poultry farm, and samples were collected up to 9 months of storage. Chicken litter inoculated with desiccation-adapted *Salmonella* cells was heat-treated at 75, 80, 85, and 150°C. *Salmonella* populations decreased in all these samples during heat treatment, and the inactivation rates became lower in chicken litter when storage time was extended from 0 to 6 months. There was no significant difference ($P > 0.05$) in thermal resistance of *Salmonella* in 6- and 9-month litter samples, indicating that a threshold for thermal resistance was reached after 6 months. Overall, the thermal resistance of *Salmonella* in chicken litter was affected by the storage time of the litter. The changes in some chemical, physical, and microbiological properties during storage could possibly contribute to this difference. Moisture and ammonia could be two of the most significant factors influencing the thermal resistance of *Salmonella* cells in chicken litter. Our results emphasize the importance of adjusting time and temperature conditions for heat processing chicken litter when it is removed from the chicken house at different time intervals.

Chicken litter is a waste by-product of poultry industry, which consists of feces, bedding materials, wasted feed, and feathers (1). More than 14 million tons of chicken litter are produced every year in the United States, most of which is spread onto arable land as organic fertilizer or soil amendment (2). Chicken litter is known to potentially harbor a variety of human pathogens, such as *Salmonella* (3). Therefore, the direct application of chicken litter to agricultural land can possibly be harmful to the environment and also the food supply (4).

Chicken litter is introduced into arable land either immediately after its removal from chicken houses, after being stockpiled for an extended period of time, or after going through a composting process (5). Agricultural practices for handling chicken litter vary widely among farms and also among economic crops grown on an individual farm. Chicken litter cleanout does not always coincide with availability of agricultural land or with suitable field conditions that allow operation of equipment or desirable nutrient uptake. Temporary storage should thus be provided until conditions are proper for direct application on land or until the litter can be composted or further heat processed. Notably, chicken litter is a heterogeneous waste product with variable compositions and differences in physical, chemical, and microbiological properties, which can be affected by storage time and other factors (6). In addition to composting, physical dry-heat treatment after composting or without composting is one of the most commonly used methods to eliminate potential pathogens in chicken litter (6). The storage time of chicken litter may, to some extent, affect the thermal inactivation of food-borne pathogens. Based on the study by Kim et al. (6), *Salmonella* survived much longer in dry or aged chicken litter than in wet or fresh litter when exposed to the same lethal temperature, suggesting that the moisture level and chemical composition (especially ammonia) of chicken litter are critical factors affecting the thermal resistance of this pathogen. However, in that study, the origins of fresh and aged chicken litter samples were different, which could place some limitations on the application of their findings.

It is known that chicken litter is a relatively hostile environ-

ment for the growth and persistence of enteric pathogens, since it is generally very dry and high in ammonia (5). However, some pathogenic cells may become acclimatized to hostile environments during stockpiling or composting, which can cross-protect them against subsequent thermal treatment (1, 7). A limitation regarding most published thermal inactivation studies on pathogens in compost and manure is that the conclusions are primarily based on using nonstressed cells (5, 6), and the data obtained from these studies can be misinformed or biased. Currently, the use of stressed cells in the challenge studies on validating the efficacy of heat-based hurdles for food-borne pathogens is still novel, and few examples of quantitative assessments are available in the published literature (8, 9). Our previous work has already demonstrated that desiccation-adapted *Salmonella* cells in chicken litter were more heat resistant than nonadapted populations during dry-heat treatment, suggesting possible survival of some heat-resistant desiccation-adapted cells due to inadequate thermal processing (8, 9). These pathogenic cells can potentially contaminate fresh produce and the environment when physically heat-treated chicken litter is applied to agricultural land as organic fertilizer or soil amendment. This concern has created a significant, industry-wide demand for establishing some practical guidelines on temperature-time combinations to thermally process chicken litter of different properties to produce *Salmonella*-free finished products. Therefore, desiccation-adapted cells should be used in order to

Received 5 June 2015 Accepted 20 July 2015

Accepted manuscript posted online 24 July 2015

Citation Chen Z, Wang H, Ionita C, Luo F, Jiang X. 2015. Effects of chicken litter storage time and ammonia content on thermal resistance of desiccation-adapted *Salmonella* spp. *Appl Environ Microbiol* 81:6883–6889. doi:10.1128/AEM.01876-15.

Editor: J. L. Schottel

Address correspondence to Xiuping Jiang, xiuping@clemson.edu.

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doi:10.1128/AEM.01876-15

simulate the real-world conditions in chicken litter during composting or stockpiling.

The objective of this study was to determine the thermal resistance of desiccation-adapted *Salmonella* spp. and how it is affected by changes in some physical, chemical, and microbiological characteristics of chicken litter during long-term storage. We believe that this novel study will elucidate the effects of various properties of stacked chicken litter on die-off dynamics of desiccation-adapted *Salmonella* spp. and provide some important new insights into the microbiological safety of heat-processed chicken litter as biological soil amendment for agricultural application.

MATERIALS AND METHODS

Sample preparation. Chicken litter was removed from the broiler house at Mendel Stone Farm, Westminster, SC, after harvesting of all the chickens. The litter was kept as a stacked round heap (ca. 1.2 m in height by 2 m in diameter) in a covered shed on the farm for 9 months (May 2013 to about February 2014). The shed was a typical roofed storage structure commonly seen on poultry farms, as described by Ogejo and Collins (10). The internal and external temperatures of the heap ranged from -2.5 to 32°C and from -15 to 34°C , respectively.

Chicken litter samples were collected aseptically at 0, 3, 6, and 9 months of storage. Subsamples of 10 kg ($n = 5$) were taken at different locations (external and internal) throughout the heap. These subsamples were thoroughly mixed using a clean shovel and placed into sample bags. Sufficient samples (ca. 30 kg) were collected for the entire experiment, screened manually to a particle size of less than 3 mm using a sieve (sieve pore size, 3 by 3 mm), and then stored at 4°C until use.

Bacterial strains and inoculum preparation. *Salmonella enterica* serovars Enteritidis H2292 and Heidelberg 21380 (kindly provided by Michael Doyle, University of Georgia, Griffin, GA) and Senftenberg ATCC 43845, and avirulent *S. enterica* serovar Typhimurium 8243 [genotype: *thyA deo polA2 zie-3024::Tn10(dTc)zag-1256::Tn10(dKm)*, derived from *S. Typhimurium* LT2 by John Foster, University of South Alabama, Mobile, AL, and provided by Roy Curtiss III, Washington University, St. Louis, MO] (8) were used. All the strains were induced to rifampin resistance ($100\text{ }\mu\text{g of ml}^{-1}$) using the gradient plate method (11).

Each *Salmonella* strain was grown overnight at 37°C in tryptic soy broth without dextrose (TSB; Becton, Dickinson and Company, Sparks, MD) supplemented with $100\text{ }\mu\text{g}$ of rifampin/ml. The overnight cultures were centrifuged and washed three times with sterile phosphate-buffered saline (PBS; pH 7.4). The final pelleted cells were resuspended in PBS to desired cell concentrations (ca. 10^9 CFU/ml) through adjusting the optical density at 600 nm to ca. 0.7. Afterwards, these resuspended cultures were further concentrated 100 times (ca. 10^{11} CFU/ml) by centrifugation. Equal volumes of four cultures were mixed as inoculum for the subsequent experiments.

Desiccation adaptation and thermal inactivation. Briefly, as described by Chen et al. (8), bacterial cultures were added (1:10, vol/wt) into 100 g of chicken litter collected at different storage times at a final concentration of ca. 10^{10} CFU/g, mixed well, and incubated at room temperature for a 24-h desiccation adaptation. After the 24-h desiccation adaptation, chicken litter with desiccation-adapted cells was further mixed (1:10, wt/wt) with 800 g of chicken litter using a blender (KitchenAid Inc., St. Joseph, MI) for subsequent heat treatment.

Twenty grams of samples in triplicate was distributed evenly inside an aluminum pan (inside diameter, 10 cm), and pans were placed in three different locations (close to the door, center, and far away from the door) on the shelf of a controlled convection oven (Binder Inc., Bohemia, NY) and then exposed to 75, 80, and 85°C for up to 6 h. Temperature was monitored constantly using T-type thermocouples (DCC Corp., Pennsauken, NJ), with one cord kept inside the oven chamber and others inserted into the bottom of litter samples at three different locations. The temperature of the oven was initially set 5°C higher than the target tem-

FIG 1 Survival of *Salmonella* spp. in chicken litter stored for 0, 3, 6, and 9 months at 75°C (A), 80°C (B), and 85°C (C). "Adjusted MC" indicates that the moisture content of 9-month litter was adjusted to equal that of 0-month litter at 85°C . The dotted line indicates that *Salmonella* was detectable only by enrichment (detection limit by plating, 1.52 log CFU/g).

TABLE 1 Survival of *Salmonella* spp. in chicken litter stored for 0, 3, 6, 9, and 9 (adjusted MC) months at 150°C

Storage time (mo)	Survival (log CFU/g) with exposure time (min) ^a			
	15	30	45	60
0	1.50 ± 0.91 Cb	+	—	—
3	2.16 ± 0.21 b	+	+	+
6	3.29 ± 0.28 a	+	+	+
9	3.63 ± 0.28 Aa	+	+	+
9 (adjusted MC) ^b	2.36 ± 0.34 B	+	+	—

^a Data are means ± SD from two trials. Means with different lowercase letters are significantly different ($P < 0.05$), while for 0, 9, and 9 (adjusted MC) months, means with different uppercase letters are significantly different ($P < 0.05$). +, detectable by enrichment; —, not detectable by enrichment.

^b Adjusted MC, moisture content of 9-month litter was adjusted to equal that of 0-month litter.

perature to reduce the come-up time. When the interior of litter sample reached target temperatures, the temperature setting was adjusted to maintain the designated experimental temperature. Chicken litter samples were taken out of the oven every 0.5 h, transferred into a Whirl-Pak bag (Nasco, Fort Atkinson, WI), and placed immediately in an ice-water bath to cool down the samples and minimize further cell death. For heat treatment at 150°C, triplicate samples were withdrawn every 15 min for up to 1 h. These experiments were performed in two separate trials.

Salmonella enumeration. For the challenge study, 10 g of samples was mixed with 90 ml of sterile PBS (pH 7.4) containing 1% sodium pyruvate, kept at room temperature for 30 min to allow recovery of heat-injured cells (12), and then diluted serially with PBS. Afterwards, diluents were spread in triplicate onto tryptic soy agar (TSA) supplemented with 100 µg rifampin/ml. In case of the possible failure of recovering any heat-injured cells by a direct plating method (detection limit, 1.52 log CFU/g) after 1 h of heat treatment, 25 g of chicken litter samples was pre-enriched in 225 ml of universal pre-enrichment broth (UPB; Neogen Corp., Lansing, MI) followed by a secondary enrichment in Rappaport-Vassiliadis broth (RV; Becton, Dickinson and Company, Sparks, MD) supplemented with 100 µg rifampin/ml. After 24 h incubation at 42°C, enriched cultures were streaked with a 10-µl inoculation loop onto xylose-lysine-Tergitol agar (XLT-4; Becton, Dickinson and Company, Sparks, MD) supplemented with 100 µg rifampin/ml. Samples taken at the beginning of heat treatment (0 h) were used to determine the initial inoculum levels. For heat treatment at 150°C, samples were plated onto TSA supplemented with 100 µg rifampin/ml and also enriched as described above to determine whether there were viable *Salmonella* cells.

Thermal inactivation kinetics. A log-linear model with shoulder and/or tailing was used to describe the thermal inactivation of desiccation-adapted *Salmonella* spp. in chicken litter (13), which can be formulated as follows:

$$\frac{dN}{dt} = -k_{\max} \cdot N \cdot \left(\frac{1}{1 + C_c} \right) \cdot \left(1 - \frac{N_{\text{res}}}{N} \right) \quad (1)$$

TABLE 2 Parameter estimates of the thermal inactivation model for desiccation-adapted *Salmonella* spp. in chicken litter^a

Temp (°C)	Storage time (mo)	$k_{\max}$ (1/h)	N_{res} (log CFU/g)	Adjusted R^2
75	0	0.17	1.59	0.9709
	3	0.11	1.72	0.9080
	6	0.12	3.95	0.9131
	9	0.08	3.95	0.8843
80	0	0.18	1.57	0.9720
	3	0.13	1.61	0.9715
	6	0.13	3.88	0.9030
	9	0.12	3.92	0.8974
85	0	0.31	1.58	0.9868
	3	0.17	1.66	0.8688
	6	0.11	1.88	0.9293
	9	0.07	1.52	0.9244

^a $k_{\max}$ is the specific inactivation rate, and N_{res} is the residual population density. Adjusted R^2 was used to evaluate the goodness of fit of the model.

$$\frac{dC_c}{dt} = -k_{\max} \cdot C_c \quad (2)$$

N is the microbial cell density (in log CFU per gram), t is the treatment time (in hours), C_c is related to the physiological state of the cells, $k_{\max}$ is the specific inactivation rate (per hour), and N_{res} is the residual population density (in log CFU per gram).

The model was fitted using averages from two experimental data sets. An adjusted regression coefficient, R^2 , was used to evaluate the goodness of fit of the proposed model as described by Chen and Zhu (14).

Analyzing physical, chemical, and microbiological characteristics of chicken litter. Moisture content was determined using a moisture analyzer (model IR-35; Denver Instrument, Denver, CO), whereas water activity (a_w) was measured with a dew point water activity meter (Aqualab series 3TE; Decagon Devices, Pullman, WA). Ammonia content and pH were measured according to the methods described by Weatherburn (15) and the U.S. Composting Council (16), respectively. Electrical conductivity was determined using the Orion Versa Star conductivity meter (Thermo Fisher Scientific Inc., Waltham, MA) based on the method described by the U.S. Composting Council (16). Each sample was analyzed in duplicate. Duplicate samples were analyzed for chemical characteristics, including nutrients and metals (total concentrations, including water-soluble and water-insoluble concentrations), based on the methods proposed by the U.S. Composting Council (16).

Prior to the challenge study, chicken litter was analyzed for background microorganisms, including mesophiles, thermophiles, *Enterobacteriaceae*, yeasts and molds, and actinomycetes, and also screened for *Salmonella* by following the microbiological detection method described by the U.S. FDA's *Bacteriological Analytical Manual* (17). The media used for mesophiles, thermophiles, *Enterobacteriaceae*, yeasts and molds, and acti-

TABLE 3 Moisture content, electrical conductivity, and microbial counts of chicken litter during 9-month storage^a

Storage time (mo)	Moisture content (%)		Electrical conductivity (mS/cm)	Count (log CFU/g) of:				
		a_w		Mesophiles	Thermophiles	<i>Enterobacteriaceae</i>	Yeasts and molds	Actinomycetes
0	29.93 ± 0.00 a	0.88 ± 0.00 a	20.25 ± 0.36 a	7.80 ± 0.07 a	7.66 ± 0.19 a	3.10 ± 0.21	3.86 ± 0.12 a	5.03 ± 0.32 a
3	19.81 ± 0.09 b	0.79 ± 0.00 b	19.12 ± 0.48 b	6.78 ± 0.33 b	6.77 ± 0.33 b	—	3.12 ± 0.01 c	3.59 ± 0.12 c
6	19.53 ± 0.08 b	0.74 ± 0.01 c	17.18 ± 0.36 c	6.66 ± 0.07 b	4.84 ± 0.01 c	—	3.43 ± 0.15 b	3.94 ± 0.12 b
9	18.20 ± 0.00 c	0.74 ± 0.00 c	15.95 ± 0.09 d	6.55 ± 0.12 b	4.10 ± 0.23 d	—	3.68 ± 0.15 a	3.40 ± 0.20 c

^a Means with different letters in the same column are significantly different ($P < 0.05$). —, not detectable (detection limit of 1.52 log CFU/g).

TABLE 4 Chemical characteristics of chicken litter during 9-month storage^a

Storage time (mo)	Concn (%) of nutrient									
	OM ^b	C	N	C/N	P	K	Ca	Mg	S	Na
0	72.35 ± 0.07 a	36.68 ± 0.21 a	3.72 ± 0.01 a	9.85 ± 0.01 b	4.10 ± 0.11 c	3.98 ± 0.11 c	2.89 ± 0.05 c	0.79 ± 0.03 c	0.88 ± 0.02 b	0.86 ± 0.02 c
3	67.65 ± 0.50 b	33.98 ± 0.57 b	3.32 ± 0.01 c	10.24 ± 0.23 a	4.78 ± 0.06 b	4.74 ± 0.15 b	3.26 ± 0.08 b	0.94 ± 0.04 b	1.03 ± 0.03 a	1.00 ± 0.01 a
6	63.65 ± 2.48 c	32.70 ± 0.29 c	3.52 ± 0.06 b	9.30 ± 0.08 c	5.21 ± 0.35 a	4.89 ± 0.08 a	3.58 ± 0.11 a	1.01 ± 0.03 a	1.01 ± 0.13 a	1.03 ± 0.01 a
9	68.30 ± 1.13 b	33.70 ± 0.54 b	3.83 ± 0.10 a	8.80 ± 0.38 d	4.56 ± 0.25 b	4.45 ± 0.13 b	3.13 ± 0.16 b	0.89 ± 0.04 b	0.84 ± 0.04 b	0.93 ± 0.02 b

(Continued on following page)

nomycetes were tryptic soy agar (TSA; Becton, Dickinson and Company, Sparks, MD), TSA, violet red bile dextrose agar (VRBD; Becton, Dickinson and Company, Sparks, MD), rose bengal chloramphenicol agar (Oxoid Ltd., Basingstoke, United Kingdom), and actinomycete isolation agar (Becton, Dickinson and Company, Sparks, MD), respectively, while the incubation temperatures used for them were 35, 55, 35, 22, and 30°C, respectively, and the incubation times were 24, 24, 24, 72, and 72 h, respectively.

The metabolic fingerprint pattern for the microbial community in chicken litter was investigated with the EcoPlate method (Biolog Inc., Hayward, CA). The EcoPlate was designed specifically for microbial community analysis and microbial ecological studies. The EcoPlate contains 31 of the most useful carbon sources for community analysis. Communities of microorganisms will display a characteristic reaction pattern called a metabolic fingerprint, which rapidly and readily provides a vast amount of information from the microplate. Litter samples were diluted in sterile PBS (pH 7.4) to reach a bacterial concentration of background microflora of approximately 10^3 CFU/ml, and then wells with different carbon sources and 3 blanks were inoculated. Plates were placed inside a plastic container with a lid containing moist towels and incubated at room temperature for up to 7 days. The development of purple color was monitored by measuring optical density at 590 nm every 24 h using the μ Quant microplate spectrophotometer (Biotek Instruments, Inc., Winooski, VT). Data collection was performed on day 5, since there was no further significant color formation after that time point.

Statistical analysis. Plate count data were converted to log CFU per gram (dry weight). Differences among samples were determined by least significant differences (LSD) using SigmaPlot 12.3 (Systat Software Inc., San Jose, CA, USA) and were considered significant when P was <0.05 . For the study on the microbial community in chicken litter, data analysis was performed with principal component analysis (PCA) using XLSTAT 2010 (Addinsoft Inc., New York, NY) to compare and analyze the changes in the microbial community reaction patterns.

RESULTS AND DISCUSSION

Effect of storage time of chicken litter on thermal resistance of desiccation-adapted *Salmonella* spp. Before artificial inoculation, chicken litter samples were all free of *Salmonella* as determined by the microbiological detection method described by the U.S. FDA's *Bacteriological Analytical Manual* (17). To determine the effect of chicken litter storage time on thermal resistance of *Salmonella*, all chicken litter samples were inoculated with desiccation-adapted *Salmonella* cells at the level of ca. $7.5 \log$ CFU/g. At 75, 80, and 85°C, *Salmonella* populations decreased in all these samples during heat treatment; however, the inactivation rates became lower in chicken litter when storage time was extended from 0 to 6 months (Fig. 1). For example, at 75°C, *Salmonella* cells were detectable only by enrichment after 2 h of heat exposure for 0-month litter, whereas up to 4 log units of *Salmonella* were recovered following 6-h heat treatment for 6- and 9-month litter samples (Fig. 1A). Interestingly, there was no significant difference in the thermal resistance of *Salmonella* in chicken litter with storage times of 6 and 9 months ($P > 0.05$), indicating that a

threshold for thermal resistance was reached after 6 months. In the chicken litter samples with the same storage time, desiccation-adapted cells were inactivated much faster when temperature was increased. For example, for 9-month litter, the population of *Salmonella* cells was still greater than $3.5 \log$ CFU/g after 6 h of heat exposure at 75°C; however, viable cells could be detected only by enrichment after 5.5 h at 85°C. In addition, compared to fresh chicken litter at day 0, the increased thermal resistance of *Salmonella* in aged chicken litter was more pronounced at 75 and 80°C than at 85°C ($P < 0.05$).

At 150°C, *Salmonella* cells in 3-, 6-, and 9-month litter had longer durations of survival than those in 0-month litter during 1-h heat treatment (Table 1). After 15 min of heat exposure, the populations of *Salmonella* in 6- and 9-month litter samples were significantly higher than those in 0- and 3-month litter samples ($P < 0.05$). Viable cells were not detectable by enrichment after 45 min of heat treatment in 0-month litter, whereas for 3-, 6-, and 9-month litter samples, they could still be detected by enrichment for at least 1 h.

Parameter estimates obtained from fitting the experimental observations into the log-linear model with shoulder and/or tailing and the adjusted R^2 values are presented in Table 2. The model used in this study was proved to be adequate in fitting all the inactivation curves and allowed modeling of an extended tail for desiccation-adapted *Salmonella*, which was supported by the good performance of goodness-of-fit experiments (adjusted R^2). Our results showed that there seemed to be a trend in temperature and storage time dependencies for $k_{\max}$ (specific inactivation rate). As temperature decreased from 85 to 75°C and storage time extended from 0 to 9 months, $k_{\max}$ tended to decrease, suggesting a slower population reduction, i.e., a lower inactivation rate. Moreover, there also seemed to be a trend in storage time dependency for N_{res} (residual population density), as N_{res} gradually increased when storage time was extended from 0 to 9 months, which reflected a higher level of population remaining viable (tail) at the end of thermal treatment. For the same storage times of 0 and 3 months, N_{res} remained relatively the same when temperature was increased from 75 to 85°C, suggesting less impact of these temperatures on those heat-resistant populations. However, when the storage time was extended from 6 to 9 months, 2 log units more of heat-resistant populations were inactivated at 85°C than at 75 and 80°C.

Effects of moisture of chicken litter on thermal resistance of desiccation-adapted *Salmonella* spp. During the extended on-farm storage, chicken litter in stockpile was subject to changes in physical, chemical, and microbiological characteristics. In this study, moisture content of chicken litter decreased gradually from 29.93 to 18.20% after 9 months of storage (Table 3). One of the factors contributing to the increased thermal resistance of *Salmonella* from 0 to 9 months could be the low moisture content in aged chicken litter, as bacteria can generally survive much longer

TABLE 4 (Continued)

Storage time (mo)	Concn (μg/g) of heavy metal					pH	NH ₄ -N concn (μg/g)
	Zn	Cu	Mn	Fe	Al		
0	605.00 ± 14.14 c	180.50 ± 4.95 c	670.50 ± 16.26 c	2,768.50 ± 48.79 c	3,832.50 ± 6.36 c	8.74 ± 0.02 a	279.13 ± 11.92 a
3	744.00 ± 18.39 a	301.00 ± 32.53 a	829.00 ± 28.28 a	3,734.00 ± 288.50 b	5,274.00 ± 309.71 b	8.61 ± 0.01 b	98.01 ± 0.26 b
6	761.00 ± 25.46 a	305.50 ± 6.36 a	881.50 ± 44.55 a	4,068.00 ± 429.92 a	6,197.50 ± 286.38 a	8.56 ± 0.00 c	65.05 ± 0.35 c
9	672.00 ± 8.49 b	225.50 ± 4.95 b	754.50 ± 34.65 b	3,428.50 ± 577.71 b	4,884.50 ± 740.34 b	8.31 ± 0.01 d	56.37 ± 0.28 d

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

^b OM, organic matter.

when being heat-treated under dry conditions (8). The results of our recent research also indicated that desiccation-adapted *Salmonella* cells in aged chicken litter became more heat resistant when moisture content decreased from 50 to 20% (8, 9). In the study by Kim et al. (6), *Salmonella* survived much longer in chicken litter with lower moisture content during dry-heat treatment. Ceustermans et al. (18) also observed a higher survival rate of *S. Senftenberg* when the moisture content of the composting materials or meat carriers was reduced. And similarly, Turner (19) found that higher moisture content of wheat straw and pig manure also played a positive role in enhancing the inactivation of *Escherichia coli* during composting. When 10 ml of *E. coli* broth was added, inactivation occurred more rapidly at both 50 and 55°C than when only 1 ml was added.

To exclude the possibility that decreased moisture content solely contributed to the increased thermal resistance of *Salmonella* with extended storage time, our research expanded on the above studies by comparing the survival profiles of *Salmonella* in 0- and 9-month chicken litter via adjusting the moisture content of 9-month litter (a_w , 0.90 ± 0.00) to the same level as that of 0-month litter at 85 and 150°C (Fig. 1C and Table 1). At 85°C, the population of *Salmonella* in 9-month litter with adjusted moisture content was higher than that in 0-month litter for at least the initial 2 h of heat treatment ($P < 0.05$) while lower than that in 9-month litter without the moisture content adjustment for at least the initial 2.5 h of heat treatment ($P < 0.05$) (Fig. 1C). Similarly, at 150°C, *Salmonella* in 9-month litter with adjusted moisture content survived for at least the initial 15 min longer compared to those in 0-month litter but shorter than those in 9-month litter without the moisture content adjustment throughout thermal exposure ($P < 0.05$) (Table 1). Therefore, it should be noted that in addition to moisture content, there could be some other explanations for the difference in the thermal resistance of *Salmonella* in chicken litter during heat exposure among four storage times.

Effects of ammonia of chicken litter on thermal resistance of desiccation-adapted *Salmonella* spp. The ammonia level was

much higher in fresh chicken litter than in aged chicken litter ($P < 0.05$), as the initial high ammonia level ($279.13 \mu\text{g NH}_4\text{-N/g}$) in 0-month litter decreased to $56.37 \mu\text{g NH}_4\text{-N/g}$ in 9-month litter (Table 4). Generally, chicken litter, high in nitrogen content, begins to decompose immediately after excretion and releases ammonia in high concentrations (20). Chicken litter also gives off ammonia due to the production of ammonia from microbial mineralization of organic nitrogen compounds in the litter at the beginning of stockpiling (21). Ammonia can cause a significant inactivation of non-spore-forming pathogens in a stacked manure heap (22, 23). In the current study, ammonia content of chicken litter decreased rapidly throughout the 9-month storage. Therefore, the initial high ammonia level could result in a large amount of ammonia emission during heat treatment, accelerating the inactivation of *Salmonella* in 0-month litter. Similar results have been obtained by Kim et al. (6), who reported that *Salmonella* cells in aged chicken litter with lower levels of ammonia showed longer survival than those in fresh chicken litter. Himathongkham and Riemann (25) evaluated the effect of drying and/or exposure to ammonia on *S. Typhimurium*, *E. coli* O157:H7, and *Listeria monocytogenes* in chicken manure. Drying to 10% moisture content followed by exposure to ammonia gas for 72 h (equivalent to 1% of the manure wet weight) led to an 8-log reduction of *S. Typhimurium* and *E. coli* O157:H7 and a 4-log reduction of *L. monocytogenes*. Thus, the dramatically decreased ammonia level in chicken litter with extended storage time could be another possible explanation for the increased thermal resistance of *Salmonella* cells.

Effects of indigenous microbial community of chicken litter on thermal resistance of desiccation-adapted *Salmonella* spp. Chicken litter was also subject to a gradual decline in mesophiles, thermophiles, and *Actinomyces*, while *Enterobacteriaceae* were not detectable after 3 months (Table 3). The microbial community changes in chicken litter were also analyzed for metabolic profiles using an EcoPlate method. Only six carbon sources (Tween 40, glycogen, *N*-acetyl-D-glucosamine, D-glucosaminic acid, glucose-1-phosphate, and D,L-α-glycerol phosphate) were

TABLE 5 Changes in OD₅₉₀ for six carbon sources after 5 days based on EcoPlate data for chicken litter stored for 0, 3, 6, and 9 months

Storage time (mo)	Change in OD ₅₉₀ for indicated carbon source ^a					
	C2	C5	C12	C13	C14	C15
0	0.601 ± 0.064 b	0.478 ± 0.011 d	0.929 ± 0.040 d	0.532 ± 0.021 c	0.674 ± 0.052 c	0.682 ± 0.127 b
3	0.766 ± 0.105 b	1.550 ± 0.009 b	1.789 ± 0.040 b	0.920 ± 0.078 b	0.935 ± 0.055 b	0.923 ± 0.093 a
6	0.599 ± 0.076 c	1.165 ± 0.045 c	1.503 ± 0.061 c	0.578 ± 0.082 c	0.616 ± 0.005 d	0.650 ± 0.103 b
9	1.063 ± 0.014 a	1.994 ± 0.045 a	2.138 ± 0.004 a	1.428 ± 0.021 a	0.996 ± 0.020 a	0.623 ± 0.009 b

^a Data are expressed as means ± SD for three samples. Means with different letters in the same column are significantly different ($P < 0.05$). C2, Tween 40; C5, glycogen; C12, *N*-acetyl-D-glucosamine; C13, D-glucosaminic acid; C14, glucose-1-phosphate; C15, D,L-α-glycerol phosphate.

FIG 2 Principal component analysis (PCA) ordination diagram of EcoPlate data for six carbon sources (C2, Tween 40; C5, glycogen; C12, *N*-acetyl-D-glucosamine; C13, D-glucosaminic acid; C14, glucose-1-phosphate; C15, D,L- α -glycerol phosphate) oxidized at the highest rates (changes in OD₅₉₀ of >0.7 after 5 days based on EcoPlate data) by microbial communities in chicken litter stored for 0 (M0), 3 (M3), 6 (M6), and 9 (M9) months.

oxidized at a higher rate by microbial communities in chicken litter during 9-month storage (changes in optical density at 590 nm [OD₅₉₀] for 0-, 3-, 6-, and 9-month samples were all higher than 0.4) were used for PCA. As shown in Table 5, these six carbon sources were all oxidized at a higher rate. Obviously, Tween 40, glycogen, *N*-acetyl-D-glucosamine, D-glucosaminic acid, and glucose-1-phosphate were oxidized at the highest rate by 9-month microflora ($P < 0.05$), while D,L- α -glycerol phosphate was oxidized at the highest rate by 3-month microflora ($P < 0.05$). Consistently, the cluster diagram based on the EcoPlate data also revealed a distinct clustering of the oxidation rates of these above six carbon sources in relation to storage times (Fig. 2). Therefore, our results showed that there were some changes in the metabolic fingerprint patterns of microbial communities during 9-month storage of chicken litter.

The use of carbon sources present in the EcoPlate was sensitive enough to detect changes in the microbial diversity in response to different storage times. By using the PCA, the different use of carbon sources allowed separation for 0-, 3-, 6-, and 9-month samples. However, particular sources should not be emphasized, since carbon sources present in the EcoPlate do not accurately reflect the sources present in the real-world chicken litter ecosystems; so they cannot be interpreted as reflecting *in situ* utilization. Therefore, the carbon sources in the EcoPlate constitute a set of compounds that permit only estimation of relative potential metabolic versatility and rapid microbial community fingerprinting.

The aged chicken litter with reduced microbiological diversity and species richness might be more susceptible to invasion by *Salmonella* due to the lack of competition (26). Millner et al. (27) reported that the inactivation of *Salmonella* during composting was not due to the activity of a single group of microorganisms, indicating that the concentrations and diversity of background microorganisms in compost may affect the fate of this pathogen.

In this study, the microbial community analysis of chicken litter revealed changes in microbial species and population during 9-month storage; however, it is difficult to draw a conclusion about the impact on thermal resistance of *Salmonella* in chicken litter, as the chicken litter was exposed to an external heat source rather than the heat generated by indigenous microbial activities.

Effects of heavy metals of chicken litter on thermal resistance of desiccation-adapted *Salmonella* spp. In addition to macro- and micronutrients, chicken litter also contains different levels of heavy metals which are related to their additions in the diet or environmental source (28). Table 4 presents data on the concentrations of five common heavy metals, including Zn, Cu, Mn, Fe, and Al. In the present study, the total concentrations of these five heavy metals in chicken litter increased, reached a maximum at 6 months, and decreased thereafter. When Sims and Wolf (29) analyzed results from studies conducted from 1969 to 1992, they found that Zn and Cu concentrations in poultry litter ranged from nondetectable to 669 $\mu\text{g/g}$ and nondetectable to 1,003 $\mu\text{g/g}$, respectively. In this study, Cu concentrations determined throughout the 9-month storage were all within the range reported by Sims and Wolf (29), although Zn concentrations became slightly higher after 3 months. Hsu and Lo (30) studied the influence of composting on the concentrations of Cu, Mn, and Zn in swine manure. Total metal concentrations increased with time, while the water-soluble fractions of metals increased at the beginning of the 122-day composting and then gradually decreased at the end. Paré et al. (31) also found that the concentrations of Zn, Cr, Cu, and Pb increased by 145, 124, 73.6, and 26.3% during 41-day cocomposting of biosolids and municipal solid wastes, respectively, whereas the concentration of Co decreased by 60% and that of Ni maintained relatively constant. The difference between results of other studies and ours may be due to the fact that the duration of the stockpiling in our study was much longer and also the difference in the composition of compost material. In this study, the heavy metal concentrations reached the highest level at the 6-month sampling time. Accordingly, desiccation-adapted *Salmonella* in chicken litter became increasingly resistant to lethal temperatures when storage time increased from 0 to 6 months. However, there was no significant difference between heat resistance characteristics of *Salmonella* in 6- and 9-month chicken litter ($P > 0.05$), even though the 9-month chicken litter had a lower moisture content and ammonia concentration than the 6-month chicken litter. Therefore, our findings at least implied that heavy metals could possibly be one of the factors enhancing the thermal resistance of *Salmonella* cells in chicken litter. Further study is needed to delineate the role of heavy metals in the thermal resistance of microorganisms in manure and compost.

We acknowledge that a limitation of this study was the use of two replicates per trial in our experimental design. Therefore, the results should be interpreted with some caution, especially when the heterogeneity of chicken litter is taken into consideration.

In conclusion, our results revealed that the thermal resistance of desiccation-adapted *Salmonella* in chicken litter was affected by storage times. Moisture and ammonia could be two of the most significant factors influencing the thermal resistance of *Salmonella* cells in chicken litter. This is a novel study in which we have demonstrated that the desiccation-adapted *Salmonella* cells in fresh chicken litter are more susceptible to thermal inactivation than those in aged chicken litter, highlighting the importance of considering the storage time of chicken litter as a critical factor when

chicken litter is heat processed. Our recommendation to the chicken litter processing industry is to thermally process chicken litter immediately after removal from chicken houses to decrease the development of heat resistance in *Salmonella* spp.

ACKNOWLEDGMENTS

We thank Mendel Stone at the Mendel Stone Farm, SC, for assisting us with stockpiling and maintenance of chicken litter heap.

This research was financially supported by a grant (201121654-01) from the Center for Produce Safety, University of California, Davis.

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