FINAL REPORT
September 7, 2016

Pilot study of applying *Salmonella*-specific bacteriophages in a rendering processing plant

Principal Investigator(s): Xiuping Jiang, Prof.
xiuping@*clemson.edu*
Department of Food, Nutrition, Packaging Sciences
228A Life Sciences Facility
Clemson University
Clemson, SC 29634
(864) 656-6932

Co-PI: Annel K. Greene, Prof.
agreene@clemson.edu
Department of Animal and Veterinary Sciences
250 Poole Agricultural Center
Clemson University
Clemson, SC 29634
(864) 656-3123

Date Submitted: 9/6/2016

Start Date: 7/5/2012

Duration of Project: 4 years

Lay Summary:

The goal of this proposed study was to determine if bacteriophage treatment can be an effective and low-cost approach for controlling *Salmonella* contamination in rendering environment. In this project, we first conducted a survey of *Salmonella* in two rendering plants, then tested the methods for producing bacteriophages in large quantities, and finally determined the efficacies of bacteriophage cocktail for reducing *Salmonella* attachment/biofilms on the surfaces under lab, greenhouse, and rendering plant conditions.

A microbiological analysis of *Salmonella* contamination was conducted in two rendering plants. Sampling locations were pre-determined at the potential areas where *Salmonella* contamination may occur including raw materials receiving, crax grinding and the finished meal loading-out areas. Among 108 samples analyzed, 79 samples (73%) were *Salmonella*-positive after enrichment. Selected *Salmonella* isolates (n = 65) were identified to 31 unique PFGE patterns, and 16 *Salmonella* serotypes including Typhimurium and Mbandaka identified as predominant serotypes, and 10 *Salmonella* strains were determined as strong biofilm formers. Raw material receiving area was found as the primary source of *Salmonella*, whereas the surfaces surrounding crax grinding and the finished meal loading-out areas harbor *Salmonella* in biofilms. The same *Salmonella* serotypes found in both raw materials receiving and the finished meal loading-out areas also suggested a potential of cross-contamination between different areas in rendering processing environment.

A mixed bacteriophage production in a single batch was developed. To scale up the production of *Salmonella*-specific bacteriophages with low cost for field study. Bacteriophage titer of mixed bacteriophage production yielded 10.3 log PFU/ml with optimized conditions of multiplicity of infection (MOI) of 0.01, agitation speed of 200 rpm, nalidixic acid at concentration of 0.06 μg/ml and incubation time of 8 h at 37°C. Additionally, final titer of bacteriophage production could reach up to 11.5 log PFU/ml with a PEG-6000 precipitation at concentration of 8% and sodium chloride at concentration of 3%.

To determine the efficacy of bacteriophage cocktail for reducing *Salmonella* attachment/biofilms on the surfaces, a mixture of 6 *Salmonella*-specific bacteriophage strains was selected among 94 bacteriophages for bacteriophage treatment based on evaluating host ranges against the 10 selected *Salmonella* isolates obtained from rendering plants. The effectiveness of bacteriophage treatment with titers of 10^4-10^8 PFU/ml was evaluated against strong *Salmonella* biofilm formers using a colorimetric method in 96-well microplate. Furthermore, the bacteriophage treatment with a titer of 10^9 PFU/ml was applied for 7 days to reduce *Salmonella* attached to the stainless steel surfaces in laboratory and different seasons under greenhouse conditions. With bacteriophage treatment in 96-well microplate, the inhibition of biofilm formation and reduction of pre-formed biofilm of *Salmonella* reached up to 90 and 66%, respectively. Bacteriophage treatment reduced up to ca. 2.9 and 3.0 log CFU/cm^2 of slightly formed biofilm/attachment of selected top 10 *Salmonella* biofilm former strains and strain 8243, respectively, under laboratory condition, as compared with 3.4, 1.4 and 3.0 log CFU/cm^2 of *Salmonella* strain 8243 in summer, fall/winter and spring seasons under greenhouse condition, respectively.

To test the efficacy of above *Salmonella*-specific bacteriophage cocktail for reducing *Salmonella* contamination on workers' rubber boots, biofilms of *Salmonella* Typhimurium strain 8243 formed on rubber templates or boots were treated with the same *Salmonella*-specific bacteriophage cocktail of 6 strains (ca. 9 log PFU/ml) for 6 h under laboratory condition. *Salmonella*-specific bacteriophage treatments combined with sodium hypochlorite (400 ppm), 10-min pre-treatment

with sodium hypochlorite (400 ppm) or brush scrubbing (30 sec) were also investigated for a synergistic effect on reducing *Salmonella* biofilms. Under laboratory condition, *Salmonella* biofilms formed on rubber templates and boots were reduced by 95.1-99.999% and 91.5-99.2%, respectively.

To reduce contamination of indigenous *Salmonella* on workers' boots, two trials of field study were conducted to apply *Salmonella*-specific bacteriophage treatments for 1 week at a rate of 3 times in rendering processing environment. In rendering processing environment (average temperature: 19.3ºC; average relative humidity: 48%), indigenous *Salmonella* populations on workers' boots were reduced by 84.5, 92.9, and 93.2% after treated with bacteriophage cocktail alone, bacteriophages + sodium hypochlorite, and bacteriophage + scrubbing for 1 week, respectively.

In conclusion, our study examined the current contamination rates of *Salmonella* in rendered animal meals and rendering processing environment, and indicated the high potential of finished meals being re-contaminated with *Salmonella* biofilms during the post-rendering process. We also optimized a scale-up production of mixed bacteriophages in a single batch with reduced cost for field application. Under both lab and greenhouse conditions, our study demonstrated that bacteriophages could reduce the *Salmonella* attachment/biofilms formed on various surfaces effectively. Furthermore, the results of field study demonstrated the effectiveness of bacteriophage treatments in reducing indigenous *Salmonella* attachment/biofilms formed on the surfaces of workers' boots in rendering processing environment.

Overall, our research findings validated bacteriophage treatment as an effective, non-corrosive and environmentally friendly biological control method to reduce *Salmonella* attachment/biofilms in rendering processing environment, thereby, helping the rendering industry to have a safe working environment for workers and produce high quality rendered animal meals free from *Salmonella* contamination.

Objective (s):

1) Identifying the sources of *Salmonella* contamination in rendering processing environment;
2) Optimizing a scale-up production of *Salmonella*-specific bacteriophages;
3) Determining the effectiveness of bacteriophage treatment on reducing *Salmonella* attachment /biofilms on the surfaces under laboratory and greenhouse conditions;
4) Applying bacteriophage treatment to reduce *Salmonella* attachment/biofilms on the surfaces in rendering processing environment.

Project Overview: (Detail project report for Objective 1 can be found in one published article as attachment)

Sampling the rendering plants (Obj. 1): Two rendering processing plants were sampled for total aerobic bacterial count and the presence of *Salmonella* from June, 2012 to August, 2012. Based on the continuous rendering process layout, the raw material receiving area was determined to be the major source of *Salmonella*, whereas the crax grinding area that grinds dried rendered materials

and finished meal loading-out areas were also selected for sampling as locations of potential cross-contamination (Meeker & Hamilton, 2006).

Different types of solid materials (ca. 500 g for each) including raw poultry by-products (n = 12), rendered crax (n = 12) and finished meal products (n = 12) made from pet food grade poultry offal, animal feed grade poultry offal and poultry feathers were aseptically collected as duplicate samples in sterile sampling bags (Whirl-Pak®, Fort Atkinson, WI, USA). Environmental surface samples (n = 40) were obtained in duplicates using 3M sampling swabs (3M, St. Paul, MN, USA) with a sterile paper grid (10 × 10 cm^2) from the surfaces of those three selected areas. Tryptic soy agar (TSA, Becton Dickinson, Sparks, MD, USA) and xylose-lysine-tergitol-4 (XLT-4, Becton Dickinson, Sparks, MD, USA) agar plates in duplicates were exposed to the air for 30 min to collect air samples (n = 32) for total aerobic bacteria and *Salmonella*, respectively. Both media were supplemented with 50 mg/l of cycloheximide (Sigma-Aldrich, St. Louis, MO, USA) to inhibit fungal growth. To detect a very low level of *Salmonella*, a pre-enrichment step was included to overcome the detection limit (0.7 log CFU/g or ml) of direct plating method. A sterile empty petri dish containing 10 ml of universal pre-enrichment broth (UPB, Neogen, Lansing, MI, USA) supplemented with 50 mg/l of cycloheximide was placed at the selected locations, left open for 30 min, and then transferred aseptically to a sterile centrifuge tube (Corning Inc., Corning, NY, USA). All samples were packed in ice, transported to laboratory and analyzed immediately upon arrival.

Sample analysis for total aerobic bacteria and* Salmonella *(Obj. 1): Solid sample (25 g) was blended with 225 ml of UPB in a stand-up bag (Whirl-Pak®, Fort Atkinson, WI, USA). Swab sample was vortexed for 5 s to release bacterial cells into broth. Blended solid samples and swab samples were serially diluted with 9 ml of 0.85% sterile saline. A 100 µl of these diluted samples and UPB broth of air samples were plated onto TSA and XLT-4 plates, supplemented with 50 mg/l of cycloheximide, followed by a 24 h incubation at 35°C for enumerating total aerobic bacteria and presumptive *Salmonella* populations, respectively. All air sample plates were directly incubated at 35°C for 24 h. To enrich *Salmonella* from those samples, solid samples blended with UPB and air samples collected in UPB broth were directly used for enrichment. Swab samples were mixed with 9 ml of UPB and vortexed for 5 s. After 24 h incubation at 35°C, 1 ml of each pre-enrichment culture was transferred to 9 ml of Rappaport-Vassiliadis broth (RV, Becton Dickinson, Sparks, MD, USA) and incubated at 42°C for 24 h. Another 1 ml of pre-enrichment culture was transferred to 9 ml of Tetrathionate broth (TT, Becton Dickinson, Sparks, MD, USA) and incubated at 43°C for 24 h. A loopful culture from each selective enrichment broth was streaked onto each of Bismuth Sulfate (BS, Becton Dickinson, Sparks, MD, USA) and XLT-4 plates and incubated at 35°C for 24 h. From each plate, 3 or 4 typical black colonies on either BS or XLT-4 plates were selected based on morphology such as size and darkness, and isolated by resteaking on XLT-4 plates twice. Further purification was performed by resteaking onto TSA plates twice. Pure *Salmonella* isolates were further confirmed by streaking on CHROMagar® plate (CHROMagar, Paris, FR) and testing for agglutination in *Salmonella* latex test (Oxoid, Basingstoke, UK).

A total of 108 samples were collected from two rendering plants and 79 samples (73%) were tested positive for *Salmonella* after enrichment. For the air samples (n = 32), total aerobic bacterial counts ranged from ca. 1-3 log CFU/plate (Tables 1 & 2). *Salmonella* were below detection limit (<1 CFU/plate) in all air samples collected from finished meal loading-out area but enumerated in 33 and 17% air samples obtained from raw material receiving and crax grinding areas, respectively.

After enrichment, an average of 53% air samples (50% for raw material receiving, 50% for crax grinding area, and 63% for finished meal loading-out area) were positive for *Salmonella*.

For the surface samples (n = 40), there were higher numbers of total aerobic bacterial counts on the surface of raw material receiving area (ca. 7-9 log CFU/cm^2) than surface samples of loading-out area (ca. 5-7 log CFU/cm^2) and crax grinding area (ca. 4-5 log CFU/cm^2) (Tables 1 & 2). *Salmonella* were enumerated with highest counts from raw material receiving area (ca. 3-4 log CFU/cm^2) followed by loading-out (ca. <0-3 log CFU/cm^2) and crax grinding areas (ca. <0-2 log CFU/cm^2).

For the solid samples (n = 36), raw materials contained the highest counts of total aerobic bacteria and *Salmonella* in the range of ca. 7-9 log and 4-6 log CFU/g, respectively (Tables 1 & 2). Total aerobic bacterial counts in crax varied in the range of ca. 1-5 log CFU/g. *Salmonella* was not enumerated in any of crax samples by direct plating, but *Salmonella* colonies were observed on 16.7% replicate plates after enrichment resulting in 33% samples positive for *Salmonella*. As for the finished meals at the loading-out area, total aerobic bacterial counts were enumerated in the range of ca. 2-5 log CFU/g. All finished meals were negative for *Salmonella* by direct plating, but *Salmonella* colonies were observed on 33% replicate plates after enrichment resulting in 66.7% samples positive for *Salmonella*.

Apparently, total aerobic bacterial counts and *Salmonella* in finished meals were higher ($P < 0.05$) than both solid and surface samples collected in crax area, indicating that the recontamination of rendered meals probably occurred in the loading-out area. Moreover, high contamination rate of *Salmonella* in surface samples, especially in crax grinding and meal loading-out areas, which cannot be cleaned by water due to dry processing environment, may suggest that the pathogen exists in the biofilms on the surfaces in rendering processing environment. Therefore, these biofilms could be the source of recontamination of finished animal meals.

Plant A had less number of *Salmonella* positive samples after enrichment as compared with plant B in both finished meal loading-out and crax processing areas for animal and pet food grade meals, although *Salmonella* contamination rate was higher in the raw materials receiving area of plant A as compared to plant B (Tables 1 & 2). During the sampling, we observed that rendering plant A had a slightly better hygienic practices, such as workers' boots being disinfected frequently, separated processing areas and routinely cleaned raw by-products receiving area, as compared to the rendering plant B. These good hygienic practices in plant A may have minimized the spread of *Salmonella* from raw materials receiving area to other processing areas.

Table 1. Microbiological analysis of total aerobic bacterial counts and *Salmonella* in plant A

Location	Meal type	Sample type	Plate count [a]		*Salmonella* after enrichment	*Salmonella* isolates
			Total aerobic bacteria	Presumptive *Salmonella*		
Raw materials receiving area	Feather	Air	2.75±0.01 (k)	<0 G	+/- [b]	0
		Surface	7.16±0.02 (d)	2.96±0.01 D	+	6
		Solid	9.11±0.10 (a)	5.28±0.31 B	+	4
	Feed grade	Air	1.57±0.01 (l)	0.74±0.04 G	+	5
		Surface	8.09±0.21 (c)	3.03±0.03 D	+	3
		Solid	8.26±0.01 (b)	5.79±0.01 A	+	6
	Pet grade	Air	1.35±0.37 (l)	0.65±0.27 G	+	5
		Surface	8.05±0.26 (c)	2.99±0.02 D	+	2
		Solid	7.85±0.03 (c)	4.88±0.01 C	+	7
Crax [c]	Feather	Air	1.83±0.01 (l)	<0 G	+	1
		Surface	5.25±0.01 (g)	1.65±0.15 F	+	12
		Solid	3.79±0.02 (i)	<0 G	-	0
	Feed grade	Air	1.77±0.04 (l)	<0 G	+/-	0
		Surface	4.80±0.03 (h)	1.38±0.22 F	+	2
		Solid	1.61±0.09 (l)	<0 G	-	0
	Pet grade	Air	1.77±0.01 (l)	<0 G	-	0
		Surface	5.93±0.07 (f)	1.72±0.03 F	+	11
		Solid	1.70±0.03 (l)	<0 G	-	0
Finished meal loading-out	Feather	Air	1.43±0.02 (l)	<0 G	+/-	0
		Surface Floor	7.05±0.12 (d)	2.90±0.20 D	+	1
		Surface Wall	7.17±0.14 (d)	2.85±0.04 D	+	1
		Solid	4.51±0.05 (h)	<0 G	+/-	0
	Feed grade	Solid	3.04±0.01 (j)	<0 G	+/-	0
	Pet grade	Air	1.54±0.01 (l)	<0 G	+/-	0
		Surface Floor	6.72±0.05 (e)	2.30±0.11 E	+	1
		Surface Wall	6.59±0.25 (e)	3.00±0.18 D	+	4
		Solid	2.68±0.03 (k)	<0 G	-	0

[a] Average of duplicate samples ± standard deviation; units for air, surface and solid samples are log CFU/plate, log CFU/cm^2 and log CFU/ g, respectively. Average numbers with different letters in the same column are significantly different ($P < 0.05$).

[b] *Salmonella* detected in either one of duplicate samples or only one of selective medium plate.

[c] Crax, rendered materials prior to grinding process.

Table 2. Microbiological analysis of total aerobic bacterial counts and *Salmonella* in plant B

Location	Meal type	Sample type		Plate count [a]		*Salmonella* after enrichment	*Salmonella* isolates
				Total aerobic bacteria	Presumptive *Salmonella*		
Raw materials receiving area	Feather	Air		1.94±0.03 (l)	<0 G	- [b]	0
		Surface		7.55±0.05 (e)	4.01±0.13 D	+	7
		Solid		9.41±0.01 (a)	6.05±0.07 A	+	6
	Feed grade	Air		1.75±0.03 (l)	<0 G	+/-	0
		Surface		9.11±0.06 (b)	4.82±0.06 C	+	6
		Solid		7.66±0.05 (e)	4.70±0.09 C	+	2
	Pet grade	Air		2.15±0.02 (l)	<0 G	-	0
		Surface		8.56±0.06 (c)	3.88±0.03 D	+	6
		Solid		8.38±0.01 (d)	5.96±0.02 B	+	5
Crax [c]	Feather	Air		0.81±0.03 (l)	<0 G	-	0
		Surface		4.70±0.10 (i)	2.30±0.24 F	+	8
		Solid		5.57±0.16 (h)	<0 G	+	4
	Feed grade	Air		1.04±0.04 (l)	0.39±0.30 G	+	1
		Surface		5.19±0.18 (h)	3.19±0.36 E	+	14
		Solid		4.89±0.01 (i)	<0 G	+	4
	Pet grade	Air		1.69±0.05 (l)	<0 G	+/-	0
		Surface		5.99±0.01 (g)	<0 G	+/-	0
		Solid		4.72±0.05 (i)	<0 G	-	0
Finished meal loading-out	Feather	Air		2.13±0.07 (l)	<0 G	+/-	0
		Surface	Floor	6.96±0.01 (f)	3.53±0.16 E	+	7
			Wall	5.06±0.40 (h)	<0 G	+/-	0
		Solid		5.03±0.11 (h)	<0 G	+	6
	Feed grade	Solid		2.97±0.07 (k)	<0 G	+	6
	Pet grade	Air		3.32±0.09 (j)	<0 G	+	1
		Surface	Floor	6.62±0.01 (f)	2.40±0.35 F	+	6
			Wall	5.31±0.16 (h)	2.54±0.42 F	+	1
		Solid		4.17±0.09 (i)	<0 G	+	5

[a] Average of duplicate samples ± standard deviation; units for air, surface and solid samples are log CFU/plate, log CFU/cm^2 and log CFU/g, respectively. Average numbers with different letters in the same column are significantly different ($P < 0.05$).

[b] *Salmonella* detected in either one of duplicate samples or only one of selective medium plate.

[c] Crax, rendered materials prior to grinding process.

PFGE analysis, serotyping and biofilm-forming ability of selected* Salmonella *isolates (Obj. 1):
Pulsed-field gel electrophoresis (PFGE) analysis was conducted according to Centers for Disease Control and Prevention (CDC, 2013) version of one-day rapid PFGE procedure. Briefly, overnight *Salmonella* culture prepared in agarose plugs was treated in cell lysis buffer at 54°C for 2 h with shaking (80 rpm). After washing with TE buffer, plugs were digested by XbaI restriction enzyme (Promega, Madison, WI, USA) at 37°C for 16 h, and then electrophoresed for 18 h in 0.5 × Tris-Borate-EDTA (TBE) buffer at 14°C using a CHEF Mapper XA System (Bio-Rad, Hercules, CA). Following ethidium bromide staining for 30 min, the banding patterns were observed under ultraviolet (UV) illumination and a digital image of the PFGE patterns was acquired using the Gel Doc system (Bio-Rad, Hercules, CA). *Salmonella* serotype Braenderup strain H9812 was used as a standard strain for DNA markers. *Salmonella* isolates with different PFGE patterns were sent to United States Department of Agriculture-National Veterinary Service Laboratory (USDA-NVSL, Ames, IA, USA) for serotype determination.

To determine the biofilm-forming ability of each *Salmonella* isolate, overnight cultures of selected *Salmonella* isolates were collected by centrifugation at 5,000 × g, washed with 0.85% sterile saline and adjusted to OD of 0.5 at a wavelength of 600 nm (ca. 9 log CFU/ml). A 20 µl of 1:10,000 diluted culture of each selected isolate was mixed with 180 µl of 10% tryptic soy broth (TSB, Becton Dickinson, Sparks, MD, USA) in eight replicate wells of a 96-well microplate (Costar®, Corning Inc., Corning, NY, USA). After static incubation at 30°C for 48 h, each well was washed with sterile distilled water and allowed to air-dry. Biofilm in each well was stained by 1% crystal violet solution (Becton Dickinson, Sparks, MD) at 22°C for 45 min followed by an elution with 95% ethanol, and then measured by a spectrometer (µQuant; Bio Tek, Winooski, VT, USA) at 600 nm. Bacterial strains of *Hafnia alvei* (S211) and *Pseudomonas aeruginosa* (ATCC® 27853) were included as reference of strong biofilm formers (Gong & Jiang, 2015).

A total of 166 *Salmonella* isolates were isolated from samples that were *Salmonella*-positive after enrichment, and confirmed using both CHROMagar® and *Salmonella* latex test. Among those *Salmonella* isolates, 70 isolates were from raw materials receiving area, while 57 isolates were obtained from crax area, particularly from surface swabs, and 39 isolates from finished meal loading-out area (Tables 1 & 2). Sixty five representative isolates were selected proportionally from different sampling locations and sample types for PFGE analysis (Figure 1). PFGE pattern of each selected *Salmonella* isolate was obtained, and the isolates sharing the same PFGE patterns were assigned to the same numbers from 1 to 31. Figure 1A-B present the patterns of *Salmonella* isolates obtained from the rendering plant A while Figure 1C-E show the isolates from plant B.

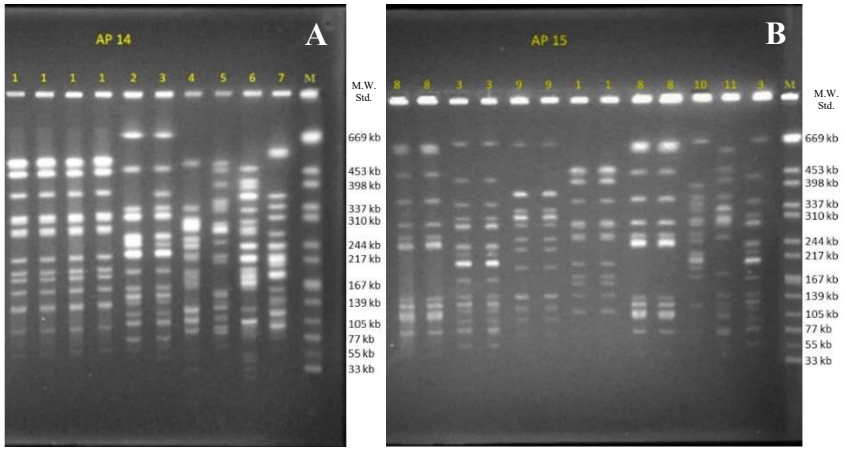
Salmonella isolates (n = 31) with different PFGE patterns were then serotyped by USDA-APHIS lab, and twenty-two isolates were identified into 16 serotypes (Table 3). Mbandaka and Typhimurium were the two most frequently isolated serotypes each including 6 *Salmonella* isolates. Each of serotypes Schwarzengrund and Ealing included 5 *Salmonella* isolates followed by serotypes Widemarsh, Infantis, Senftenberg, Johannesburg, Kentucky, and Heidelberg including 4, 3, 3, 3, 2, and 2 *Salmonella* isolates, respectively. There was only one *Salmonella* isolate identified for each of the rest serotypes.

The biofilm forming ability of *Salmonella* was measured using a colorimetric method in 96-well microplate with a cut-off optical density (ODc) value set as 0.042. The biofilms formed by 22 selected *Salmonella* isolates were determined with average optical density (OD) value of 0.37 in a range of 0.19-0.62 (Figure 2). There were 13 *Salmonella* isolates formed at more or the same level of biofilms as compared to a strong biofilm former *Hafnia* strain S211 in our previous study (Gong

& Jiang, 2015). The average OD value of top 10 isolates was 0.47, which was higher than *Hafnia* strain S211 (OD of 0.37).

To measure the ability of bacteria to form biofilm using a colorimetric method, the following criteria have been used widely: no biofilm producer = OD ≤ ODc, weak biofilm producers = ODc < OD ≤ 2 × ODc, moderate biofilm producer = 2 × ODc < OD ≤ 4 × ODc, and strong biofilm producer = OD > 4 × ODc (Stepanovic et al., 2003). In this study, all tested *Salmonella* isolates formed biofilms having OD values greater than 4 × ODc, i.e. 0.168 (Figure 2). Therefore, these selected *Salmonella* isolates are considered as strong biofilm producers suggesting a high potential of biofilm formation by this pathogen on the surfaces in rendering plant.

In conclusion, above results from Obj. 1 analyzed the *Salmonella* contamination levels and total aerobic bacterial counts of air, surface and solid samples collected from protective sites for pathogen persistence in two typical rendering processing plants. Raw material receiving area was found as the primary source of *Salmonella*, whereas surfaces surrounding the crax grinding area and finished meal loading-out area also harbor *Salmonella*, probably in biofilms that may re-contaminate the finished meals. *Salmonella* serotypes Typhimurium, Infantis and Senftenberg found in both raw materials receiving area and finished meal loading-out area indicated a potential of cross-contamination between different areas in a rendering processing environment. Moreover, strong biofilm formers of *Salmonella* isolates such as Typhimurium and Senftenberg found in the rendering processing plants may consistently challenge the microbiological safety of animal feed and pet food by reducing the effectiveness of sanitation practices currently employed in rendering industry. This also suggests that good cleaning practices and development of more effective disinfection methods are urgently needed for rendering industry.



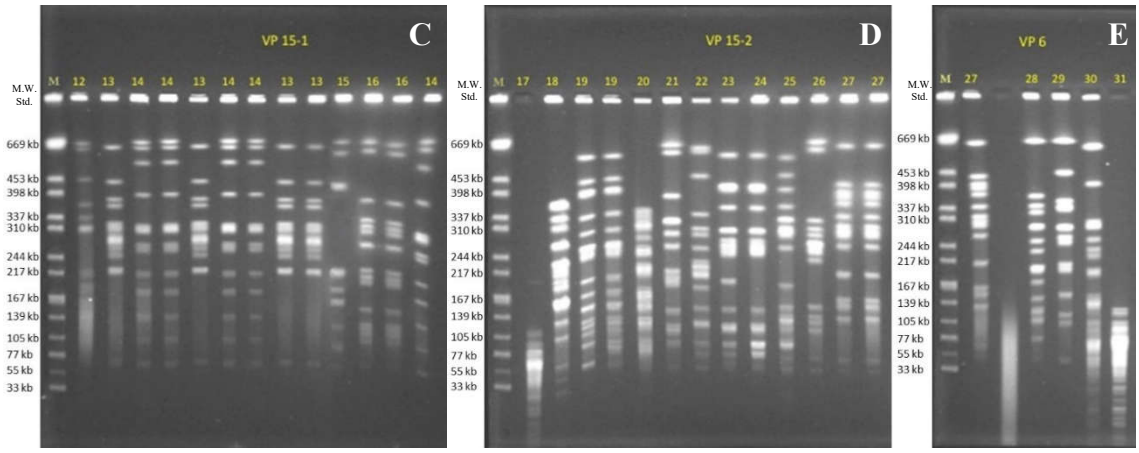


Figure 1. PFGE patterns of selected *Salmonella* isolates obtained from rendering plant A (Panels A and B) and B (Panels C, D and E). Different numbers at the top of each panel indicate different PFGE patterns. On panel A, pattern numbers 1, 2 and 3 represent *Salmonella* serotypes Mbandaka, Schwarzengrund and Schwarzengrund, respectively. On panel B, pattern numbers 8, 9, 10 and 11 represent *Salmonella* serotypes Typhimurium, Infantis, Urbana and Kentucky, respectively. On panel C, pattern numbers 12, 13, 14, 15 and 16 represent *Salmonella* serotypes Uganda, Widemarsh, Ealing, Pomona and Senftenberg, respectively. On panel D, pattern numbers 19, 21, 22, 23, 24, 25, 26 and 27 represent *Salmonella* serotypes Heidelberg, Senftenberg, Ouakam, Typhimurium, Typhimurium, Kentucky, Hadar and Johannesburg, respectively. On panel E, pattern numbers 28 and 29 represent *Salmonella* serotypes Infantis and Tennessee, respectively. Pattern numbers 4, 5, 6, 7, 17, 18, 20, 30 and 31 represent untypable serovars of *Salmonella*. Pattern M represents standard DNA marker of *Salmonella* serotype Braenderup strain H9812.

Table 3. Serotypes of *Salmonella* isolates associated with sampling locations

***Salmonella* Serotype**	**Location[a]**	**No. of *Salmonella* Isolates**	***Salmonella* Serotype**	**Location**	**No. of *Salmonella* Isolates**
Mbandaka	CG & LO[b]	6	Ealing	CG	5
Schwarzengrund	RR	5	Pomona	LO	1
Kentucky	RR	2	Senftenberg	RR & LO[b]	3
Urbana	LO	1	Heidelberg	RR	2
Typhimurium	RR & LO[b]	6	Ouakam	RR	1
Infantis	RR & LO[b]	3	Hadar	RR	1
Uganda	CG	1	Johannesburg	LO	3
Widemarsh	CG	4	Tennessee	LO	1

[a] LO, finished meal loading-out area; CG, crax grinding area; RR, raw material receiving area.
[b] Indicates a potential of cross-contamination.

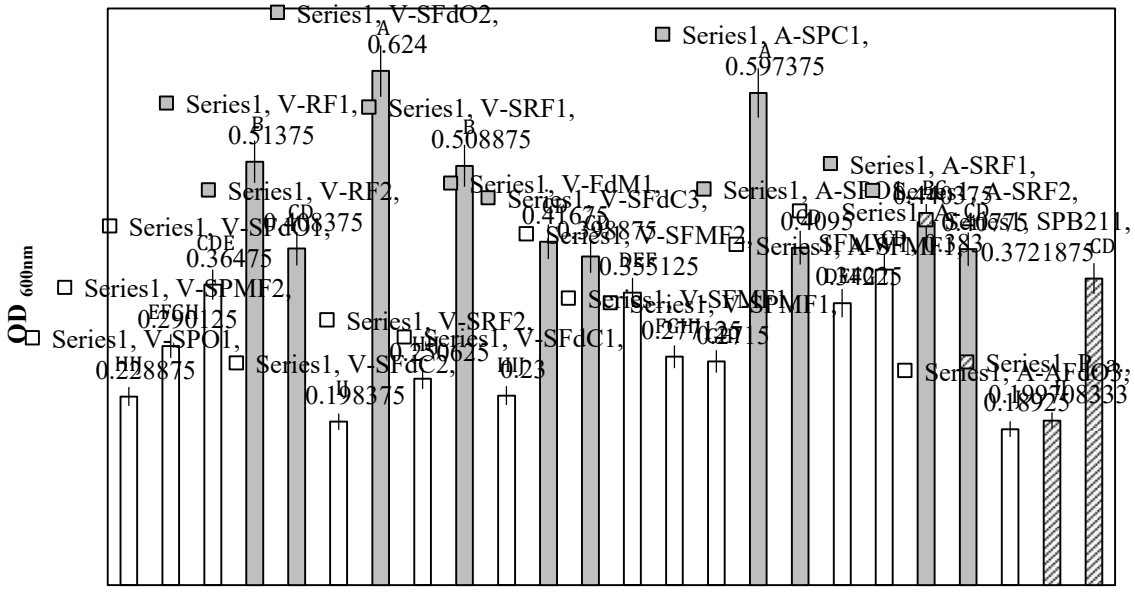


Figure 2. Biofilm formation of selected *Salmonella* isolates obtained from rendering plants in 96-well microplate. The top 10 biofilm producing *Salmonella* isolates are indicated with "■" namely: V-RF1 (Typhimurium), V-RF2 (Typhimurium), V-SFdO2 (Ouakam), V-SRF1 (Kentucky), V-FdM1 (Johannesburg), V-SFdC3 (Ealing), A-SPC1 (Mbandaka), A-SPO1 (Kentucky), A-SRF1 (Schwarzengrund), and A-SRF2 (Schwarzengrund). Reference strains *Pseudomonas aeruginosa* (P. a.; ATCC® 27853) and *Hafnia alvei* (SPB 211) are indicated with "▨". Bars labeled with different letter are significantly different ($P < 0.05$).

Host ranges determination of bacteriophages against selected* Salmonella *isolates (Obj. 2):

Salmonella-specific bacteriophages isolated from various sources were selected for this study based on host ranges of bacteriophages against above 10 *Salmonella* isolates. Host range determination was performed using a soft agar overlay method (Gong & Jiang, 2015). Briefly, 3 ml of 0.6% melted agar (Becton Dickinson, Sparks, MD, USA) containing a *Salmonella* overnight culture at a concentration of 10^7 CFU/ml was overlaid onto a tryptic soy agar (TSA; Becton Dickinson, Sparks, MD, USA) plate. After the overlaid agar plate solidified, a 10-µl drop of bacteriophage suspension was spotted onto the surface. Due to the broad host ranges, six bacteriophage strains JC1, S5p2, 29, 52, 1PB and VCA1 were selected for bacteriophage treatment of *Salmonella* in the following experiments (Table 4). Bacteriophage stock solutions were prepared according to Heringa et al. (2010). Prior to each experiment, bacteriophage stocks were incubated at 37°C for 30 min to reduce clumping and then diluted to the desired concentrations using sodium magnesium (SM) buffer [100 mM NaCl, 8 mM $MgSO_4 \cdot 7H_2O$, 50 mM Tris-HCl (pH 7.5)]. Bacteriophage titers were determined by the double agar layer plaque assay according to Heringa et al. (2010). The bacteriophage cocktail consisted of equal amounts of bacteriophages at a final titer of 1×10^4, 10^5, 10^6, 10^7 10^8 or 10^9 PFU/ml for following studies.

Table 4. Host ranges of selected bacteriophages against selected *Salmonella* isolates.

***Salmonella* isolates**	***Salmonella* serotype**	**Bacteriophages**					
		JC1	**S5p2**	**29**	**52**	**1PB**	**VCA1**
V-RF1[ab]	Typhimurium	+++[c]	+++	+++	+++	+	+
V-SFdO2[ab]	Ouakam	++	+++	+++	+++	+	++
V-SRF1[ab]	Kentucky	-	-	++	++	+	++
V-SRF2	Hadar	+	+	-	+	-	-
V-FdM1[ab]	Johannesburg	+++	+++	+++	+++	+	++
A-SRF1[ab]	Schwarzengrund	-	-	-	-	-	++
V-SFdO1[b]	Senftenberg	++	++	+++	+++	+	+
V-RF2[b]	Typhimurium	+++	+++	+++	+++	-	++
A-SFMW1[b]	Typhimurium	++	++	+++	+++	++	+++
A-SPO1[b]	Kentucky	-	-	-	-	+	+++
A-SRF2[b]	Schwarzengrund	++	++	+++	+++	+	++
8243	Typhimurium	++	++	+++	+++	++	+++

[a] Selected as top 5 strong *Salmonella* biofilm formers.
[b] Selected as top 10 strong *Salmonella* biofilm formers.
[c] Lysis results were recorded as decreasing lysis effect as follows: +++, ++, +; -, no lysis.

Scale-up production and PEG precipitation of* Salmonella-*specific bacteriophages (Obj. 2): An overnight culture of *Salmonella* strain 8243 was inoculated into a 4-liter flask (Fisherbrand™, Pittsburgh, PA, USA) or 8-liter carboy (Pyrex®, Corning, NY, USA) containing 2 liters of tryptic soy broth (TSB) at a final concentration of 7 log CFU/ml. To increase bacteriophage yield by slightly inhibiting the growth of bacterial host or enhancing bacteriophage attachment, either Mg^{2+} (50mM, $MgSO_4$, Fisher Scientific, Pittsburgh, PA, USA), ampicillin (0.003 or 0.06 μg/ml, Sigma, St. Louis, MO, USA) or nalidixic acid (0.003 or 0.06 μg/ml, Sigma, St. Louis, MO, USA) was added to the culture media separately. After a 30-min pre-incubation, a single bacteriophage S5p2 was inoculated with a MOI of 0.1 or 0.01, and the bacteriophage production was conducted at 37°C with an agitation speed of 200 rpm. Bacterial population and bacteriophage titer were enumerated every 2 h until 12 h using direct plating method on TSA plates and soft agar overlay method, respectively. In an effort to reduce the cost of bacteriophage production, a mixed bacteriophages production method was tested through inoculating a mixture of 6 bacteriophages (JC1, S5p2, 29, 52, 1PB and VCA1) into the 4-liter flask production system. A 5-liter bioreactor (New Brunswick BioFlo®/CelliGen® Model BF-115, Enfield, CT, USA) containing 4 liters of TSB was also employed for optimizing bacteriophage production since parameters such as temperature and agitation speed could be exactly controlled and monitored.

To increase the final titer of bacteriophages, polyethylene glycol (PEG) method was studied to precipitate the bacteriophage particles. PEG with molecular weight of 6000 or 8000 (Alfa Aesar®, Ward Hill, MA, USA) at concentrations of 4, 6, 8 or 10% (w/v) and 3% sodium chloride (NaCl, Fisher Scientific, Pittsburgh, PA, USA) were added into bacteriophage lysate and mixed thoroughly until completely dissolved. The mixture was incubated at 4°C for 24 h to precipitate the bacteriophage particles and then centrifuged at 14,000 × g for 30 min. Supernatant was poured off and the bacteriophage pellet was resuspended in SM buffer. Final bacteriophage titer was enumerated using a soft agar overlay method. All tests above were conducted in two trials and duplicate samples were obtained from each trial.

Based on bacteriophage growth curve (data not shown), an incubation of 8 h was required for reaching high yield of bacteriophages. In the 8-liter carboy and 4-liter flask production systems containing 2 liters of TSB, bacteriophage titers ranged 10.5-10.7 and 10.4-10.9 log PFU/ml, respectively, under different conditions such as growth medium concentrations, MOIs, and addition of antibiotics and Mg^{2+} (Table 5). In the mixed bacteriophages production, the titer of bacteriophage lysate resulted in up to 10.3 log PFU/ml with optimized conditions of MOI of 0.01, nalidixic acid concentration of 0.06 μg/ml, agitation speed of 200 rpm and incubation time of 8 h at 37°C. In the 5-liter bioreactor, bacteriophage production yielded a titer of 10.0 log PFU/ml. Among the single bacteriophage productions, the highest bacteriophage titer was yielded with addition of nalidixic acid at concentration of 0.003 μg/ml followed by addition of ampicillin at concentration of 0.06 μg/ml, and there was no significant difference among other conditions ($P > 0.05$). Although lower bacteriophage titers were enumerated in the mixed bacteriophage productions, the bacteriophage cocktail containing 6 different strains was produced in a single batch with a reduced cost for field application. It was also found that use of bioreactor did not enhance the yield of bacteriophage production, although the conditions of temperature, agitation speed and oxygen level were exactly controlled.

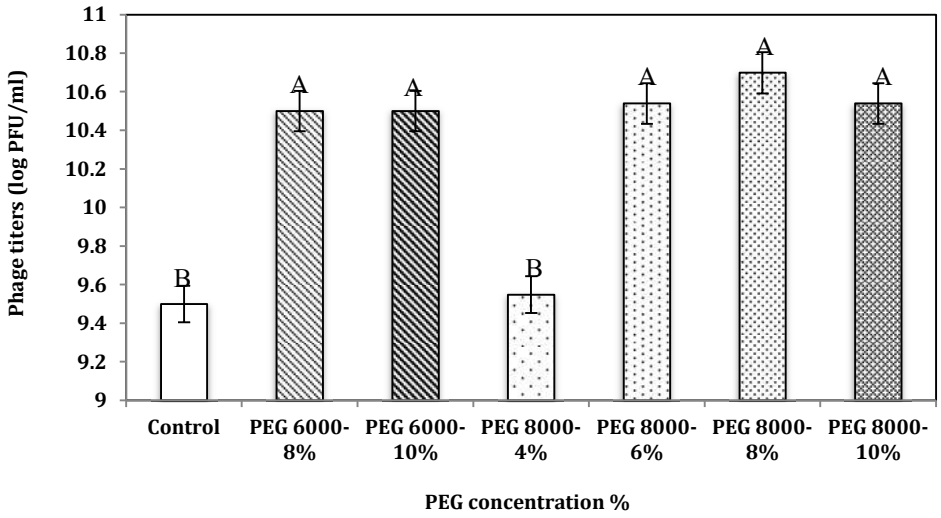


Figure 3. PEG precipitation of bacteriophage lysate with PEG 6000 or 8000 at concentrations of 4, 6, 8, or 10%. Data column with different letters are significantly different ($P < 0.05$).

Table 5. Summary of scale-up bacteriophage productions.

Production system	Volume (l)	Phage type[a]	Media (%)	MOI	Ampicillin (μg/l)	Nalidixic acid (μg/l)	Mg^{2+} (mM)	Phage titer[b] (log PFU/ml)
8-liter Carboy	2	Single	50	0.1	0	0	0	10.5±0.09C
			100	0.01	0	0	0	10.5±0.08C
			100	0.1	0	0	0	10.5±0.05C
					0.003	0	0	10.5±0.06C
					0.06	0	0	10.7±0.05B
4-liter Flask	2	Single	100	0.1	0	0	0	10.4±0.08CD
						0.003	0	10.9±0.02A
					0.06	0	50	10.5±0.05C
		Mix	100	0.1	0	0	0	9.6±0.04G
						0.06	0	9.7±0.03F
				0.01	0	0.06	0	10.3±0.08D
5-liter Bioreactor	4	Single	100	0.1	0	0	0	10.0±0.13E

[a] Single: only one bacteriophage S5p2 used for production; Mix: mixture of 6 bacteriophages (JC1, S5p2, 29, 52, 1PB and VCA1) used for production. [b] Average count of four plates from duplicate samples ± standard deviation. Numbers with different letters are significantly different ($P < 0.05$).

In the study of PEG precipitation, bacteriophage titers were further increased by ca. 1-1.2 log PFU/ml with PEG-6000 or 8000 at concentrations of 6, 8 or 10% as compared to the control with bacteriophage titer of 9.5 log PFU/ml (Figure 3), although there was no significant increase ($P > 0.05$) with PEG 8000 at concentration of 4%. Therefore, PEG-6000 at concentration of 8% was determined as the optimal conditions due to a lower cost as compared to PEG 8000.

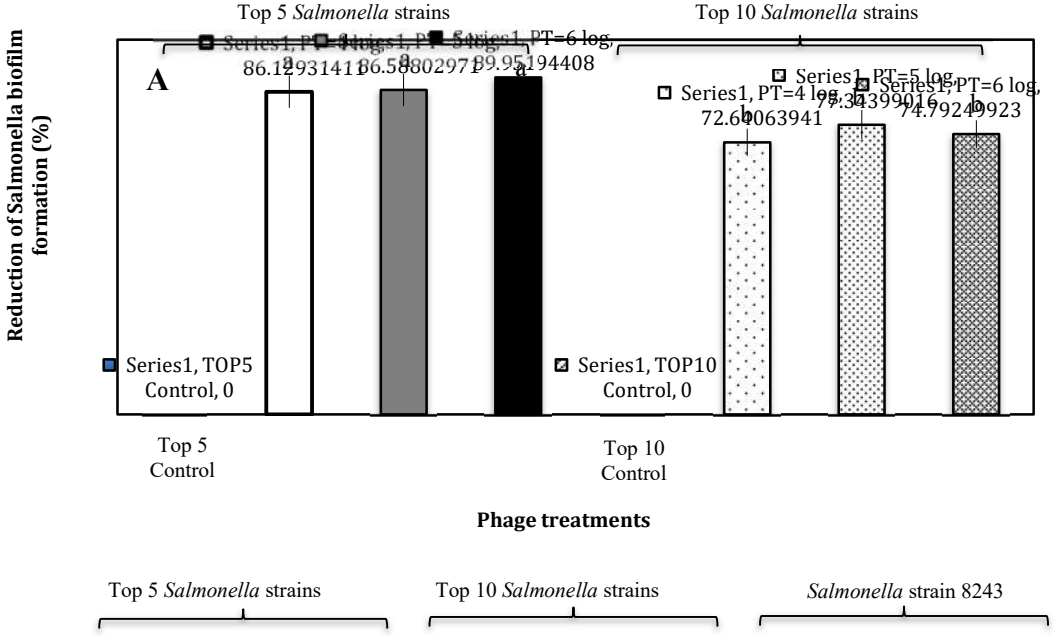
Overall, the final titer of optimized scale-up production of mixed bacteriophages could reach up to 11.5 log PFU/ml by combining the addition of nalidixic acid at concentration of 0.06 μg/ml and PEG-6000 precipitation at concentration of 8%.

Phage treatment of* Salmonella *biofilms formed in 96-well microplate (Obj. 3): A colorimetric method using 96-well microplate (Costar®, Corning Inc., Corning, NY, USA) was used to quantitatively determine the effectiveness of bacteriophage treatment on inhibiting biofilm formation and reducing pre-formed biofilm of *Salmonella*. Overnight culture of each selected *Salmonella* isolate was prepared as described above. In each well of 96-well microplate, a bacterial mixture of 5 or 10 selected *Salmonella* isolates was inoculated into 20% TSB at a final concentration of 10^4 CFU/ml. For inhibiting biofilm formation, *Salmonella*-specific bacteriophage cocktail was added into the bacterial mixtures at a final titer of 10^4, 10^5 or 10^6 PFU/ml followed by a static incubation at 30°C for 48 h. For reducing pre-formed biofilm, *Salmonella* was incubated at the same conditions as described above for 48 h to allow biofilm formation, and then followed by the same bacteriophage treatment at a final titer of 10^5, 10^6, 10^7 or 10^8 PFU/ml. SM buffer was

used as control. After bacteriophage treatment, each well was rinsed with sterile distilled water for 5 times and allowed to air-dry. Bacteriophage treated biofilms in each well was stained by 1% crystal violet solution (Becton Dickinson, Sparks, MD) at 22°C for 45 min followed by an elution with 95% ethanol, and then measured by a spectrometer (μQuant; BioTek, Winooski, VT, USA) at a wavelength of 600 nm.

A colorimetric method using 96-well microplate was employed to assess the biofilm-forming ability of *Salmonella* isolates and effectiveness of bacteriophage treatment on inhibiting biofilm formation and reducing pre-formed biofilm of *Salmonella* (Figure 4). Bacteriophage treatments with 6 bacteriophages (PT) with different titers (10^4, 10^5 or 10^6 PFU/ml) caused inhibition of *Salmonella* biofilm formation by 86.1-90.0 and 72.6-77.3% against top 5 and 10 strong *Salmonella* biofilm formers, respectively (Figure 4A). Bacteriophage treatments against top 5 strong *Salmonella* biofilm formers were more effective than against top 10 strong *Salmonella* biofilm formers ($P < 0.05$). The highest inhibition of 90% was observed in PT at a titer of 10^6 PFU/ml against top 5 strong *Salmonella* biofilm formers, although there was no significant difference as compared to bacteriophage treatment with titers of 10^4 and 10^5 PFU/ml.

For reducing the pre-formed biofilms, bacteriophage treatments of 6 bacteriophages with different titers (10^5, 10^6, 10^7 or 10^8 PFU/ml) reduced pre-formed biofilms of selected top 5, 10 strong biofilm formers strains and strain 8243 of *Salmonella* by 22.0-60.2, 28.5-62.1 and 20.0-65.9%, respectively (Figure 4B). The highest reduction of 65.9% was observed in PT at a titer of 10^8 PFU/ml against *Salmonella* strain 8243 and a dose-response effect was also observed among different titers. As compared to the test of inhibiting biofilm formation, lower effectiveness of bacteriophage treatment against pre-formed *Salmonella* biofilms was observed in all titers and strains. This could be explained by the complex structure of pre-formed biofilms that obstacle the bacteriophage treatment through multiple ways such as physical barrier, increased number of insensitive cells and decreased metabolic rate of biofilm cells.



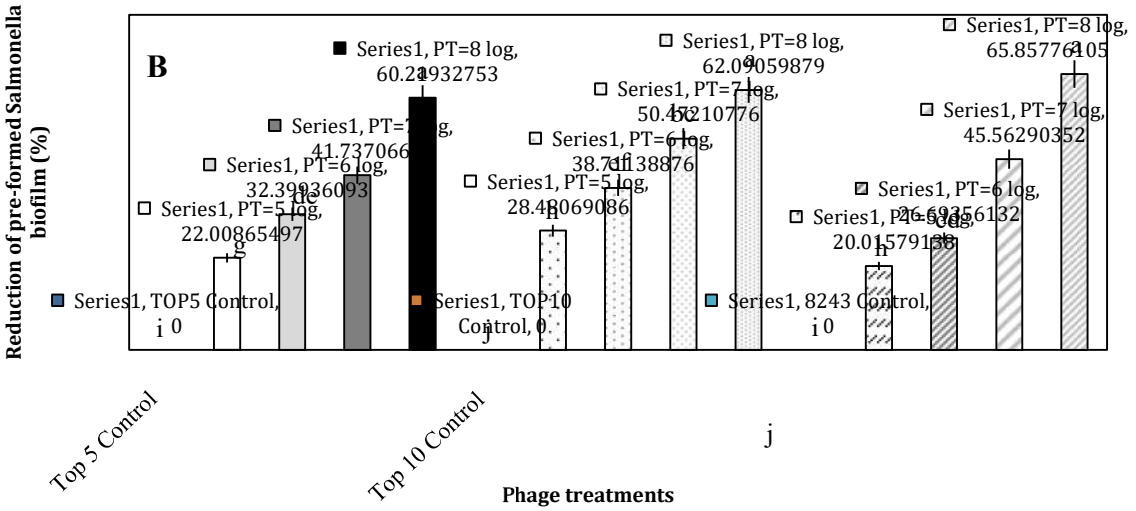


Figure 4. Bacteriophage treatment of *Salmonella* biofilm formation (A) and pre-formed *Salmonella* biofilm (B) in a 96-well microplate. PT=4 log, PT=5 log, PT=6 log, PT=7 log or PT=8 log indicates bacteriophage treatment (titer of ca. 10^4, 10^5, 10^6, 10^7 or 10^8 PFU/ml, respectively) containing 6 bacteriophages (JC1, Φ52, Φ29, S5p2, 1PB and VCA1). The control indicates the treatment with SM buffer. Solid columns indicate bacteriophage treatment against a mixture (concentration of 10^4 CFU/ml) of top 5 strong biofilm former *Salmonella* isolates obtained from rendering plants including V-RF1 (Typhimurium), V-SFdO2 (Ouakam), V-SRF1 (Kentucky), V-FdM1 (Johannesburg), and A-SRF1 (Schwarzengrund). Dotted columns indicate bacteriophage treatment against a mixture (concentration of 10^4 CFU/ml) of top 10 strong biofilm former *Salmonella* isolates obtained from rendering plants including V-SFdO1 (Senftenberg), V-RF2 (Typhimurium), A-SFMW1 (Typhimurium), A-SPO1 (Kentucky), A-SRF2 (Schwarzengrund) and those top 5 strong biofilm former *Salmonella* isolates above. Data column with different letters are significantly different ($P < 0.05$).

Phage treatment of* Salmonella *attached to stainless steel surface under laboratory and greenhouse conditions (Obj. 3): Ground chicken meat (ca. 1,000 g containing 50% saline, w/v) was artificially inoculated with a bacterial mixture of 10 selected *Salmonella* isolates for laboratory study or an avirulent *Salmonella* Typhimurium strain 8243 (kindly provided by Dr. Roy Curtis III, Washington University, St. Louis, MO) for greenhouse study at a concentration of ca. 10^8 CFU/g, and evenly spread onto the surface of four sterile stainless steel trays (50 cm × 20 cm). After 6 h of attachment under laboratory (Temperature 23±0.5°C; Relative humidity 48±3%) or greenhouse (summer, fall/winter and spring seasons) conditions, chicken meat was washed off using 200 ml of 0.85% (w/v) saline once. A 50 ml of the bacteriophage cocktail with a titer of 10^9 PFU/ml was sprayed onto the surface of stainless steel trays on which *Salmonella* attachment/slight biofilm was formed. The same volume of SM buffer was sprayed onto other 2 stainless steel trays as control. On 6 h and days 2, 4 and 7, the populations of attached *Salmonella* and slightly formed biofilm within an area of 10 × 10 cm were sampled using sterile cotton swabs (Puritan® Medical Products LLC., Guilford, ME, USA) and transferred into 10 ml of 0.85% (w/v) saline. Duplicate samples were obtained from both treatment and control groups. Samples were centrifuged at 5,000 × *g* for 10 min and supernatants were poured off to separate residual bacteriophages. Bacterial pellets were resuspended in 0.85% (w/v) saline and spread-plated onto XLT-4 plates for *Salmonella* enumeration.

Under greenhouse conditions, chicken meat was only inoculated with avirulent *Salmonella* strain 8243 for all trials and a multiple-dose bacteriophage treatment was investigated in spring season

only. In this approach, the same bacteriophage cocktail was sprayed repeatedly on sampling days 2 and 4 to enhance the effectiveness of bacteriophage treatment. The values of temperature and relative humidity in each trial under greenhouse conditions were recorded by a remote monitoring system (Argus Control System Ltd., White Rock, BC, Canada). Duplicate trials were conducted for all the settings in both laboratory and greenhouse conditions.

In order to determine if the *Salmonella* in contaminated chicken meat formed the biofilm on the surfaces, *Salmonella* strain 8243 was inoculated into the same ground chicken meat as described above and spread onto the surface of the same stainless steel tray. Sterile saline was used as blank control. After 6 h incubation under the same laboratory condition, the surface of stainless steel tray was rinsed with 200 ml sterile distilled water for 5 times to remove non-biofilm cells, allowed to air-dry and stained by crystal violet as described above. The stained biofilm within an area of 10 × 10 cm was swabbed in triplicates and then transferred into a sterile centrifuge tube containing 1 ml of 95% ethanol followed by vortex for 30 sec to elute the stained biofilm cells. The amount of formed biofilms was measured in a 96-well microplate as described above. The entire assay was performed in duplicate trials.

For bacteriophage treatment against 10 selected *Salmonella* isolates, population of *Salmonella* attached to the surface of stainless steel increased by 0.53 log CFU/cm^2 (71%) within first 6 h after removing chicken meat (Figure 5A), and slightly increased again by 2 days followed by a steady reduction to day 7. For bacteriophage treatment, the population of *Salmonella* attachment/slightly formed biofilm had a quick drop of 0.62 log CFU/cm^2 (76%) in first 6 h and started to decrease steadily to day 7. The highest reduction of ca. 2.93 log CFU/cm^2 (99.88%) was observed on day 7 as compared with the control.

For bacteriophage treatment against *Salmonella* strain 8243, the population of *Salmonella* attached to the surface of stainless steel increased by 0.84 log CFU/cm^2 (86%) in first 6 h after removing the chicken meat (Figure 5B), and slightly increased again by 2 days followed by steady reduction to day 7. In bacteriophage treatment group, the population of *Salmonella* attachment/slightly formed biofilm had a quick drop of 0.66 log CFU/cm^2 (78%) in first 6 h and started to decrease steadily to day 7. The highest reduction of ca. 3.0 log CFU/cm^2 (99.9%) was observed on day 7 as compared with the control.

We also determined if *Salmonella* biofilm was formed during those 6 h incubation with contaminated chicken meat on the stainless steel surfaces. The amount of formed *Salmonella* biofilms resulted in an average OD value of 0.40, which was significantly higher ($P < 0.05$) than the control (average OD value of 0.14). Clearly, the biofilm was formed on the surfaces during the 6 h incubation time at room temperature.

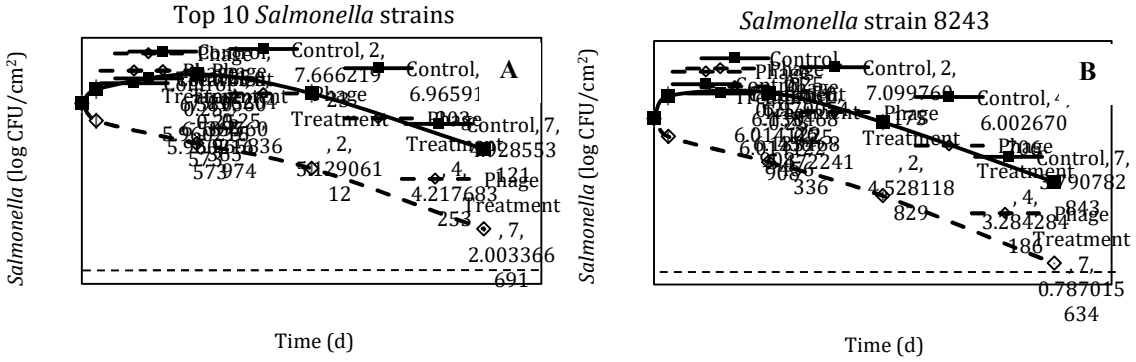


Figure 5. Bacteriophage treatment (titer of ca. 10^9 PFU/ml) of *Salmonella* biofilm/attachment formed by top 10 strong *Salmonella* biofilm formers (A) and *Salmonella* strain 8243 (B) on stainless steel surface under laboratory conditions. Symbols " " and " " represent *Salmonella* populations in control and bacteriophage treatment, respectively. The error bars represented standard error of each data point from the average of duplicate trials. Dotted line represents the detection limit of 0.4 log CFU/cm^2.

Bacteriophage treatment was conducted in a greenhouse to simulate conditions in rendering processing environment as the temperature and relative humidity fluctuate throughout a day and in different seasons.

In summer trial, average temperature through 24 h in a day and 7 days ranged 24.7-31.3 and 27.9-28.7°C, respectively, and average relative humidity through 24 h in a day and 7 days ranged 55.4-77.3 and 65.2-70.3%, respectively (Figure 6A). Population of *Salmonella* strain 8243 attached to the surface of stainless steel increased by 1 log CFU/cm^2 (90%) in first 6 h after removing the chicken meat (Figure 7A), and slightly decreased by 4 days followed by a rapid drop on day 7. In bacteriophage treatment group, the population of *Salmonella* attachment/slightly formed biofilm started to decrease steadily from the beginning. As compared with the control, the highest reduction of ca. 3.4 log CFU/cm^2 (99.96%) was observed on day 7 resulting in a population below detection limit of 0.4 log CFU/cm^2. There was only one positive result from duplicate enriched samples taken on day 7.

In fall/winter trial, average temperature through 24 h in a day and 7 days ranged 18.7-26.9 and 19.3-21.3°C, respectively, and average relative humidity through 24 h in a day and 7 days ranged 24.3-32.6 and 16.1-48.3%, respectively (Figure 6B). Population of *Salmonella* attached to the surface of stainless steel increased by 0.35 log CFU/cm^2 (55%) within first 6 h after removing chicken meat (Figure 7B), and slightly decreased by 4 days followed by a rapid drop on day 7. For bacteriophage treatment, the population of *Salmonella* attachment/slightly formed biofilm had a quick drop of 0.97 log CFU/cm^2 (89%) in first 6 h and started to decrease steadily to day 7. The highest reduction of ca. 1.44 log CFU/cm^2 (96.4%) was observed on day 7 as compared with control.

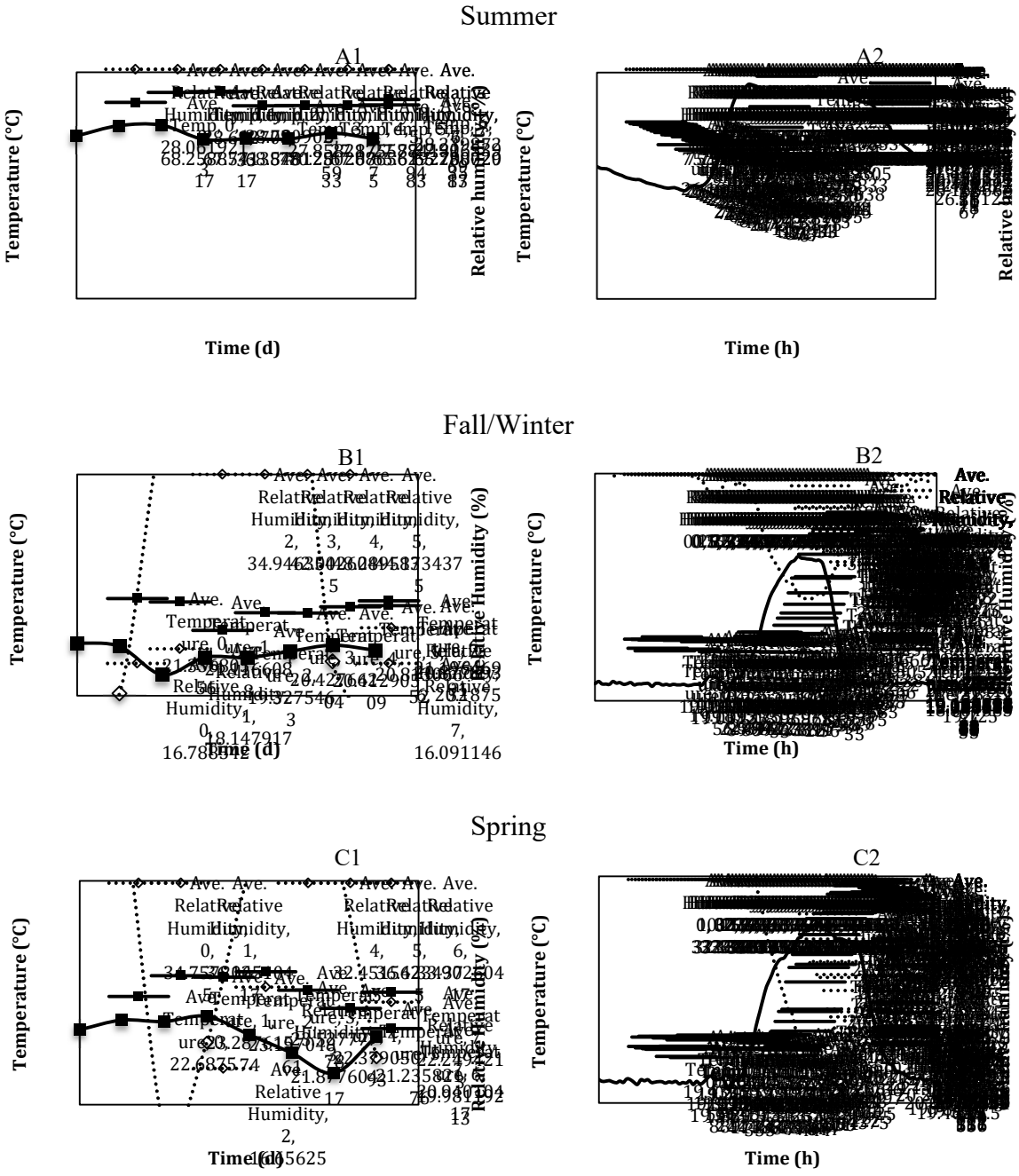


Figure 6. Temperature (solid line) and relative humidity (dotted line) values recorded during summer trial through 7 days (A1) and 24 h in a day (A2), fall/winter trial through 7 days (B1) and 24 h (B2) and spring trial through 7 days (C1) and 24 h in a day (C2).

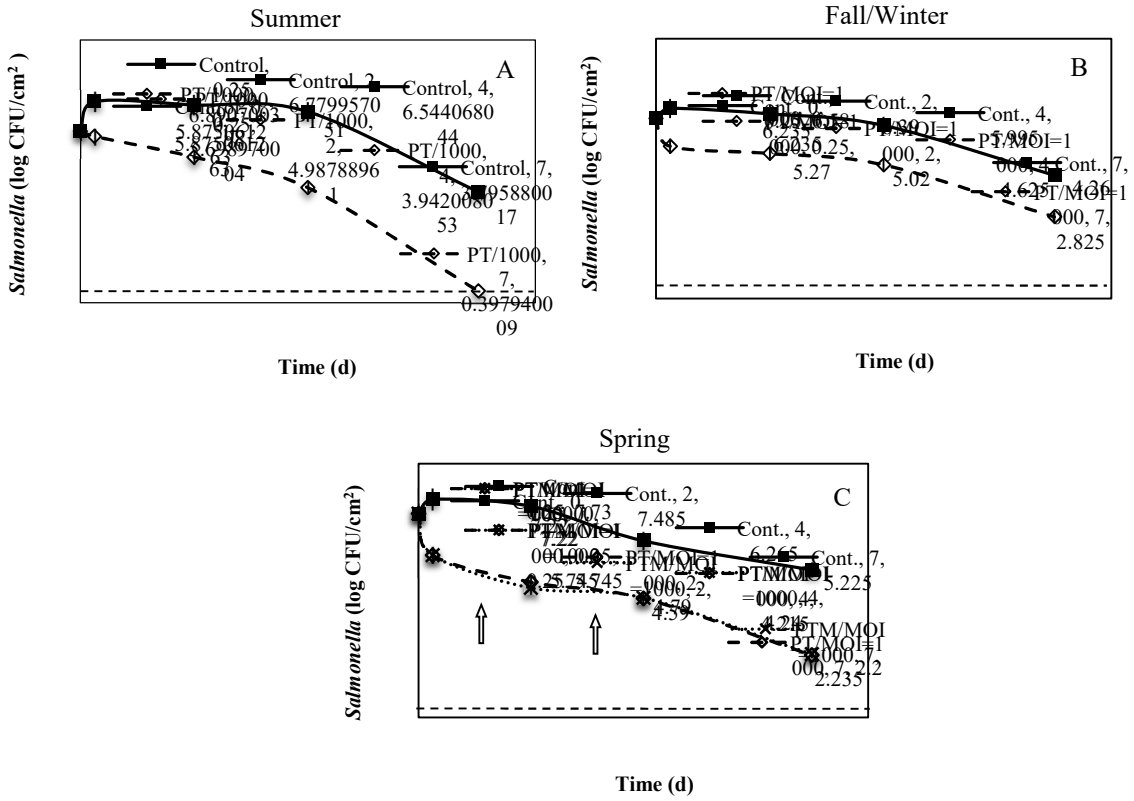


Figure 7. Bacteriophage treatment (titer of ca. 10^9 PFU/ml) of *Salmonella* strain 8243 biofilm/attachment formed on stainless steel surface in summer (A), fall/winter (B) and spring (C) seasons under greenhouse conditions. Arrows indicate additional doses of bacteriophage treatment. Symbols " ", "■ " and "◇× " represent *Salmonella* populations in control, bacteriophage treatment, and multiple-dose bacteriophage treatment (spring trial only), respectively. The error bars represented standard error of each data point from the average of duplicate trials. Dotted line represents the detection limit of 0.4 log CFU/cm^2

In spring trial, average temperature through 24 h in a day and 7 days ranged 18.8-29.0 and 20.0-23.5°C, respectively, and average relative humidity through 24 h in a day and 7 days ranged 19.5-36.2 and 16.6-36.4%, respectively (Figure 6C). Population of *Salmonella* attached to the surface of stainless steel (control) increased by 0.51 log CFU/cm^2 (69.1%) within first 6 h after removing chicken meat (Figure 7C), and slightly decreased by 2 days followed by a steady reduction to day 7. For bacteriophage treatment, the population of *Salmonella* attachment/slightly formed biofilm had a quick drop of 1.48 log CFU/cm^2 (96.7%) in first 6 h and started to decrease steadily to day 7. The highest reduction of ca. 3.0 log CFU/cm^2 (99.9%) was observed on day 7 as compared with the control. For the bacteriophage treatment in multiple-dose, there was no significant improvement ($P > 0.05$) was observed in spring season (Figure 7C).

Overall, our bacteriophage treatment reduced 1.44-3.4 log CFU/cm^2 (96.4-99.96%) of *Salmonella* attachment/slightly formed biofilms on the surface of stainless steel with the effectiveness depending on the seasons.

Phage survival in different concentrations of sodium hypochlorite (Obj. 4). To investigate the feasibility of a combinational treatment of bacteriophage cocktail and sodium hypochlorite for reducing the *Salmonella* biofilms formed on the boots, survival times of bacteriophages in freshly diluted sodium hypochlorite product, Clorox® (Oakland, CA, USA) were measured. Briefly,

bacteriophage cocktail of 100 µl at a titer of ca. 10^9 PFU/ml was mixed with 900 µl sodium hypochlorite solution at concentrations of 200, 400 and 800 ppm, respectively. At pre-determined intervals, sodium thiosulfate ($Na_2S_2O_3$, Fisher Scientific, Pittsburgh, PA, USA) at a final concentration of 2% (w/v) was added as a neutralizer to stop the reaction and then bacteriophage titers were determined using a soft agar overlay method.

Sodium hypochlorite concentrations were determined using an iodimetric titration method (Clarkson et al., 2001). Briefly, sodium hypochlorite sample of 10 ml was transferred into a volumetric flask (Pyrex®, Corning, NY, USA) mixed with 10 ml of 3% (w/v) sulfuric acid (H_2SO_4, Spectrum®, New Brunswick, NJ, USA) and 20 ml of 100 g/l potassium iodine (KI, Fisher Scientific, Pittsburgh, PA, USA). The flask was incubated in the dark at room temperature for 5 min, and then titrated with 0.1 M $Na_2S_2O_3$ with 1 ml soluble starch (1g/l, Difco®, Becton Dickinson, Sparks, MD, USA) as indicator for titration end point. The sodium hypochlorite concentration was calculated using the volume of $Na_2S_2O_3$ consumed. A standard curve of chlorine concentration (ppm) and sodium hypochlorite concentration (%) was generated with regression coefficient $R^2 = 0.9999$ (data not shown).

In the first 2 h incubation, bacteriophages were rapidly inactivated to below detection limit in 800-ppm sodium hypochlorite solution (Figure 8). After 8 h incubation at room temperature, the titers of bacteriophages decreased by 5.0, 5.5 and >6.7 log PFU/ml in sodium hypochlorite solution at concentrations of 200, 400 and 800 ppm, respectively. Therefore, sodium hypochlorite concentration of 400 ppm was selected for a combinational treatment of bacteriophage cocktail and sodium hypochlorite in following tests.

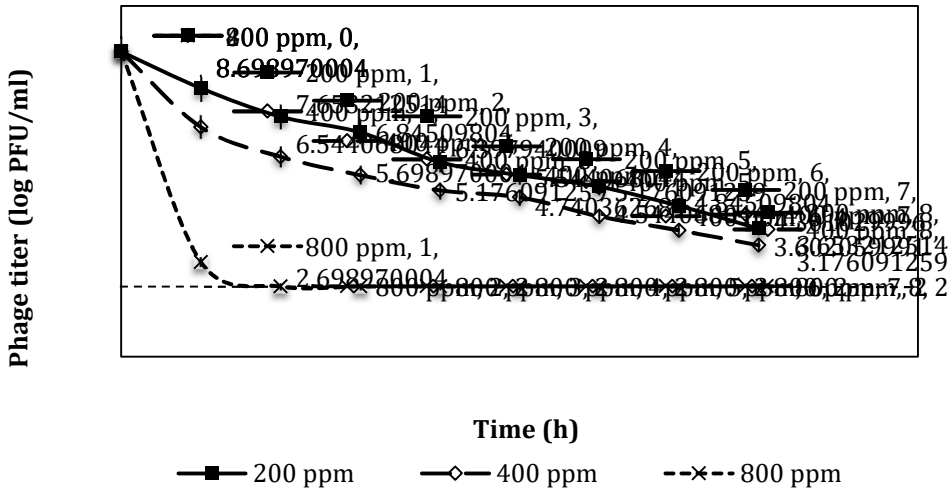


Figure 8. Survival times of bacteriophages in sodium hypochlorite at different concentrations. Symbols "■", "◇" and " × " represent chlorine concentrations of 200, 400 and 800 ppm, respectively. Dotted line represents the detection limit of 2 log PFU/ml.

Formation of* Salmonella *biofilm on rubber templates and boot soles (Obj. 4). Prior to each experiment, rubber templates (5 × 5 cm, n = 12 for each trial) or boots (size 9, n = 4 for each trial, lightly used condition, LaCrosse®, Portland, OR, USA) were washed with disinfectant detergents Simply Right® (0.13% benzalkonium chloride, Toronto, ON, Canada) and Clorox® (6% sodium

hypochlorite, Oakland, CA, USA), rinsed with 100 ml sterile distilled water and air-dried inside a biological safety hood. These templates or boots were then placed into a stainless steel tray (25 × 30 cm, Bloomfield, St. Louis, MO, USA) containing 500 ml tryptic soy broth (TSB) inoculated with *Salmonella* strain 8243 at a final concentration of ca. 7 log CFU/ml. After 48 h incubation at 30°C for *Salmonella* biofilms formation, the surfaces of templates or boots were rinsed with 0.85% sterile saline to remove non-biofilm bacterial cells and sampled using cotton swabs (Puritan® Medical Products LLC., Guilford, ME, USA) for determining initial *Salmonella* populations.

Phage treatment of* Salmonella *biofilms formed on rubber templates and boot soles under laboratory condition (Obj. 4). Bacteriophage treatments were carried out by submerging the templates in petri dishes (Falcon®, Becton Dickinson, Sparks, MD, USA) or soaking the boots in stainless steel trays (25 × 30 cm, Bloomfield, St. Louis, MO, USA) containing bacteriophage cocktail at a final titer of 1×10^9 PFU/ml. Combinational treatments of the same bacteriophage cocktail with sodium hypochlorite (400 ppm), a 10-min sodium hypochlorite pre-treatment or a 30-sec scrubbing with a brush (8 × 12 cm head with 55 cm handle, Blue Hawk, Gilbert, AZ) were also tested. SM buffer, sodium hypochlorite alone and 10-min sodium hypochlorite pre-treatment alone were used as controls. After 6 h incubation at room temperature (ca. 22°C), treated *Salmonella* biofilms on the entire templates or boot soles were swabbed and enumerated on xylose lysine tergitol-4 (XLT-4, Hardy Diagnostics, Santa Maria, CA, USA) plates. At the end of each treatment, $Na_2S_2O_3$ was added as described above to neutralize remaining sodium hypochlorite in all samples. The titers of residual bacteriophages were enumerated using a soft agar overlay method. Two trials of above experiments were conducted and duplicate samples were enumerated at each time point for each trial.

Initial population of *Salmonella* biofilms on surfaces of rubber templates was ca. 6.9 log CFU/template (Table 6). After 6 h incubation at room temperature, the population of *Salmonella* biofilms decreased by ca. 0.08 log CFU/template in control. The populations of *Salmonella* biofilms were reduced by 1.31 log (95.1%), 4.89 log (99.998%), 1.24 log (94.2%), 2.35 log (99.6%) and 5.34 log (99.9995%) CFU/template after treated with bacteriophage cocktail alone, 400-ppm sodium hypochlorite alone, a 10-min sodium hypochlorite pre-treatment, a 10-min sodium hypochlorite pre-treatment combined with bacteriophages, and a mixture of bacteriophage cocktail and sodium hypochlorite for 6 h, respectively. The combinational treatment of bacteriophage cocktail with 400-ppm sodium hypochlorite for 6 h resulted in a significantly higher reduction ($P < 0.05$) as compared to either the combinational treatment of bacteriophage cocktail with a 10-min sodium hypochlorite pretreatment or treatment of sodium hypochlorite alone for 6 h (Table 6), and there was no significant difference ($P > 0.05$) between the reductions of *Salmonella* biofilms treated with bacteriophage treatment alone or 10-min sodium hypochlorite pretreatment alone. The titers of residual bacteriophages in petri dishes after 6 h treatment were ca. 10^9, 10^7 and 10^6 PFU/ml in the treatments of bacteriophage cocktail alone, a 10-min sodium hypochlorite pre-treatment combined with bacteriophages, and a mixture of bacteriophage cocktail and sodium hypochlorite, respectively.

Initial populations of *Salmonella* biofilms on the soles of rubber boots were ca. 6.9 log CFU/boot on both control and treatment boots (Table 6). After 6 h incubation at room temperature, the population of *Salmonella* biofilms increased by approximately 0.08 log CFU/boot in control. The population of *Salmonella* biofilms was reduced by 1.07 log (91.5%), 1.18 log (93.4%), 1.52 (97.0%) and 2.08 (99.2%) log CFU/boot after treated with bacteriophage treatment alone, 400-ppm sodium hypochlorite alone, a mixture of bacteriophage cocktail and 400-ppm sodium

hypochlorite for 6 h and bacteriophage treatment following 30-sec brush scrubbing, respectively. The titers of residual bacteriophages were ca. 10^6 PFU/ml. These results indicate that the combinational treatments of bacteriophage cocktail mixed with 400-ppm sodium hypochlorite or following brush scrubbing were significantly more effective ($P < 0.05$) on *Salmonella* biofilms formed on the surfaces of rubber templates and boots.

Table 6. Bacteriophage treatment of *Salmonella* biofilms on rubber templates and boots under laboratory condition.

Material	Treatment Method[a]	*Salmonella* Biofilm Population[b] (log CFU/surface or boot)	
		0 h	6 h
Rubber Template	Control	6.91±0.05	6.83±0.03A
	Phage Alone	6.91±0.05	5.60±0.07B
	Sodium Hypochlorite Alone	6.91±0.05	2.02±0.19D
	Phage + Sodium Hypochlorite	6.91±0.05	1.57±0.15E
	10-min Sodium Hypochlorite	6.91±0.05	5.67±0.09B
	Phage + 10-min Sodium Hypochlorite	6.91±0.05	4.56±0.11C
Rubber Boot	Control	6.93±0.04	7.01±0.06a
	Phage Alone	6.93±0.04	5.86±0.06b
	Sodium Hypochlorite Alone	6.93±0.04	5.75±0.14b
	Phage + 30-sec Scrubbing	6.93±0.04	4.85±0.03d
	Phage + Sodium Hypochlorite	6.93±0.04	5.41±0.04c

[a] Control: SM buffer; Phage Alone: bacteriophage treatment at final titer of 9 log PFU/ml; Sodium Hypochlorite Alone: 400 ppm. Phage + Sodium Hypochlorite: bacteriophage cocktail (9 log PFU/ml) mixed with sodium hypochlorite (400 ppm); 10-min Sodium Hypochlorite: pretreatment with sodium hypochlorite (400 ppm) for 10 min followed by SM buffer; Phage + 10-min Sodium Hypochlorite: pretreatment with sodium hypochlorite (400 ppm) for 10 min followed by bacteriophage treatment (9 log PFU/ml); Phage + 30-sec Scrubbing: scrubbing with a brush for 30 sec followed by bacteriophage treatment (9 log PFU/ml).
[b] Average count of four replicate samples from two trials ± standard deviation. Numbers with different letters in uppercase or lowercase are significantly different ($P < 0.05$) in each column for each material.

Phage treatment of* Salmonella *contamination on the soles of workers' boots in rendering processing environment (Obj. 4). Workers' boots (size 9-11, 3 pairs for each trial, 1 pair for each treatment, medium to heavily contaminated condition, ACE Work Boots™, Shoes For Crews Corp., West Palm Beach, FL, USA) each from grinding room, processing room or finished meal loading-out area were selected. A half sole of each boot was sampled using Q-Swab® (Hygiena, Camarillo, CA, USA) for enumerating initial *Salmonella* populations upon arrival at rendering plant. Bacteriophage treatments were applied by soaking one boot of each pair in aluminum foil trays (25 × 30 cm, Hefty®, Reynolds Consumer Products, Lake Forest, IL, USA) containing 500 ml of bacteriophage cocktail (1×10^9 PFU/ml) alone, mixed with sodium hypochlorite (400 ppm) or combined with 30-sec brush scrubbing (back and forth on the sole of

boot for multiple times). The other boot of each pair was soaking in 500 ml of SM buffer as control. The same bacteriophage treatments were applied for 3 times within 1 week (6 h, day 2 and day 5) and *Salmonella* populations on the workers' boots were swabbed in two halves as duplicate samples at 6 h after the first treatment and on the day 7. The reductions of *Salmonella* contamination on worker's boots were determined by subtracting the populations on the boots treated with bacteriophage or other treatments from those on the boots treated with SM buffer. Two sampling trials were conducted in Nov. 2015 and Dec. 2015. Temperature and relative humidity data were recorded using a portable hygro-thermometer (VWR, Radnor, PA, USA).

The populations of total aerobic bacteria and fecal coliforms were enumerated by directly plating on TSA plates and *E. coli*/coliforms Petrifilms™ (3M Food Safety Division, Cartersville, GA, USA), respectively. To compare the sensitivity and specificity of different *Salmonella*-selective media, XLT-4, Millar-Mallinson (M-M; Mallinson et al., 2000) and CHROMagar™ (Chromagar Inc., Paris, France) plates were employed for enumerating *Salmonella* populations on workers' boots, respectively. The presumptive *Salmonella* colonies grown on these selective agar plates were confirmed using a real-time PCR method (Malorny et al., 2003).

In the first trial conducted from 11/18 to 11/25 in 2015, the temperatures were 23, 20 and 17ºC on the treatment days 0, 2 and 5, respectively, and the relative humidity were 57, 42 and 34% on the treatment days 0, 2 and 5, respectively. In the second trial conducted from 12/16 to 12/23 in 2015, the temperatures were 23, 16 and 17°C on the treatment days 0, 2 and 5, respectively, and the relative humidity were 44, 45 and 65% on the treatment days 0, 2 and 5, respectively.

A total of 72 swab samples were collected from works' boots in those two trials. On day 0, the average initial numbers of total aerobic bacteria, *E. coli*, presumptive *Salmonella* on M-M, XLT-4 and CHROMagar™ were 6.49, 3.23, 2.87, 1.83 and 2.86 log CFU/boot, respectively. After phage treatments for 6 h, the average reductions of total aerobic bacteria, fecal coliforms, and presumptive *Salmonella* on M-M, XLT-4 and CHROMagar™ were 0.30, 0.55, 0.53, 1.05 and 0.57 log CFU/boot, respectively (Table 7). After phage treatments for 1 week, the average reductions of total aerobic bacteria, fecal coliforms, presumptive *Salmonella* on M-M, XLT-4 and CHROMagar™ were 0.62, 0.83, 1.04, 1.29 and 0.79 log CFU/boot, respectively. The titers of residual phages left in the aluminum foil trays from different treatments were enumerated ranging ca. 8.0-8.4 log PFU/ml on day 7 of each trial.

By comparing *Salmonella* counts on three selective agar media, XLT-4 resulted in significant lower counts ($P < 0.05$) than other two selective media due to presence of the weak H_2S producing *Salmonella* in rendering processing environment. In addition, the interference of non-*Salmonella* species grown on CHROMagar™ was also observed. Therefore, M-M agar was the better choice for enumerating *Salmonella* in rendering processing environment, and the following results and discussion of *Salmonella* reductions were based on the plate count data using M-M agar.

Among the different phage treatments after 6 h and 1 week, the combinational treatment of phage cocktail and 30-sec scrubbing resulted in slightly high average *Salmonella* reductions of 0.61 and 1.17 log CFU/boot, respectively, as compared with 0.54 and 1.15 log CFU/boot, respectively (Table 7), for the combinational treatment of phage cocktail and sodium hypochlorite. Furthermore, both combinational treatments reduced more *Salmonella* populations ($P < 0.05$) than the phage cocktail alone (average reductions of 0.44 and 0.80 log CFU/boot after 6 h and 1 week treatments, respectively). The following is the summary of our field study results:

- M-M agar was chosen for enumerating indigenous *Salmonella* in rendering processing environment as ultra-weak H_2S producing *Salmonella* is able to form black colony.
- There was enhanced reduction of *Salmonella* on the workers' boots using combinational phage treatments with either sodium hypochlorite or brush scrubbing.
- High titers of residual phages at the end of each trial indicate the stability of phage virions in rendering plant.
- The reductions of total aerobic bacteria and fecal coliforms on the workers' boots could be due to the, destruction of indigenous biofilms treated by phage cocktail, broad antimicrobial spectrum of disinfectant sodium hypochlorite or physical removal of brush scrubbing.
- Combinational phage treatments could reduce not only the *Salmonella* contamination on the workers' boots but also total aerobic bacteria and fecal coliforms populations resulting in an overall improvement in microbiological safety of rendered meals in rendering processing plant.

Table 7. Phage treatments of *Salmonella* biofilms on workers' boots in rendering plant.

Time	Microorganism[a]	Microbial Reductions (log CFU/boot)[b]					
		Trial 1			Trial 2		
		Phage[b] Alone	Phage + NaClO	Phage + Scrubbing	Phage Alone	Phage + NaClO	Phage + Scrubbing
6 h	Total bacteria	0.18	0.45	0.26	0.28	0.31	0.31
	Fecal coliforms	0.51	0.78	0.54	0.32	0.51	0.66
	Salmonella	0.41	0.38	0.54	0.48	0.69	0.68
7 d	Total bacteria	0.63	0.38	0.52	0.85	0.85	0.51
	Fecal coliforms	1.13	0.92	0.78	0.77	0.67	0.71
	Salmonella	0.72	1.11	1.12	0.88	1.19	1.22

[a] Total bacteria, on TSA; Fecal coliforms, on Petrifilm™; *Salmonella*, on M-M agar; [b] Average of duplicate samples; [c] Phage Alone: Phage cocktail (9 log PFU/ml); Phage + NaClO: Phage cocktail (9 log PFU/ml) + sodium hypochlorite (400 ppm); Phage + Scrubbing: brush scrubbing for 30 sec followed by phage cocktail (9 log PFU/ml).

Impacts and Significance: *Salmonella* has long been established as a contaminant of rendered animal by-products and animal feed. The biological control method using *Salmonella*-specific bacteriophages being developed in this project will have the potential for reducing bacterial populations within rendering processing environment, thereby reducing the chances for the finished animal meals being re-contaminated with *Salmonella* prior to shipment out. Bacteriophages are superior to chemical disinfectants since they are not corrosive and hazardous to the environment, humans and other animals, and self-replicating meaning lower cost. Elimination of this pathogen by bacteriophage approach will ensure the microbiological safety of rendered animal meal products.

Publications:

1) Gong, C., A.K. Greene and X. Jiang. (2016) Application of Bacteriophage to Reduce *Salmonella* Contamination on the Workers' boots in Rendering Processing Plant. *Poult. Sci.* (In preparation)
2) Gong, C. and X. Jiang. (2016) Application of Bacteriophage to Reduce *Salmonella* Biofilm or Attachment on Hard Surfaces. *Poult. Sci.* (Under review)
3) Gong, C. and X. Jiang. (2016) Identifying the Sources of *Salmonella* Contamination in Rendering Processing Environment. *J. Food Prot*. (in press)
4) Gong, C. and X. Jiang. (2016) Bacteriophage Treatment of *Salmonella* Contamination on Workers' Boots in a Rendering Processing Environment. *International Association for Food Protection Annual Meeting*, St. Louis, MO.
5) Gong, C. and X. Jiang. (2015) Pilot study of applying *Salmonella*-specific bacteriophages in a rendering processing plant. ASM South Carolina Regional Annual Meeting. April 11, 2015, Furman University.
6) Gong, C. and X. Jiang. (2015) Pilot study of applying *Salmonella*-specific bacteriophages in a rendering processing plant. ACREC Annual spring meeting, March 2015.
7) Gong, C., A.K. Greene and X. Jiang. (2014) Biological Control of *Salmonella* Biofilm on Stainless Steel Surfaces. *International Association for Food Protection Annual Meeting.* Indianapolis, IN, USA.

Outside funding: None

Future Work: Further studies such as optimizing bacteriophage cocktails to be more effective against indigenous *Salmonella* biofilm formers under the conditions with broader temperature and humidity ranges, and conducting a large scale of field study for bacteriophage treatment are critically needed.

Acknowledgments: This research was a part of graduate student, Chao (James) Gong's Ph.D. dissertation. We would like to thank Hongye (Maple) Wang for her assistance on sampling the rendering plants. We also would like to give thanks to participating rendering plants in this project.

The following is a journal manuscript that is currently under review:
Gong, C. and X. Jiang. (2016) Application of Bacteriophage to Reduce *Salmonella* Biofilm or Attachment on Hard Surfaces. *Poult. Sci.*

Gong, C. and X. Jiang. (2016) Application of Bacteriophage to Reduce *Salmonella* Biofilm or Attachment on Hard Surfaces. *Poult. Sci.* (under review)

Running title: *Salmonella* prevalence in rendering plant

Characterizing *Salmonella* Contamination in Two Rendering Processing Plants

Chao Gong[1] and Xiuping Jiang[2, *]

[1] Department of Biological Sciences, [2] Department of Food, Nutrition and Packaging Sciences, Clemson University, Clemson, SC, 29634, USA.

Keywords: *Salmonella*, Rendering Processing Environment, Cross-contamination, Biofilm

[*]Corresponding Author: xiuping@clemson.edu

Address: 228A Life Science Facility, Clemson University, Clemson, SC, 29634, USA

Tel: 864-656-6932

Fax: 864-656-0331

ABSTRACT

A microbiological investigation on *Salmonella* contamination was conducted in two rendering plants in order to investigate the potential cross-contamination of *Salmonella* in rendering processing environment. Sampling locations were pre-determined at the areas where *Salmonella* contamination may potentially occur including raw materials receiving, crax grinding and the finished meal loading-out areas. *Salmonella* were either enumerated directly on xylose-lysine-tergitol-4 (XLT-4) agar plates or enriched in Rappaport-Vassiliadis (RV) and Tetrathionate (TT) broths. The presumptive *Salmonella* isolates were confirmed using CHROMagar® and latex test, and then characterized using pulsed field gel electrophoresis (PFGE), serotyping and biofilm-forming determination. Among 108 samples analyzed, 79 samples (73%) were *Salmonella*-positive after enrichment. Selected *Salmonella* isolates (n = 65) were assigned to 31 unique PFGE patterns with 16 *Salmonella* serotypes including Typhimurium and Mbandaka identified as predominant serotypes, and 10 *Salmonella* strains were determined as strong biofilm formers. Based on our results, raw material receiving area was found as the primary source of *Salmonella*, whereas the surfaces surrounding crax grinding and the finished meal loading-out areas harbor *Salmonella* in biofilms that may re-contaminate the finished meals. The same *Salmonella* serotypes found in both raw materials receiving and the finished meal loading-out areas also suggested a potential of cross-contamination between different areas in rendering processing environment.

Salmonella is one of the major human pathogens causing foodborne illnesses in the U.S. and many other countries around the world. Both *Salmonella enterica* serovars Enteritidis and Typhimurium are the most commonly identified serotypes in the incidence of salmonellosis (*10*). Human infection with *Salmonella* typically occurs when people consume the contaminated food products or contact with the contaminated pet food when feeding pets (*5*, *18*).

The animals become infected with *Salmonella* often through being fed with the contaminated animal feeds, which is at the starting point of the food safety chain "from farm to fork" and can be easily contaminated with the pathogen during the manufacturing and transportation processes (*5*, *18*). Several studies have determined the contamination rates and most prevalent serovars of *Salmonella* in a variety of animal feeds and feed ingredients samples collected from animal feed facilities and rendering plants as well as through retrospective analysis based on surveillance data (*7*, *12*, *23*, *28*). Overall, *Salmonella* prevalence in animal feeds ranged from 12.5 to 22.9% at a low contamination level (less than 10 MPN/g) in the U.S.; with higher contamination rates up to 34.4% observed in some feed ingredients such as animal bone meals and blood meals (*11*, *12*, *23*). Prevalence of *Salmonella* in animal-derived feed ingredients was higher than in plant-derived feed ingredients (*8*, *12*). Also, the mash meals had higher contamination rate of *Salmonella* than pellet feeds (*19*). In a study conducted in Great Britain, Papadopoulou *et al.* (*28*) found that *Salmonella* contamination rate in animal feeds and feed ingredients ranged from 8.4 to 11.2%. In two recently conducted surveys, 8.3 to 8.7% of the rendered animal product samples collected from rendering plants across the U.S. were positive for *Salmonella* (*17*, *22*).

The principle of rendering process has not been changed over years with sizing, heating, pressing and grinding as the major procedures. Although heating process is accurately controlled and monitored with advanced technologies nowadays, which can guarantee the inactivation of

Salmonella from raw animal by-products, animal meals may still be recontaminated by *Salmonella* in the form of biofilm persisting in the processing environments such as surfaces of manufacturing equipment, floor and storage tanks of finished animal meals as well as aerosol flowing through processing areas (*6*, *24*, *27*). For environmental swab samples collected from the surfaces of machines, walls and floor in rendering plants, *Salmonella* contamination rate ranged from 25.6 to 50.0% (*6*, *21*, *24*, *27*). Orthoefer *et al.* (*27*) reported that 26.6% aerosol samples collected from a rendering plant were positive for *Salmonella*. Also, swab samples taken from raw materials area in a rendering processing plant had higher *Salmonella* contamination rate up to 95.0% as compared to 15.2% in finished product area (*6*, *21*, *24*, *27*). Therefore, monitoring *Salmonella* contamination in rendering processing environment is important for controlling the *Salmonella* in rendered animal products, whereas the *Salmonella* contamination level in current rendering processing environment has not been updated for decades.

In order to sufficiently reduce *Salmonella* levels in foods, feeds and processing environment, the Food Safety Inspection Service (FSIS) of United States Department of Agriculture (USDA) published Hazard Analysis and Critical Control Point (HACCP) guidance (*33*). Moreover, Food Safety Modernization Act (FSMA) proposed new rules on Current Good Manufacturing Practices (CGMPs) with sanitation preventive controls in order to preventively control *Salmonella* contamination in the foods for animals, these rules will be applied to pet food, animal feed and raw materials (*34*). We have explored using bacteriophages as the biological control agent to reduce harmful bacteria in the rendering processing environment (*13*). In order to apply bacteriophage for controlling *Salmonella* more effectively, it is critical to understand the ecology of this pathogen in rendering processing environment. Therefore, the objectives of our study were to investigate the contamination of *Salmonella* in current rendering processing

environment, identify the potential sources and routes of *Salmonella* cross-contamination in the plants, and determine the biofilm forming ability of *Salmonella* isolates from the rendering processing plants.

MATERIALS AND METHODS

Sampling the rendering plants. Two US rendering processing plants were sampled for total aerobic bacterial count and the presence of *Salmonella* from June 2012 to August 2012, and these two plants were not significantly different in terms of processing volume, feed types and geographical location. Based on the continuous rendering process layout, the raw material receiving area was determined to be the major source of *Salmonella*, whereas the crax grinding area that grinds dried rendered materials and finished meal loading-out areas were also selected for sampling as locations of potential cross-contamination (*25*).

Different types of solid materials (ca. 500 g for each) including raw poultry by-products (n = 12), rendered crax (n = 12) and finished meal products (n = 12) made from pet food grade poultry offals, animal feed grade poultry offals and poultry feathers were aseptically collected as duplicate samples in sterile sampling bags (Whirl-Pak®, Fort Atkinson, WI, USA). Different types of raw materials destined for pet food, animal feed and feather protein meals were processed in separated processing lines, thus air and surfaces associated with specific processing lines were sampled separately. Environmental surface samples (n = 40, animal food-non-contact) were obtained in duplicates using 3M sampling swabs (3M, St. Paul, MN, USA) with a sterile paper grid (10 × 10 cm^2) from the surfaces of those three selected areas. The sampled surfaces of raw material receiving, grinding and loading-out areas are made of steel, steel, and concrete, respectively. Tryptic soy agar (TSA, Becton Dickinson, Sparks, MD, USA) and xylose-lysine-tergitol-4 (XLT-

4, Becton Dickinson, Sparks, MD, USA) agar plates in duplicates were exposed to the air for 30 min to collect air samples (n = 32) for total aerobic bacteria and *Salmonella*, respectively. Both media were supplemented with 50 mg/l of cycloheximide (Sigma-Aldrich, St. Louis, MO, USA) to inhibit fungal growth. To detect a very low level of *Salmonella*, a pre-enrichment step was included to overcome the detection limit (0.7 log CFU/g or ml) of direct plating method. A sterile empty petri dish containing 10 ml of universal pre-enrichment broth (UPB, Neogen, Lansing, MI, USA) supplemented with 50 mg/l of cycloheximide was placed at the selected locations, left open for 30 min, and then transferred aseptically to a sterile centrifuge tube (Corning Inc., Corning, NY, USA). All samples were packed in ice, transported to laboratory and analyzed immediately upon arrival.

Sample analysis for total aerobic bacteria and *Salmonella*. Serially diluted solid (25 g blended in 225 ml UPB), swab and air (undiluted in UPB) samples were plated onto TSA and XLT-4 plates (50 mg/l of cycloheximide), plus all air sample plates, followed by a 24-h incubation at 35°C for enumerating total aerobic bacteria and presumptive *Salmonella* populations. Remaining samples in UPB were directly incubated for 24 h at 35°C for *Salmonella* enrichment, followed by 24-h selective enrichment in Rappaport-Vassiliadis broth (RV, Becton Dickinson, Sparks, MD, USA) and Tetrathionate broth (TT, Becton Dickinson, Sparks, MD, USA) at 42 and 43°C, respectively. Selective enrichment broths were streaked onto Bismuth Sulfate (BS, Becton Dickinson, Sparks, MD, USA) and XLT-4 plates and incubated at 35°C for 24 h. From each plate, 3 or 4 typical black colonies were selected based on morphology such as size and darkness and resteaked onto XLT-4 plates twice for isolation, and further resteaked onto TSA plates twice for purification. Pure *Salmonella* isolates were further confirmed by streaking on CHROMagar® plate

(CHROMagar, Paris, FR) and testing for agglutination in *Salmonella* latex test (Oxoid, Basingstoke, UK).

PFGE analysis, serotyping and biofilm-forming ability of selected *Salmonella* isolates. Pulsed-field gel electrophoresis (PFGE) analysis was conducted according to Centers for Disease Control and Prevention (CDC) version of one-day rapid PFGE procedure (*3*). Briefly, *Salmonella* cultures prepared in agarose plugs were treated in cell lysis buffer, washed with TE buffer and then digested by XbaI restriction enzyme (Promega, Madison, WI, USA) followed by electrophoresis using a CHEF Mapper XA System (Bio-Rad, Hercules, CA). Digital images of the PFGE patterns was acquired using the Gel Doc system (Bio-Rad, Hercules, CA). *Salmonella* serotype Braenderup strain H9812 was used as a standard strain for DNA markers.

Salmonella isolates with different PFGE patterns were sent to United States Department of Agriculture-National Veterinary Service Laboratory (USDA-NVSL, Ames, IA, USA) for serotype determination.

To determine the biofilm-forming ability of each *Salmonella* isolate, overnight cultures of selected *Salmonella* isolates were collected by centrifugation at 5,000 × g, washed with 0.85% sterile saline and adjusted to OD of 0.5 at a wavelength of 600 nm (ca. 9 log CFU/ml). A 20 µl of 1:10,000 diluted culture of each selected isolate was mixed with 180 µl of 10% tryptic soy broth (TSB, Becton Dickinson, Sparks, MD, USA) in eight replicate wells of a 96-well microplate (Costar®, Corning Inc., Corning, NY, USA) (*31*). After static incubation at 30°C for 48 h, each well was washed with sterile distilled water and allowed to air-dry. Biofilm in each well was stained by 1% crystal violet solution (Becton Dickinson, Sparks, MD) at 22°C for 45 min followed by an elution with 95% ethanol, and then measured by a spectrometer (µQuant; Bio Tek, Winooski, VT, USA) at 600 nm. Bacterial strains of *Hafnia alvei* (S211) and *Pseudomonas aeruginosa*

(ATCC® 27853) were included as reference of strong biofilm formers (*13*).

Statistical Analysis. Bacterial plate count data were converted to $\log_{10}$ CFU per plate, ml, g or cm^2 for statistical analysis. An analysis of variance (ANOVA) for a completely randomized design was conducted to determine if general differences existed between bacterial count means using the general linear model (GLM) procedure. Specific comparisons among different samples were accomplished with Tukey's test. Correlation Coefficient (*r*) values were also generated to present the relationship between aerobic bacterial counts and *Salmonella* populations.

RESULTS AND DISCUSSION

Analyzing *Salmonella* and total aerobic bacterial populations in two rendering plants. A total of 108 samples were collected from two rendering plants and 79 samples (73%) were tested positive for *Salmonella* after enrichment. For the air samples (n = 32), total aerobic bacterial counts varied in a narrow range (Table 1). *Salmonella* were below detection limit (<1 CFU/plate) in all air samples collected from finished meal loading-out area but enumerated in 33 and 17% air samples obtained from raw material receiving and crax grinding areas, respectively. After enrichment, an average of 53% air samples were positive for *Salmonella*. For the surface samples (n = 40), there were higher numbers of total aerobic bacterial counts on the surface of raw material receiving area than surface samples of loading-out area and crax grinding area (Table 1). *Salmonella* were enumerated with highest counts from raw material receiving area followed by loading-out and crax grinding areas. For the solid samples (n = 36), raw materials contained the highest counts of total aerobic bacteria and *Salmonella* (Table 1). Total aerobic bacterial counts in crax (rendered materials before grinding process) varied in a broad range. *Salmonella* was not enumerated in any of crax samples by direct plating, but *Salmonella* colonies were observed on

16.7% replicate plates after enrichment. As for the finished meals at the loading-out area, total aerobic bacterial counts varied in different samples, and all finished meals yielded zero number for *Salmonella* by direct plating, but *Salmonella* colonies were observed on 33% replicate plates after enrichment.

Several studies were conducted more than 45 years ago on *Salmonella* prevalence and recontamination of rendered animal products in rendering processing plants. Kaufmann & Feeley (*21*) collected 95 swab samples from two rendering plants including the surfaces of machines, walls and floor resulting in 48.4% *Salmonella*-positive samples. Orthoefer *et al.* (*27*) reported that overall 34.0% samples from a rendering plant contained *Salmonella*, with 25.6% environmental swabs and 26.6% aerosol samples as *Salmonella*-positive as compared with 65.5% solid samples positive for *Salmonella*. Magwood *et al.* (*24*) found 25% solid samples from grinding line and storage tanks were *Salmonella*-positive as compared with a positive rate of 50% for environmental samples. In a relatively recent study, Davies *et al.* (*6*) found 3.7% finished product samples have *Salmonella*, despite treated with 1.5% solution of formic and propionic acids. There were 95.0 and 15.2% *Salmonella*-positive samples in raw material and finished product areas, respectively. Overall, 28.8% samples were *Salmonella*-positive in their entire study. In agreement with above studies, we found that 92 to 94% swab samples collected from the surfaces of crax grinding and finished meal loading-out areas of rendering plants were *Salmonella*-positive, which is much higher than *Salmonella* contamination rate (33-66.7%) of solid samples (Table 1). As compared to above studies, higher contamination rates observed in our study can be explained by using the selective media such as XLT-4 agar that are more sensitive and accurate for enumerating low numbers of *Salmonella* from the environment as well as an pre-enrichment step of UPB employed for recovering those injured *Salmonella* cells in this study. Moreover, sampling spots were selected

based on high potential of *Salmonella* contamination which were not be cleaned for a long time and some of them were located in the hidden areas in rendering processing environment. Therefore, these results represented the "worst case scenario" in the microbial contamination of rendering processing plants. Additionally, our findings demonstrated that the accumulation of dusts and material residues around processing equipment and floor may harbor low level of *Salmonella* within the rendering plant, although the correlations of total aerobic bacterial counts and *Salmonella* populations were weak for plant A ($r = 0.38$) and moderate for plant B ($r = 0.74$).

Apparently, total aerobic bacterial counts and *Salmonella* in finished meals were higher ($P < 0.05$) than both solid and surface samples collected in crax area, indicating that the recontamination of rendered meals probably occurred in the loading-out area. Tittiger & Alexander (*32*) stated that the recontamination occurred during the post-cooking process in the processing environment since *Salmonella* could be destroyed during the proper cooking process. Moreover, high contamination rate of *Salmonella* in surface samples, especially in crax grinding and meal loading-out areas, which cannot be cleaned by water due to dry processing environment, may suggest that the pathogen exists in the biofilms on the surfaces in rendering processing environment. Therefore, these biofilms could be the source of recontamination of finished animal meals.

Plant A had less number of *Salmonella* positive samples after enrichment as compared with plant B in both finished meal loading-out and crax processing areas for animal and pet food grade meals, although *Salmonella* contamination level was higher in the raw materials receiving area of plant A as compared to plant B (Table 1). During the sampling, we observed that rendering plant A had a slightly better hygienic practices, such as workers' boots being disinfected frequently, separated processing areas and routinely cleaned raw by-products receiving area, as compared to

the rendering plant B. These good hygienic practices in plant A may have minimized the spread of *Salmonella* from raw materials receiving area to other processing areas.

PFGE analysis and serotyping of selected *Salmonella* isolates. A total of 166 *Salmonella* isolates were isolated from samples that were *Salmonella*-positive after enrichment, and confirmed using both CHROMagar® and *Salmonella* latex test. Among those *Salmonella* isolates, 70 isolates were from raw materials receiving area, while 57 isolates were obtained from crax area, particularly from surface swabs, and 39 isolates from finished meal loading-out area (Table 1). Sixty five representative isolates were selected proportionally from different sampling locations and sample types for PFGE analysis. PFGE pattern of each selected *Salmonella* isolate was obtained, and the isolates sharing the same PFGE patterns were assigned to the same numbers from 1 to 31.

Salmonella isolates (n = 31) with different PFGE patterns were then serotyped by USDA-APHIS lab, and twenty-two isolates were identified into 16 serotypes (Table 2). Mbandaka and Typhimurium were the two most frequently isolated serotypes each including 6 *Salmonella* isolates. Each of serotypes Schwarzengrund and Ealing included 5 *Salmonella* isolates followed by serotypes Widemarsh, Infantis, Senftenberg, Johannesburg, Kentucky, and Heidelberg including 4, 3, 3, 3, 2, and 2 *Salmonella* isolates, respectively. There was only one *Salmonella* isolate identified for each of the rest serotypes. Serotypes such as Kentucky, Heidelberg, Mbandaka, Infantis, Tennessee, Senftenberg and Typhimurium identified in this study were also frequently reported in previous studies conducted in rendering plants (*6*, *21*, *24*, *27*). Among the identified *Salmonella* serotypes from animal feeds, Tenseness, Mbandaka and Senftenberg were the most frequently isolated, whereas human associated serovars Typhimurium and Enteritidis were isolated at percentages of 1.6-2.4% and 0.5-0.6%, respectively (*28*). In our study, those

serotypes including Mbandaka, Typhimurium, Infantis and Senftenberg found in both raw materials receiving or crax grinding areas and finished meal loading-out area indicated a potential of cross-contamination between different areas in rendering plant, however, *Salmonella* Johannesburg is the another serotype frequently isolated from finished meal products suggesting other possible routes such as birds, rodents and flies may have transported *Salmonella* to the finished meals (*18*, *29*).

Determination of biofilm-forming ability of *Salmonella* isolates. The biofilm forming ability of *Salmonella* was measured using a colorimetric method in 96-well microplate with a cut-off optical density (ODc) value set as 0.042. The biofilms formed by 22 selected *Salmonella* isolates were determined with average optical density (OD) value of 0.37 in a range of 0.19-0.62. There were 13 *Salmonella* isolates formed at more or the same level of biofilms as compared to a strong biofilm former *Hafnia* strain S211 in our previous study (*13*). The average OD value of top 10 isolates was 0.47, which was higher than *Hafnia* strain S211 (OD of 0.37). According to the widely used criteria for determining biofilm forming ability (*31*), all tested *Salmonella* isolates formed biofilms having OD values greater than 4 × ODc, i.e. 0.168. Therefore, these selected *Salmonella* isolates are considered as strong biofilm producers suggesting a high potential of biofilm formation by this pathogen on the surfaces in rendering plant.

Salmonella has been reported as one of the persistent bacteria by forming biofilms in rendering processing plants (*2*, *27*). Although the ability to form biofilm may vary among different serotypes due to the differences in flagella or fimbriae, serotypes Typhimurium, Tennessee, Agona, Montevideo, Senftenberg, Enteritidis and Gallinarum were reported as stronger biofilm formers than other serotypes (*1*, *9*). *Salmonella* was able to form rigid biofilms in a high population ranging 5-7 logs CFU/cm^2 on a variety of surfaces such as stainless steel, glass, rubber and

polymeric plastic commonly used in poultry processing plants and other food processing facilities (*1*, *4*, *9*, *20*). Once a biofilm is formed, normal cleaning and disinfecting procedures such as washing with chlorine or iodine sanitizers may not remove the entire biofilm (*20*). It was reported that *Salmonella* could survive as long as 28 days on stainless steel under tempered and dry condition and some strains of *Salmonella* could even survive in a biofilm for longer time up to 6 months in a dry environment (*15*, *16*). Moreover, the rendering environment, especially the post-heat processing environments such as crax grinding, finish meal storage, and loading-out areas may provide favorable conditions for *Salmonella* to form biofilm due to the ambient temperature (20-40°C), limited nutrient and less effective cleaning for dry processing environment (*30*, *31*). The roughness of the surfaces in processing plant may also allow the biofilm to form and exhibit resistance to sanitizers and adverse environment (*4*, *26*). Additionally, the resident microflora including a variety of microorganisms on the surfaces may also be able to form multiple-species biofilm with *Salmonella* and provide the protection for its growth or survival (*14*).

As only two rendering plants were surveyed in this study, our research findings may not represent all rendering facilities, esp. those with different raw animal by-products, equipment design (continuous vs. batch cookers), facility layout, hygienic practices, etc. Therefore, a large and comprehensive microbiological survey of rendering plants is needed in order to determine current status on *Salmonella* prevalence in the rendering processing environment.

In conclusion, this study analyzed the *Salmonella* contamination levels and total aerobic bacterial counts of air, surface and solid samples collected from protective sites for pathogen persistence in two typical rendering processing plants. Raw material receiving area was found as the primary source of *Salmonella*, whereas surfaces surrounding the crax grinding area and finished meal loading-out area also harbor *Salmonella*, probably in biofilms that may re-

contaminate the finished meals. *Salmonella* serotypes Typhimurium, Infantis and Senftenberg found in both raw materials receiving area and finished meal loading-out area indicated a potential of cross-contamination between different areas in a rendering processing environment. Moreover, strong biofilm formers of *Salmonella* isolates such as Typhimurium and Senftenberg found in the rendering processing plants may consistently challenge the microbiological safety of animal feed and pet food by reducing the effectiveness of sanitation practices currently employed in rendering industry. This also suggests that good cleaning practices and development of more effective disinfection methods are urgently needed for rendering industry.

ACKNOWLEDGMENTS

We'd like to thank, Dr. Annel Greene at Clemson University, for helping us to recruit those two rendering plants for our study. This project was partially funded by a grant from Animal Co-Product Research and Education Center.

REFERENCES

1. Agarwal, R. K., S. Singh, K. N. Bhilegaonkar, and V. P. Singh. 2011. Optimization of microtiter plate assay for the testing of biofilm formation ability in different *Salmonella* serotypes. *Int. Food Res. J.* 18:1493-1498.
2. Bensink, J. 1979. *Salmonella* contamination of meat and bone meal. *Aust. Vet. J.* 55:13-15.
3. Centers for Disease Control and Prevention (CDC). 2013. Standard operating procedure for PulseNet PFGE of *Escherichia coli* O157:H7, *Escherichia coli* non-O157 (STEC), *Salmonella* serotypes, *Shigella sonnei* and *Shigella flexneri.* Available at: http://www. cdc.gov/pulsenet/PDF/ecoli-shigella-salmonella-pfge-protocol-508c.pdf (Accessed Feb. 2016)
4. Chia, T., R. Goulter, T. McMeekin, G. Dykes, and N. Fegan. 2009. Attachment of different *Salmonella* serovars to materials commonly used in a poultry processing plant. *Food Microbiol.* 26:853-859.
5. Crump, J. A., P. M. Griffin, and F. J. Angulo. 2002. Bacterial contamination of animal feed and its relationship to human foodborne illness. *Clin. Infect. Dis.* 35:859-865.
6. Davies, R. H., R. A. J. Nicholas, I. M. McLaren, J. D. Corkish, D.G. Lanning, and C. Wray. 1997. Bacteriological and serological investigation of persistent *Salmonella* Enteritidis infection in an integrated poultry organization. *Vet. Microbiol.* 58:277-293.
7. Davies, R. H. and A. D. Wales. 2010. Investigations into *Salmonella* contamination in poultry feed mills in the United Kingdom. *J. Appl. Microbiol.* 109:1430-1440.
8. Davies, R. H. and A. D. Wales. 2013. *Salmonella* contamination of cereal ingredients for animal feeds. *Vet. Microbiol.* 166:543-549.

9. Díez-García, M., R. Capita, and C. Alonso-Calleja. 2012. Influence of serotypes on the growth kinetic and the ability to form biofilms of *Salmonella* isolates from poultry. *Food Microbiol.* 31:173-180.
10. Fatica, M. K. and K. R. Schneider. 2011. *Salmonella* and produce: Survival in the plant environment and implications in food safety. *Virulence.* 2:573-579.
11. Franco, D. A. 2005. A survey of *Salmonella* serovars and most probably numbers in rendered-animal-protein meals: inferences for animal and human health. *J. Environ. Health.* 67:18-22.
12. Ge, B., P. C. LaFon, P. J. Carter, S. D. McDermott, J. Abbott, A. Glenn, S. L. Ayers, S. L. Friedman, J. C. Paige, and D. D. Wagner. 2013. Retrospective analysis of *Salmonella*, *Campylobacter*, *Escherichia coli*, and *Enterococcus* in animal feed ingredients. *Foodborne Pathog. Dis*. 10:684-691.
13. Gong, C. and X. Jiang, 2015. Application of bacteriophages to reduce biofilms formed by hydrogen sulfide producing bacteria on surfaces in a rendering plant. *Can. J. Microbiol.* 61:1-6.
14. Habimana, O., E. Heir, S. Langsrud, A. W. Åsli, and T. Møretrø. 2010. Enhanced surface colonization by *Escherichia coli* O157: H7 in biofilms formed by an *Acinetobacter calcoaceticus* isolate from meat-processing environments. *Appl. Environ. Microbiol*. 76:4557-4559.
15. Habimana, O., T. Moretro, S. Langsrud, L. K. Vestby, L. L. Nesse, and E. Heir. 2010. Micro ecosystems from feed industry surfaces: a survival and biofilm study of *Salmonella* versus host resident flora strains. *BMC Vet. Res.* 6:48-57.

16. Iibuchi, R., Y. Hara-Kudo, A. Hasegawa, and S. Kumagai. 2010. Survival of *Salmonella* on a polypropylene surface under dry conditions in relation to biofilm-formation capability. *J. Food. Prot*. 73:1506-1510.

17. Jiang, X. 2016. Prevalence and characterization of *Salmonella* in animal meals collected from rendering operations. *J. Food. Prot.* In press.

18. Jones, F. 2011. A review of practical *Salmonella* control measures in animal feed. *J. Appl. Poult. Res.* 20,102-113.

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

20. Joseph, B., S. Otta, and I. Karunasagar. 2001. Biofilm formation by *Salmonella* spp. on food contact surfaces and their sensitivity to sanitizers. *Int. J. Food Microbiol*. 64:367-372.

21. Kaufmann, A. F. and J. C. Feeley. 1968. Culture survey of *Salmonella* at a broiler-raising plant. *Public Health Rep*. 83:417-422.

22. Kinley, B., J. Rieck, P. Dawson, and X. Jiang. 2010. Analysis of *Salmonella* and enterococci isolated from rendered animal products. *Can. J. Microbiol.* 56:65-73.

23. Li, X., L. Bethune, Y. Jia, R. Lovell, T. Proescholdt, S. Benz, T. Schell, G. Kaplan, and D. McChesney. 2012. Surveillance of *Salmonella* prevalence in animal feeds and characterization of the *Salmonella* isolates by serotyping and antimicrobial susceptibility. *Foodborne Pathog. Dis*. 9:692-698.

24. Magwood, S. E., J. Fung, and J. L. Byrne. 1965. Studies on *Salmonella* contamination of environment and product of rendering plants. *Avian Dis.* 9:302-308.

25. Meeker, D., and C. Hamilton. 2006. An overview of the rendering industry, p.1-16. *In* D. Meeker and C. Hamilton, Essentials of Rendering: All about the animal by-product industry. National Renderers Association, Arlington, VA.

26. O'Leary, D., E. M. M. Cabe, M. P. McCusker, M. Martins, S. Fanning, and G. Duffy. 2013. Microbiological study of biofilm formation in isolates of *Salmonella enterica* Typhimurium DT104 & DT104b cultured from the modern pork chain. *Int. J. Food Microbiol*. 161:31-43.

27. Orthoefer, J. G., M. Schrieber, J.B. Nichols, and N. Schneider. 1968. *Salmonella* contamination in a rendering plant. *Avian Dis.* 12:303-311.

28. Papadopoulou, C., J. Carrique-Mas, R. Davies, and A. Sayers. 2009. Retrospective analysis of *Salmonella* isolates recovered from animal feed in Great Britain. *Vet. Rec*. 165:681-688.

29. Pulido-Landínez, M., P. Washington, J. K. Thornton, Y. Zhang, R. Sánchez-Ingunza, A. Banda, J. Guard, V. P. Nascimento, D. L. Magee, and M. J. Mauel. 2014. Serotype and antimicrobial resistance patterns of *Salmonella* isolates from commercial birds and poultry environment in Mississippi. *Avian Dis.* 58:64-70.

30. Speranza, B., M. R. Corbo, and M. Sinigaglia. 2011. Effects of nutritional and environmental conditions on *Salmonella* spp. biofilm formation. *J. Food Sci*. 76:M12-M16.

31. Stepanovic, S., I. Cirkovic, V. Mijac, and M. Svabic-Vlahovic. 2003. Influence of the incubation temperature, atmosphere and dynamic conditions on biofilm formation by *Salmonella* spp. *Food Microbiol*. 20:339-343.

32. Tittiger, F. and D. C. Alexander. 1971. Recontamination of products with *Salmonella* after rendering in Canadian plant. *Can. Vet. J.* 12:200-203.
33. USDA-FSIS. 1996. Pathogen Reduction/Hazard Analysis Critical Control Point systems final rules. *Pathogen Reduction & HACCP Guidance Documents.* Available at: http://www.fsis.usda.gov/wps/portal/fsis/topics/regulatory-compliance/haccp/pr-and-haccp-guidance-documents (Accessed Feb. 2016)
34. US-FDA. 2013. CGMPs and preventive controls for food for animals. *FDA Food Safety Modernization Act.* Available at: http://www.fda.gov/Food/GuidanceRegulation/FSMA/ucm366510.htm (Accessed Feb. 2016)

TABLE 1.

Microbiological analysis of total aerobic bacterial counts and *Salmonella* in plants.

Location	Meal type	Sample type		Plate count [a]				*Salmonella* after enrichment	*Salmonella* isolates
				Total aerobic bacteria		Presumptive *Salmonella*			
				Plant A	Plant B	Plant A	Plant B		
Raw materials receiving area	Feather	Air		2.75±0.01 (k)	1.94±0.03 (l)	<0 G	<0 G	+/- [b]	0
		Surface		7.16±0.02 (d)	7.55±0.05 (e)	2.96±0.01 D	4.01±0.13 D	+	13
		Solid		9.11±0.10 (a)	9.41±0.01 (a)	5.28±0.31 B	6.05±0.07 A	+	10
	Feed grade	Air		1.57±0.01 (l)	1.75±0.03 (l)	0.74±0.04 G	<0 G	+/-	5
		Surface		8.09±0.21 (c)	9.11±0.06 (b)	3.03±0.03 D	4.82±0.06 C	+	9
		Solid		8.26±0.01 (b)	7.66±0.05 (e)	5.79±0.01 A	4.70±0.09 C	+	8
	Pet grade	Air		1.35±0.37 (l)	2.15±0.02 (l)	0.65±0.27 G	<0 G	+/-	5
		Surface		8.05±0.26 (c)	8.56±0.06 (c)	2.99±0.02 D	3.88±0.03 D	+	8
		Solid		7.85±0.03 (c)	8.38±0.01 (d)	4.88±0.01 C	5.96±0.02 B	+	12
Crax [c]	Feather	Air		1.83±0.01 (l)	0.81±0.03 (l)	<0 G	<0 G	+/-	1
		Surface		5.25±0.01 (g)	4.70±0.10 (i)	1.65±0.15 F	2.30±0.24 F	+	20
		Solid		3.79±0.02 (i)	5.57±0.16 (h)	<0 G	<0 G	**+/-**	4
	Feed grade	Air		1.77±0.04 (l)	1.04±0.04 (l)	<0 G	0.39±0.30 G	+/-	1
		Surface		4.80±0.03 (h)	5.19±0.18 (h)	1.38±0.22 F	3.19±0.36 E	+	16
		Solid		1.61±0.09 (l)	4.89±0.01 (i)	<0 G	<0 G	**+/-**	4
	Pet grade	Air		1.77±0.01 (l)	1.69±0.05 (l)	<0 G	<0 G	+/-	0
		Surface		5.93±0.07 (f)	5.99±0.01 (g)	1.72±0.03 F	<0 G	+/-	11
		Solid		1.70±0.03 (l)	4.72±0.05 (i)	<0 G	<0 G	-	0
Finished meal loading-out	Feather	Air		1.43±0.02 (l)	2.13±0.07 (l)	<0 G	<0 G	+/-	0
		Surface	Floor	7.05±0.12 (d)	6.96±0.01 (f)	2.90±0.20 D	3.53±0.16 E	+	8
			Wall	7.17±0.14 (d)	5.06±0.40 (h)	2.85±0.04 D	<0 G	+/-	1
		Solid		4.51±0.05 (h)	5.03±0.11 (h)	<0 G	<0 G	+/-	6
	Feed grade	Solid		3.04±0.01 (j)	2.97±0.07 (k)	<0 G	<0 G	+/-	6
	Pet grade	Air		1.54±0.01 (l)	3.32±0.09 (j)	<0 G	<0 G	+/-	1
		Surface	Floor	6.72±0.05 (e)	6.62±0.01 (f)	2.30±0.11 E	2.40±0.35 F	+	7
			Wall	6.59±0.25 (e)	5.31±0.16 (h)	3.00±0.18 D	2.54±0.42 F	+	5
		Solid		2.68±0.03 (k)	4.17±0.09 (i)	<0 G	<0 G	**+/-**	5

[a] Average of duplicate samples ± standard deviation; units for air, surface and solid samples are log CFU/plate, log CFU/cm^2 and log CFU/ g, respectively. Average numbers with different letters in the same column are significantly different ($P < 0.05$).

[b] *Salmonella* detected in either one of duplicate samples or only one of selective medium plate.

[c] Crax, rendered materials prior to grinding process.

TABLE 2.

Serotypes of *Salmonella* isolates associated with sampling locations.

Salmonella Serotype	Location[a]	No. of *Salmonella* Isolates	PFGE Pattern Numbers[c]
Mbandaka	CG & LO[b]	6	1
Typhimurium	RR & LO[b]	6	8, 23, 24
Ealing	CG	5	14
Schwarzengrund	RR	5	2, 3
Widemarsh	CG	4	13
Johannesburg	LO	3	27
Infantis	RR & LO[b]	3	9, 28
Senftenberg	RR & LO[b]	3	16, 21
Heidelberg	RR	2	19
Kentucky	RR	2	11, 25
Hadar	RR	1	26
Ouakam	RR	1	22
Pomona	LO	1	15
Tennessee	LO	1	29
Urbana	LO	1	10
Uganda	CG	1	12

[a] LO, finished meal loading-out area; CG, crax grinding area; RR, raw material receiving area.
[b] Indicates a potential of cross-contamination.
[c] Different numbers indicate different patterns observed in PFGE analysis.