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Research Paper

A Comparison of *Salmonella* Survival and Detection Using an Enrichment Technique in Dry- and Wet-Inoculated Rendered Chicken Fat Treated with Sodium Bisulfate

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ABSTRACT

The differences in the recovery of *Salmonella* from rendered chicken fat treated with sodium bisulfate (SBS) when inoculated with a dry versus wet inoculum were evaluated. Food-grade rendered chicken fat was inoculated with a dry inoculum and a wet inoculum containing a cocktail of *Salmonella* serovars (Enteritidis, Heidelberg, and Typhimurium). In addition, the effect of an antimicrobial treatment (SBS) against *Salmonella* in both the aqueous phase and fat phase of the chicken fat was evaluated. The untreated control samples in the aqueous phase had a consistent level of *Salmonella* (~7 log) when both the dry and wet inocula were used. In the SBS-treated aqueous phase, *Salmonella* pathogens were not detectable after 6 h when the wet inoculum was used; when the dry inoculum was used, *Salmonella* pathogens were not detectable at 24 h. *Salmonella* pathogens were detected for up to 6 h in the SBS-treated fat phase when the dry inoculum was used compared with 2 h with the wet inoculum. The 24-h fat samples that failed to show growth on Trypticase soy agar were enriched for *Salmonella* isolation, followed by confirmation by PCR using primers for the *invA* gene. SBS-treated and control samples from the dry-inoculated rendered chicken fat and the inoculated control from the wet-inoculated rendered chicken fat tested positive for *Salmonella*. However, the SBS-treated sample from the wet-inoculated fat was negative for *Salmonella*. The use of dry SBS powder against dry *Salmonella* inoculum in the fat matrix caused only ~2.8-log reduction after 24 h compared with ~2.2-log reduction in the positive control. However, the recovery of *Salmonella* from untreated control fat was lower and was not different ($P > 0.05$) from that recovered from the SBS-treated fat. The results suggest that viable but nonculturable states of *Salmonella* may develop in rendered chicken fat or that injured cells may be present, which indicates that testing should include an enrichment and appropriate molecular confirmation instead of agar plating alone.

HIGHLIGHTS

- Aqueous SBS was effective against *Salmonella* in rendered chicken fat.
- Fat phase control and dry-inoculated samples failed to recover *Salmonella* on TSA.
- Fat phase *Salmonella* did not grow on agar but was positive upon enrichment and PCR.
- *Salmonella* could potentially develop VBNC cells in fat.

Key words: Dry inoculation; Rendered chicken fat; *Salmonella*; Sodium bisulfate; Wet inoculation

The products of rendering are used in a wide array of applications, such as animal feed, pet food, and pharmaceuticals *(9)*. Rendered animal fats are very popular ingredients in the pet food industry and are often coated onto dry pet food kibbles to increase energy density and enhance palatability. The rendering industry follows a rigorous thermal processing procedure that heats the products to very high temperatures for extended cooking times; however, cross-contamination from pathogens still occurs. This cross-contamination has been considered a major factor in the presence of *Salmonella* in rendered products *(19)*. Despite having low water activity for microbial growth, low-moisture foods are periodically reported to contribute to foodborne infections and outbreaks. For example, low-moisture foods such as peanut butter *(2),* dried paprika spice *(20),* and chocolate *(11)* are known to harbor *Salmonella*. Although it is widely assumed that animal fats and oils do not harbor pathogens such as *Salmonella,* they may contain some small level of moisture, which can become a reservoir for *Salmonella* growth and later contamination.

In recent years, there has been an increase in *Salmonella*-related recalls in pet foods. During 2018 to 2019 there were over two dozen *Salmonella*-related recalls in the pet food industry, and most of these were associated

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with raw pet foods and treats *(29)*. A detailed investigation regarding the *Salmonella* Schwarzengrund outbreak during 2006 to 2007 in dry dog food led to the finding that the enrobing and flavoring room in the manufacturing facility was positive for the outbreak strain *(1)*.

Rendered chicken fats are normally stored in bulk tanks in rendering facilities and in pet food processing plants, and this fat is transported by truck between the two. The rendering process is effective in killing most of the pathogens due to its high processing temperature. However, this point-in-time mitigation offers no long-term residual antimicrobial effect. Cross-contamination during handling, storage, and transportation of the rendered products is considered to be the primary factor for *Salmonella* contamination in the final product *(5, 17)*.

During pet food production, the coating of fat and flavoring agents is done after thermal processing. Hence, any cross-contamination at this step from *Salmonella* in rendered fats can cause a substantial loss to the pet food industry via recalls and for pet owners through potential food supply disruption or ill health. Finding a way to prevent cross-contamination and/or to mitigate *Salmonella* in the coated fat is important. Rendered fats and oils that are used to coat dry pet foods are commonly treated with acidulants and medium chain fatty acids to mitigate the *Salmonella* that could potentially come from postprocessing cross-contaminations *(6, 8)*. Limited research has been done on the survival of *Salmonella* in low-moisture ingredients like fats and oils, and there are no studies in which the use of antimicrobial application on a dry *Salmonella* inoculum has been evaluated. In a previous study, we reported an acidulant, sodium bisulfate (SBS) and lactic acid, to have an antibacterial effect against *Salmonella (7)* in a rendered chicken fat system. But that work was conducted solely with a wet inoculation procedure. SBS is a known food-grade antimicrobial agent against *Salmonella* and *Campylobacter (21)* and is commonly used in animal feed *(4, 8)* to control *Salmonella*. SBS is also used in pet diets for preservation of soft-moist treats and to acidify feline urine.

As a follow-up to our previous study, in this work we hypothesized that the airborne particulates containing *Salmonella* from the rendering vicinity and surroundings could also be the source of pathogen in rendered chicken fat. Hence, the objective was to inoculate with a *Salmonella* cocktail in both a dry and a wet form in a rendered chicken fat system and evaluate *Salmonella* survival during SBS treatment over time. In this study, fat that tested negative for *Salmonella (8)* on nutrient agar was also enriched for *Salmonella* isolation. In addition, considering the recent approaches of dry sanitation and risk reduction in low-moisture equipment *(13, 14, 25),* we also evaluated the effect of dry SBS powder against a dry *Salmonella* inoculum in low-moisture (no external moisture from washing) conditions for transporting chicken fat.

MATERIALS AND METHODS

***Salmonella* serotypes, sources of antimicrobials, and chicken fat.** *Salmonella* serovars Enteritidis (ATCC 4931), Heidelberg (ATCC 8326), and Typhimurium (ATCC 14028) were maintained in tryptic soy broth (TSB)–glycerol (7:3) at –80°C *(31)*. Before use, the frozen cultures were streaked onto tryptic soy agar (TSA) plates and incubated at 37°C for 24 h. A single colony of each *Salmonella* strain was inoculated to 10 mL of TSB and incubated at 37°C for 18 to 24 h. SBS was provided by Jones-Hamilton Co. (Walbridge, OH). Rendered chicken fat was provided by an established poultry rendering company (Darling International Inc., Kansas City, MO).

Preparation of the *Salmonella* dry-inoculum cocktail. The dry inoculum of *Salmonella* was prepared by a method previously described *(10)*. Briefly, talc powder (Carolina Biological Supply, Burlington, NC) was oven heated at 140°C for 4 h and verified for the absence of any microorganisms by plating on TSA. Equal volumes (~9 log each) of the three serotypes were composited to form a final volume of 18 mL. This composite was added to 25 g of sterile talc powder placed on a sterile Pyrex glass petri plate. The culture was then mixed with the talc powder using a sterile stainless steel spatula. The mixture was uniformly spread and covered with sterile gauze and then was incubated at 37°C for 24 h. After removing the plate from the incubator, it was dried at room temperature for an additional 24 h to obtain a final water activity of 0.28. Water activity was measured using a Decagon CX-2 meter (Meter Group, Pullman, WA). The inoculated talc was then broken down into fine powder and stored in a tightly sealed sterile plastic container in a refrigerator (4°C) for up to a week. The bacterial inoculum concentration in talc was found to be between 7 and 8 log.

***Salmonella* inoculation of chicken fat.** The rendered chicken fat was sterilized by autoclave and transferred to 50-mL sterile plastic centrifuge tubes. A total of four samples, two for dry inoculation (SBS and control) and two for wet inoculation (SBS and control), were maintained. Based on our previously established MIC *(8),* 0.5% (w/v) SBS was used as an antimicrobial treatment. A similar amount of sterile distilled water was added to the two controls to maintain the same moisture level in both dry- and wet-inoculated fat systems. The moisture percentage in chicken fat was maintained at ~3% (v/v). The overnight cultures of each *Salmonella* serotype were centrifuged for 10 min at 5,000 rpm (Thermo Scientific, Waltham, MA) at room temperature. The pellets were resuspended in 0.1% presterilized peptone water (BD Difco, Sparks, MD), and an equal volume of each serotype was mixed to obtain the cocktail. *Salmonella* cultures (~7 log CFU/g of dry and ~7 log CFU/mL of wet inoculum, final concentration) were added to the overnight chemically treated chicken fat. To a fat system (total 40 mL), 0.6 mL of SBS and 0.6 mL of aqueous *Salmonella* cocktail (~9 log) were added for wet inoculum; for dry inoculum, 1.2 mL of SBS solution and 1 g of dry *Salmonella* cocktail (8.5 log/g) were added. After vortex mixing, the tubes were incubated at 42°C. Microbiological analyses were conducted separately for the fat-phase and aqueous-phase treatments at predetermined time intervals (0, 2, 6, 12, and 24 h). The liquid fat and aqueous phases were gently removed by pipetting and were diluted in 0.1% (w/v) presterilized peptone water; then, 0.1 mL was spread plated onto TSA. For plating, the fat-phase samples were drawn, followed by vigorous vortex mixing (Vortex Genie, Scientific Industries Inc., Bohemia, NY) of the tubes and removal of 100 μL while the suspension was still well mixed. The plates were incubated at 37°C for 24 h, and then colonies were counted.

Detection of *Salmonella* in fat phase. For the qualitative assessment of *Salmonella* in the fat phase, liquid that showed nondetectable *Salmonella* on TSA agar after 6 h was separately processed for isolation of *Salmonella* using a modified *Bacteri-*

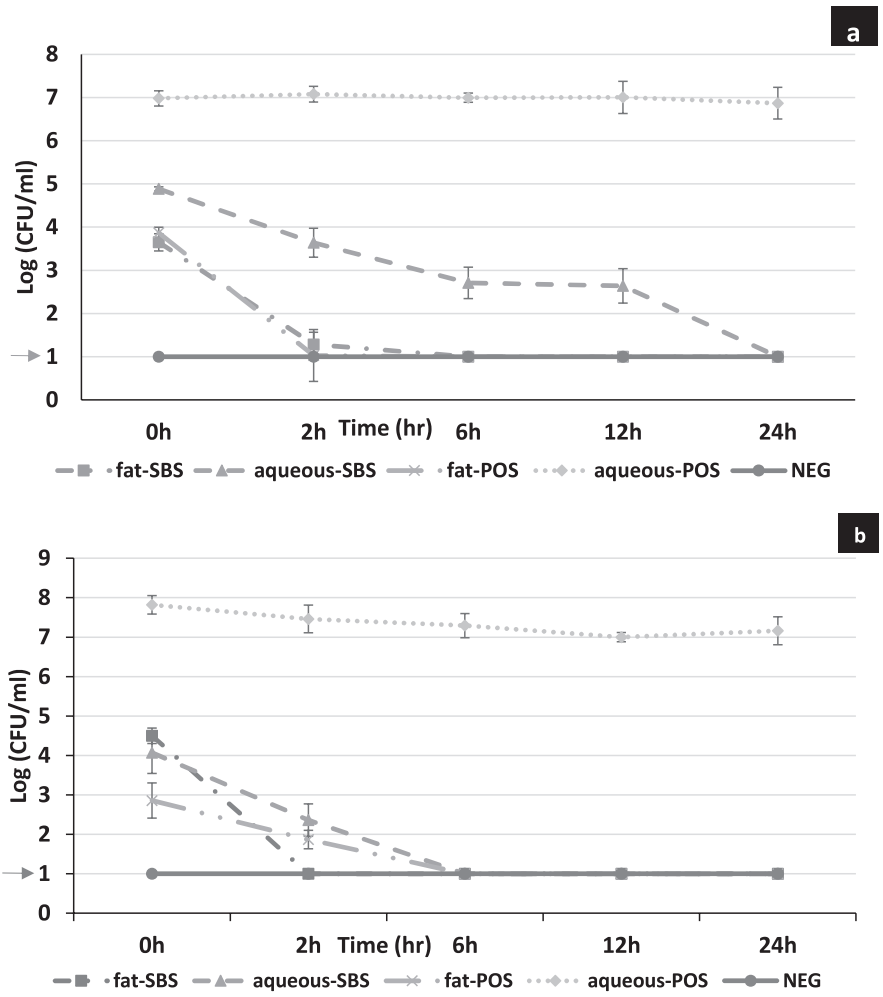

FIGURE 1. *Treatment means ± standard errors for fat phase plus sodium bisulfate (fat-SBS, ■), aqueous phase plus SBS (aqueous-SBS, ▲), fat phase plus positive control with* Salmonella *only and no added SBS (fat-POS, ×), aqueous phase plus positive control with* Salmonella *only and no SBS added (aqueous-POS, ♦), and negative control with no* Salmonella *or SBS (NEG, ●) on dry inoculum (a) and wet inoculum (b) of a cocktail of* Salmonella *serovars (Enteritidis, Heidelberg, and Typhimurium) in a fat-phase and aqueous-phase chicken fat system at 42°C on TSA plates over 24 h. The arrow indicates the detection limit (1.0 log CFU/mL) of sampling using TSA plates.*

ological Analytical Manual (28) procedure. In brief, 10 mL of the fat samples was preenriched in 90 mL of buffered peptone water for 24 h, followed by selective enrichment in tetrathionate broth and Rappaport-Vassiliadis broth for another 24 h. The enriched samples were then plated on xylose lysine desoxycholate (XLD) and brilliant green sulfa agar. The typical colonies from the XLD agar were used for DNA isolation, and a PCR amplification (Bio-Rad, Des Plaines, IL) was conducted for the *invA* gene of *Salmonella* with a nucleotide sequence 5′ GTG AAA TTA TCG CCA CGT TCG GGC AA-3′ and 5′ TCATCG CAC CGT CAAAGG AAC C-3′. Reactions were carried out in a 50-µL mixture consisting of 25 µL of PCR master mix (Thermo Scientific), 2 µL of each primer, 19 µL of molecular-grade water, and 2 µL of extraction for each isolate used in the reaction *(24)*. The amplified DNA products were run on agarose gel and observed for the DNA band. A 100-bp DNA ladder was used as a marker.

Use of dry SBS against dry *Salmonella* inoculum in chicken fat. SBS, in a dry powdered form, was applied in rendered chicken fat at 0.5% (w/v). SBS (50 mg) was added in 10 mL of fat system. After overnight incubation at 42°C, the fat sample was inoculated with 1 g of dry *Salmonella* cocktail inoculum (8.5 log/g). A positive control fat with no SBS powder was maintained. After vortex mixing, the tubes were incubated at 42°C. Microbiological analyses were conducted from the mid-stream fat phase at predetermined time intervals (0, 2, 6, 12, and 24 h). The fat sample was gently pipetted from the middle part of the test tube. The liquid fat was gently removed by pipetting, diluted in 0.1% (w/v) presterilized peptone water, and plated onto TSA. For plating, samples were drawn after vigorously mixing the tubes with a Vortex Genie mixer and removing 100 µL while the suspension was still well mixed. The plates were incubated at 37°C for 24 h, and then colonies were counted.

The GLM procedure of SAS version 9.4 (SAS Institute, Cary, NC) was used to perform analyses of variance. The means were separated using the least significant difference test. The treatments and controls were considered significantly different at $P \leq 0.05$. The log means and standard deviations for counts were plotted over time for each of the treatments. All the experiments were run in triplicate. The detection of *Salmonella* in fat phase was conducted in duplicate. Comparisons between treatments and the positive and negative controls were identified; primary results were evaluated with the limit of detection at <1 log/mL of sample.

RESULTS AND DISCUSSION

Comparison of dry inoculum versus wet inoculum. *Salmonella* recovery over time in both the fat and aqueous phases using the dry and wet inocula is shown in Figure 1. When the fat samples were subjected to aqueous SBS treatment and subsequently were inoculated with *Salmonella,* the aqueous phase in the dry-inoculated fat system sustained *Salmonella* for up to 24 h (Fig. 1a). Whereas, in the aqueous phase of the wet-inoculated fat system, *Salmonella* was not detectable at 6 h (Fig. 2b). In the fat phase of the SBS-treated fat system, an extended presence

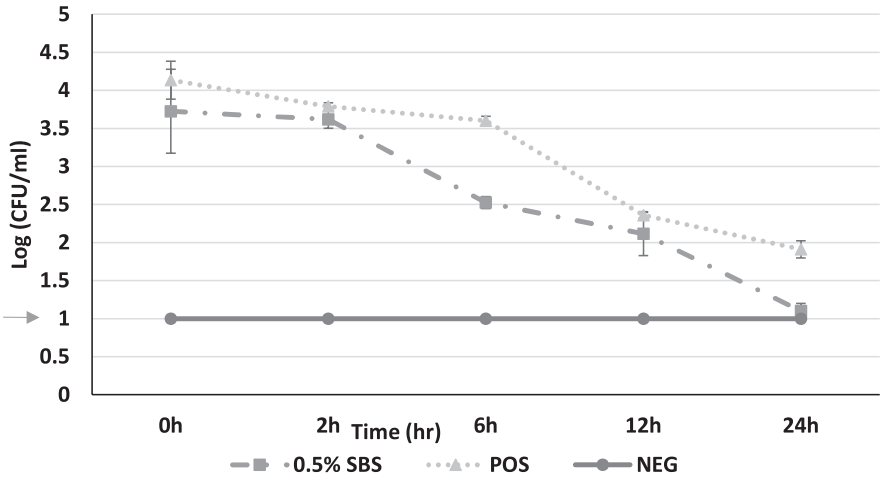

FIGURE 2. *Treatment means ± standard errors for dry sodium bisulfate (0.5% SBS, ■), positive control with* Salmonella *only and no added SBS (POS, ▲), and a negative control with no* Salmonella *or SBS (●, NEG) on a dry inoculum cocktail of* Salmonella *serovars (Enteritidis, Heidelberg, and Typhimurium) in a fat-phase system at 42°C on TSA plates over 24 h. The arrow indicates the detection limit (1.0 log CFU/mL) of sampling using TSA plates.*

of *Salmonella* (nondetectable at 6 h) when the dry inoculum was used was observed relative to the wet inoculum (nondetectable at 2 h). The dry inoculation method mimics the contamination of rendered fat from a dry dust aerosol and insoluble fractions. In conditions where the storage tanks and trucks contain no moisture or very little moisture (likely from the condensation of hot rendered fat), use of the dry inoculation method may be a more representative approach. Whereas, the wet inoculation method simulates the aqueous contamination of *Salmonella*. In a study by Trinetta et al. *(26),* the dry inoculation of a *Salmonella* cocktail in rendered chicken fat at 3% moisture caused >1-log decline within 24 h and >7-log reduction after day 4. The difference in these results and our findings may be due to the sampling technique. We performed the microbial analysis of fat and aqueous phases separately, whereas the authors in the abovementioned study vortexed and combined the phases of the sample before sampling.

Salmonella pathogens in control fat, in both dry and wet inoculations, were detected for up to 6 h. In addition, for both dry and wet inoculations, the aqueous phase of the control samples without SBS treatment maintained the original *Salmonella* concentration (~7 log) throughout the incubation period. This finding is similar to that of Trinetta et al. *(26),* in whose study the *Salmonella* cocktail remained constant for up to 6 days when 5.5% (v/w) of liquid inoculum was added in rendered chicken fat. Kiel et al. *(16)* reported a sharp decline (from ~7 log to ~1 log) in the *Salmonella* population in rendered chicken fat when 3% moisture was incorporated and after storage at 45°C for 48 h. The recovery procedure of *Salmonella* in their study also differs from ours; we analyzed the fat phase and aqueous phase separately to mimic one potential industry scenario for bulk fat storage and (or) transportation.

***Salmonella* isolation from fat phase.** The fat phase of both the SBS-treated and nontreated control samples in both dry and wet inoculum treatments were below the *Salmonella* detection limit (detection limit 1×10^{-1} CFU/mL) on TSA at 6 h and afterward (Fig. 1a and 1b). However, when the fat samples were subjected to *Salmonella* isolation using the modified FDA bacteriological enrichment method *(28),* both the SBS-treated and control fat of the dry-inoculum–treated fat system, and the control fat of the wet-inoculum–treated fat system, were *Salmonella* positive on XLD agar. The typical black colonies from the XLD agar were further confirmed by PCR amplification of the *invA* gene and by visualizing a 284-bp DNA fragment in agarose gel electrophoresis. This result corroborates the finding of Shanmugasamy et al. *(24)*. When a fat phase was *Salmonella* positive after enrichment and PCR confirmation, this indicates that either the *Salmonella* cells were injured and were not able to grow on TSA agar plates, or the cells entered into a viable but nonculturable (VBNC) state, or the detection limit of the enrichment method is lower than that of the quantitative approach. It is common for *Salmonella* cells to get injured in a fat medium due to low water activity and buffering capacity *(19)*. A further plating of the fat-phase suspension on TSA plates supplemented with sodium pyruvate is also suggested as a way to resuscitate the potentially injured *Salmonella* cells *(30)*.

Note that the fat phase of the wet-inoculum–treated fat system tested negative for *Salmonella*. This supports the conclusion that the aqueous SBS treatment was effective at mitigating *Salmonella* in both the fat phase and aqueous phase of the bulk fat system. In the study by Kiel et al. *(16),* when a low-moisture (<1%) fat was plated on TSA, no *Salmonella* was detected at 2 h; when medium (2.1 to 3.0%) and high (3.9 to 4.8%) moisture were used, no *Salmonella* was detected on TSA at 6 h and thereafter. This result aligns with our findings, wherein the fat phases of both dry and wet inocula were negative for *Salmonella* when plated on TSA plates. Kiel et al. *(16)* used a fluorescently tagged *Salmonella* to detect the location of *Salmonella* in a long burette system. The limitation of their study was that even the nonviable cells tended to fluoresce *(12),* giving a false positive for the presence of viable cells in the fat system. In our study, we sampled the midstream fat from the sample tube and processed it for enrichment and PCR confirmation; we found that the fat-phase samples have viable *Salmonella* cells that grow in XLD agar after preenrichment in buffered peptone water and enrichment in tetrathionate and Rappaport-Vassiliadis broth. There could be multiple reasons for our study's low recovery (~4 log) of *Salmonella* and

nondetectable *Salmonella* after ~6 h from the fat phase (with both dry and wet inocula) compared with the aqueous phase. The free fatty acids from oleic acid present in the rendered fats have known antimicrobial activity *(32)*. Sedimentation of the aqueous-phase *Salmonella* to the bottom could be another possible explanation for the low, or no, recovery of *Salmonella* from the fat phase. In a study using fats and oils, Lamb *(19)* found a loss of about 3 log CFU/mL of *Salmonella* cocktail after 24 h. However, the 24-h fat phase on the inoculated control that was positive for *Salmonella* after PCR confirmation may indicate that *Salmonella* could remain viable in the fat phase but is not in culturable in TSA. *Salmonella* pathogens are known to enter a VBNC state to evade unfavorable nutritional and environmental conditions; they fail to display any growth on nutrient agar *(3)* and revert back to a normal resuscitated state under favorable conditions *(22)*. *Salmonella* pathogens that fail to grow on nutrient agar are known to resuscitate when enrichment cultivation techniques are used *(23)*. This method of *Salmonella* isolation from rendered chicken fat provides a viable approach for the rendering industry and pet food companies to ensure that the fat does not carry injured or VBNC *Salmonella*. Thereby, it reduces the risk from cross-contamination in the pet food system that may occur during and after the fat-coating step.

Use of dry SBS powder against dry *Salmonella* in rendered chicken fat. In fat rendering and pet food processing plants, transport trucks and tanks with a residual water layer in the bottom are widely considered to be the source of *Salmonella* in dry pet food. Hence, a new approach to develop a dry-cleaning method has been discussed in the scientific community *(13, 14, 25)*. In this study, we avoided the water layer (~3%) in the bottom of the fat and used dry antimicrobial (SBS) and dry *Salmonella* inoculum. The results (Fig. 2) show that *Salmonella* recovery at 0 h was ~4 log in both treatment and control. The *Salmonella* count kept declining over time and was ~2 log in SBS-treated fat after 24 h. There was no difference ($P > 0.05$) in *Salmonella* recovery from SBS-treated and nontreated control fat, except at 6 h. The authors do not have an explanation for why one time point differs in the reduction, but possible causes include variability in the counting method, nonhomogeneity of the cell population, or uneven cell clumping. The reason for the consistently lower recovery of *Salmonella* throughout the incubation could be either the sedimentation of the dry form of *Salmonella* or the toxic effect of fat *(15)*. A more plausible reason could be the entry of *Salmonella* into a VBNC phase, as we verified in this study (mentioned above). The recovery of ~2 log of *Salmonella* on TSA plates in the control fat after 24 h contradicts our own finding of failure to detect *Salmonella* on TSA agar in the control fat in the dry-inoculum–treated fat system (Fig. 1a). One explanation could be that, after addition of dry *Salmonella* inoculum in the former case, vortexing tubes may have caused the *Salmonella* cells to sediment with the aqueous phase of water (~3%). In addition, *Salmonella* recovery from the SBS-treated fat sample indicates that the dry SBS is less effective than aqueous SBS. This finding is consistent with the results reported by Cochrane et al. *(4),* who observed that dry SBS was not effective in mitigating *Salmonella* Typhimurium when applied to animal feed. The dry and wet inoculation methods have significant influence on the survival of *Salmonella (18, 27)*. Unlike previous reports *(8, 16, 26),* in this study we used dry inoculum and dry antimicrobial without any external added moisture in rendered fat. This method could be a more practical approach to study the viability of *Salmonella* in a fat system that contains low, or no, moisture.

In conclusion, the fat phase in bulk rendered chicken fat may harbor *Salmonella* pathogens in their VBNC state, and fat processing and pet food industry stakeholders must be aware of the extensive detection techniques necessary to ensure that fat contains no *Salmonella*. The study also showed that *Salmonella* contamination via aerosol or insoluble fractions in fat is harder to mitigate than *Salmonella* that is introduced through aqueous contamination.

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