Effects of moisture and temperature on *Salmonella* survivability in beef tallow, white grease and chicken rendered fat

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SIGNIFICANCE AND IMPACT

Bulk fats and oils used as palatant coatings are rendered free of pathogens in most instances. Nevertheless, they can be re-contaminated with *Salmonella* by handling practices that allow for contaminated contact surfaces and uncontrolled ingress of water from environmental sources and cleaning water. This presents a significant hazard given the application of these fats post the lethality step. The data from this study would imply that holding the fats at temperatures of 76°C or greater should result in minimal detectable *Salmonella* spp. after 24 hours. Most commercial operations do not typically hold liquid fats for more than a few hours at a time during peak production times, therefore, relying on temperature alone may not be adequate to control potential *Salmonella* cross contamination. At the moisture levels, times and temperatures of the experimental setup, no pathogen presence was observed. Lethality was a function of time and temperature. The time and temperature required for significant lethality are not practical conditions for routine use at factory sites.

The data from this study will help end users of these fats better understand the potential impacts of their storage parameters of time and temperature on potential *Salmonella* lethality. More research is needed to help identify more robust ways to eliminate *Salmonella* from the fat matrix.
ABSTRACT

High moisture levels introduced to fats after the rendering process can lead to *Salmonella* presence and growth. Limited research on strategies to eliminate pathogens in these environments are available. Rendered fat characteristics, such as water activity and fatty acids composition, may contribute to *Salmonella* survivability. The purpose of this research was to evaluate the effects of moisture levels (0%, 0.5%, 1%, and 3%), storage temperatures (48°C and 76°C), and fat characteristics on the growth and survival of *Salmonella* in beef tallow, white grease and poultry fat samples. Samples were inoculated with a high (~10⁸ CFU/mL) and a low (~10⁵ CFU/mL) *Salmonella* cocktail (S. Sentfenberg, S. Newport, S. Thompson and S. Infantis). Samples were stored for up to 5 days at 48 and 76 °C. Remaining population was evaluated daily with and without enrichment step. Death rates were calculated using Weibull model for each temperature and moisture level. Only temperature had an effect (*P* < 0.05) on *Salmonella* inactivation, while no effect between moisture and/or inoculum level were observed. When all products were challenged at 76 °C, counts were below detectable limits after 6 hours. At 48°C a progressive decline in *Salmonella* population was observed within 3 days for both beef tallow and white grease when high inoculum was used for the challenge study. *Salmonella* was below detectable limit within 4 days for both fat types when a low inoculum was instead applied. This research identified the effect of moisture and temperature in rendered fat samples contaminated with *Salmonella* and underlined the need to use time-moisture-temperature data to minimize microbial growth during transportation and storage.

KEYWORDS

*Salmonella*, moisture, temperature, survival, rendered fat
INTRODUCTION

Rendering is defined as the process that converts waste animal tissues into stable and usable materials. It is estimated that the rendering industry collects and processes approximately 25 million tons of animal by-products each year in the United States (Meeker and Meisinger, 2015). Livestock and pet food make up 50% of the rendered fat market, followed by industrial chemicals, soaps, food industry, and biodiesel (Jekanowski, 2011, NRA, 2008). The most common animal species used in this industry are beef, pork or poultry. Poultry fat consists of fats derived exclusively from poultry offal. Blended feed fat is a category that includes blends of tallow, grease, poultry fat, and restaurant grease/cooking oils. Blended animal and vegetable fats include blends of feed grade animal fats, poultry fats, vegetable fats, and/or restaurant grease/cooking oil.

The process of rendering involves chopping products into uniform sizes and cooking them through cycles of 40 to 90 min at 115.6 to 143.3° C (NRA, 2008). The purpose of this continuous cooking process is to separate useful by-products and eliminate bacteria (Meeker and Hamilton, 2006). Continued improvements within the industry have been implemented to ensure proven cook times and temperatures inactivate specific microorganisms deemed to be a food safety hazard (Meeker and Hamilton, 2006). Despite the aggressive thermal processing step, *Salmonella* spp. has been linked to several outbreaks in the animal and pet food industry. Cross-contamination, in which finished rendered products become re-contaminated, has been proposed as the primary factor for pathogen presence (Troutt et al., 2001; Denton et al., 2005; Kinley et al., 2010; Vidyarthi, 2021).
The Food and Drug Administration (FDA, 2019) reported that *Salmonella* was the major contributor to 14.3% of the cases associated with pet food recalls from 2008 to 2014. *Salmonella enterica* serotype Infantis and *Salmonella enterica* serotype Schwarzengrund were identified in the contaminated dry dog food, causing 53 and 70 illnesses, respectively (Deasy, et al, 2008; Imanishi et al, 2014). In 2018, 22 of the reported pet food recalls were linked to *Salmonella* (FDA, 2019). Though the source of contamination for some of these outbreaks was not determined, the use of rendered products in pet treats formulation was identified as a concern. Rendered by-products are commonly used as an incorporated ingredient and/or an outer coating for dry pet food pieces after the thermal cooking step (Thompson, 2008). The formulation of livestock feeds uses a similar process of adding rendered fats and meals (Crump, 2002). Outbreaks of *Salmonella enterica* which have been traced back to animal feeds, such as poultry and cattle, have identified the use of rendered products as the source of contamination (Crump, 2002).

*Salmonella* strains were observed to be resistant to desiccation and were observed to survive in low-moisture foods for extended periods of time (Subedi and Roopesh, 2020). Even though low water activity ingredients, such as animal fat, were once thought impossible to harbor *Salmonella* spp., it is now scientifically acknowledged that small quantities of water in these ingredients may lead to microbial contamination (Subedi and Roopesh, 2020). If residual water from wet cleaning of tankers or trucks is accumulated, *Salmonella* spp. can survive and thrive during transportation. Since this ubiquitous pathogen quickly adapts to new environmental conditions (Gwyther et al., 2012), contamination prevention is key.

The investigation of factors and parameters that can influence, and favor *Salmonella* growth in low-moisture food, such as animal fat, is important. Previous data collected by our group (Trinetta et al., 2019) suggested that residual moisture in containers during transportation of poultry fat did not impact
Salmonella spp. growth. Nevertheless, if the level of Salmonella spp. contamination levels were high (~10^8 cfu/mL) and the storage temperature was 48°C, moisture had a role towards pathogen thermal death. In the present study we compared the effects of moisture levels (0%, 0.5%, 1.0% and 3.0%) and temperatures (48°C and 76°C) on the growth and survival of Salmonella spp. in other types of animal fat (beef tallow and white grease) overtime.

RESULTS AND DISCUSSION

Table 1 shows lipid, moisture percentage, and water activity measurements for rendered fat samples selected in this study. Rendered animal fatty acid composition is instead reported in Table 2. In all fat types, the major fatty acid was oleic acid followed by palmitic acid and linoleic acid in respective amounts. Although there was no difference (P > 0.05) in their total saturated fat composition, the major saturated fatty acid (palmitic acid) was decreased (%) in chicken fat, as compared to choice white grease (Table 2) (P < 0.05). A difference (P < 0.05) was also observed in mono- and poly-unsaturated fatty acid fractions for the different fat sample types, with oleic acid greater in choice white grease as compared to chicken fat. As expected, in addition to the major fatty acids of animal fats, low to moderate amounts of C_{18:3} and C_{20:4} fatty acids were reported in our samples, and they were generally reflective of normal industry beef tallow, choice white grease, and poultry fat (Alm, 2013). Beef tallow was found to have a increased percentage of arachidonic acid (20:4) compared to choice white grease and chicken fat (P <0.05). Choice white grease was found to have a statistically increased percentage of myristic (14:0), stearic (18:0), oleic acid (18:1), eicosenoic acid (20:1) and eicosadienoic acid (20:2), while low percentage of palmitoleic (16:1) and alpha-linoleic acid (18:3) were observed (P <0.05). Increased percentages of palmitic acid (16:0) and linoleic acid (18:2) were instead observed in chicken fat (P <0.05).
A difference (P < 0.05) was observed among water activity, with choice white grease having higher values as compared to beef tallow and chicken fat (Table 3). These differences are probably attributed to variations in residual moisture introduced during handling and transportation of various rendered fats, rather than differences in animal species origin (Alm, 2013). Subsequently, fat samples were challenged at different temperatures and moisture levels. Wet inoculation wanted to mimic cross contamination from moisture during transportation and storage. No variations were observed between moisture levels and/or inoculum type at 76 °C (data not reported). All counts were below detectable limits after 24 hours (1 CFU/ml). These results were reported for each different type of animal fat analyzed in this study. Only, temperature was identified as a factor on Salmonella inactivation (P < 0.05). Similar results were also reported in our previous study, when chicken fat samples were stored at 76 °C (Trinetta et al., 2019).

Figure 1 reports the data obtained when choice white grease and beef tallow were challenged with a high and low level of Salmonella wet inoculum during the challenge study at 48 °C. After 3 days, Salmonella population was below detectable limit in all the samples (P < 0.05) (Figure 1A). The control samples (no addition of water) followed a similar trend. When a low wet inoculum was used for the challenge study a progressive decline in Salmonella population was observed in all samples within 4 days, indicating extended survival (Figure 1B). No statistical difference was observed between moisture levels (P > 0.05). No variations were observed between moisture and/or inoculum level since all counts were below detectable limits after 48 hours also for beef tallow (Figure 1 C and D).

The Weibull model was fit to mechanistically explain the effect of water content on Salmonella survival
kinetics for samples challenged with high and low inoculum levels. The model parameters $\beta$ (shape parameter related to heat resistance) and $\alpha$ (hazard rate or scale parameter) are given in Table 4. When the heat resistance of cells increases, the survival kinetics show a concave upward shape ($\beta < 1$), while a concave downward survival curve ($\beta > 1$) indicates the heat resistance of cells decreases with heating time (van Boekel, 2002). The strong correlation between the model parameters $\alpha$ and $\beta$ is also a good indication of the reliability of the analysis and performance of the model. Larger death rate kinetic values were found in the previous poultry fat study, compared to death rates found for beef tallow and white grease in this study (Trinetta et al, 2019). The Weibull model indicated that the inactivation kinetics show first order kinetic behavior. A low temperature inactivation is dependent by time, confirmed by a large $\alpha$ parameter (a characteristic time): at concentrations higher than 1% the survival kinetics approaches to first order kinetics (i.e., heat resistance does not depend on time) with a decreasing hazard rate.

Previous data collected by our group suggests that moisture percentage "largely does not affect Salmonella spp. growth" in rendered fats (Trinetta et al, 2019). Water activity ($a_w$) is the scale of measurement (0.1-1.0) for the amount of water in an environment available to microorganisms, bacteria generally require 0.86 $a_w$ and above (Jay, Loessner & Golden, 2005). Many studies have been conducted to determine factors that influence Salmonella survival in low $a_w$ foods (below 0.7). Farakos, Schaffner and Frank (2014) concluded that the major parameters affecting Salmonella survival are: 1) food composition, 2) water activity and 3) temperature. According to their study, Salmonella exhibited increasing persistence with decreased water activity and the presence of fat protected bacteria cells from inactivation (Farakos, Schaffner & Frank, 2014). The protective effect of fat was also observed by Li et al. (2014), who hypothesized that Salmonella may persist in microenvironments within high protein and high fat food mixes.
Few studies have been conducted on the effect of fatty acid compositions and pathogen growth. Zhang et al. (2016) measured the antimicrobial effect of sophorolipids with and without the addition of a palmitic, steric or oleic acid base (2%) on *Salmonella* and *Listeria* spp. populations. It was observed that the inclusion of palmitic, steric and oleic acid resulted in no difference in antimicrobial efficiency (Zhang et al., 2016). This observation might be valid also in our study, indicating that the difference observed between beef tallow, white grease and chicken fat levels of palmitic, steric and oleic fatty acids likely do not have an effect on *Salmonella* survivability. A rapid reduction in *Salmonella* population was observed as a function of increased temperature. Regardless of moisture level, inoculum level, or contamination level, holding fat at 76°C resulted in minimal detectable *Salmonella* spp. after 24 hours. This is in agreement with other research which measure bacteria presence in rendered fat products at various temperatures with bacteria levels becoming undetectable in less than 1 hour (Trinetta et al., 2019; Ramirez-Hernandez et al. 2018). Conversely, moisture percentages had an effect in *Salmonella* survival in rendered fat when samples were held at 48°C, but no differences among moisture % were observed.

The data collected in our research suggests that residual moisture in containers during transportation of white grease and beef tallow fat largely does not impact *Salmonella* spp. growth. If contaminated with a high level of *Salmonella* spp. (10⁸ cfu/mL) and held at a low temperature (48°C), moisture may influence the thermal death due to differences in water activity and water mobility kinetics.
MATERIAL & METHODS

Samples. Beef tallow, choice white grease, and chicken fat samples were obtained from a local supplier (Manhattan, KS).

Physical and chemical properties. Fat composition was calculated using a chloroform/methanol lipid extraction (Rice, et al., 2019). The fatty acid composition of each rendered fat type was determined at the Kansas Lipidomics Research Center, Kansas State University using fatty acid methyl esters (FAME) method (Christie, 1993). The transmethylation was performed using methanolic hydrochloric acid (3M) at 78 °C for 30 min. FAME were extracted using hexane:chloroform (4:1 v/v) mixture, and injected (0.5 µL) to a GC-FID system (Agilent 6890N system). Fatty acid percentages were calculated using corresponding peaks, which were identified based on relative retention times in the FAME standard mix with known concentration. Moisture percentage was calculated by oven drying at 137 ºC for 2 hours (AOAC, 2005; method 930.15). Water activity was measured using a benchtop Aqualab machine (4TE Aqualab, Meter Group, USA).

Microorganisms. Salmonella enterica Thompson (ATCC 13311), Salmonella enterica Newport (ATCC 6962), Salmonella enterica Infantis (ATCC 51741) and Salmonella enterica Senftenberg (ATCC 8400), previously kept in Cryobeads at -80 ºC, were streaked onto tryptic soy agar (TSA, BD Difco, Sparks, MD). For the duration of the study, cultures were stored at refrigerated temperatures and transferred into fresh media periodically. S. Thompson, S. Newport, and S. Infantis were selected because frequently
linked to pet food outbreaks (Crowe et al, 2005, Imanishi et al, 2014, Pitout et al, 2003). S. Senftenberg was selected based on thermal tolerance and association with rendering raw products (Kinley et al, 2010, Gong & Jiang, 2017). A low and high inoculum (~10^5 CFU/mL and ~10^7 CFU/mL initial cocktail concentration) were prepared for the inoculation procedure as explained below.

**Inoculation procedure.** One colony from each strain plate was grown overnight in 10-mL Tryptic Soy Broth (TSB, BD Difco, Sparks, MD) at 37°C. Then, 500 µl of each solution was transferred into 50 ml of TSB and grown overnight. To prepare the low inoculum, 250 µl of each overnight culture was freshly transferred into 25 ml of sterile 0.1 % peptone water (PW, BD Difco, Sparks, MD) and equal amounts of each strain was mixed in order to obtain a low inoculum of ~10^5 CFU/mL. For the high inoculum preparation, overnight cultures were centrifuged for 10 min at 4000 rpm. Supernatants were then discarded, and pellets suspended in 25 mL 0.1% PW. Pellets were completely dissolved by vortexing, and equal amounts of each strain were mixed in order to obtain a high inoculum of ~10^7 CFU/mL. Counts were confirmed by serial dilution on Tryptic Soy Agar (TSA, BD Difco, Sparks, MD). Fat samples were warmed to approximately 35 °C to increase fluidity using a bucket heater (WG05 Insulated pail band heater, WarmGuard, Salt Lake City, UT). Either low or high inoculum was then combined with samples at a liquid-to-fat ratio of 25 ml per 450 g of fat. For each type of fat (white grease and beef tallow), inoculated sample was then divided in 4 different beakers of ~ 100 g. Four different moisture levels were obtained by adding Deionized (DI) water: 0, 0.5, 1 and 3 % moisture level. The amount (ml) of DI water to add was calculated based on the mass balance equation (Lang & Steinberg, 1980). Samples were stored for 7 days at 48 and 76 °C and Salmonella population was evaluated daily. Chicken fat samples without any added water was used for comparison purposes.
**Salmonella enumeration.** At each sampling time, 10 ml of the sample was pre-enriched in 90 mL of Brain Heart Infusion Agar (BHI, BD Difco, Sparks, MD) at 35 ± 2°C for 24 ± 2 h and then enriched in Rappaport-Vassiliadis (BD Difco, Sparks, MD) broth at 42°C for 24 h. Serial dilutions were then performed on Xylose Lysine Deoxycholate Agar (BD Difco, Sparks, MD). Typical *Salmonella* black colonies were counted, and remaining population reported.

**Survival kinetics determination.** OriginPro Lab Software (version 8) was used to determine the parameters of Weibull model (Equation 1) based on our earlier work (Trinetta et., 2019):

\[
\log S(t) = -\frac{1}{2.303} \left( \frac{t}{\alpha} \right)^\beta
\]

where the parameters \(\alpha\) and \(\beta\) represent a characteristic time and curve shape, respectively. The goodness of the fit of the model was assessed using regression coefficients and least square errors. This model was applied only for samples challenged at 48 °C, since at 76 °C the count are below detectable limits after 24 hours.

**Statistical analysis.** Each experiment was conducted in triplicate. All the data collected were converted in log CFU/ml. Means and standard deviations were obtained using Excel (Microsoft Corp., Redmond, WA). Statistical differences were evaluated using SAS® software PROC GLIMMEX (SAS Institute, Cary, NC, USA). A completely randomized design was used to compare values. Differences were considered statistically significant at \(P < 0.05\).
CONCLUSION

The present research suggests that residual moisture in containers during transportation of white grease and beef tallow fat largely does not impact *Salmonella* spp. If contaminated with a high level of *Salmonella* spp. \(10^8\) cfu/mL and held at a low temperature (48°C), moisture may influence the thermal death due to differences in water activity and water mobility kinetics.
AUTHOR CONTRIBUTION

VT and CJ plan the experimental design and help analyze data. AM conducted the experiments. UY took care of the kinetic model and data analysis, while JV reviewed the manuscript.

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Figures Legend

**Figure 1.** *Salmonella* cocktail remaining population in white grease samples challenged at 48°C with different wet inocula levels (high (A) and low (B)), and moisture level (0%, 0.50%, 1% and 3%) over time and in beef tallow samples challenged at 48°C with different wet inocula levels (high (C) and low (D)), and moisture level (0%, 0.50%, 1% and 3%) over time.
**Table 1.** Chemical and physical characteristics of rendered animal fats selected for this study prior to start experiments.

|                     | Beef Tallow          | Choice White Grease | Chicken Fat |
|---------------------|----------------------|---------------------|-------------|
| **Lipid %**         | 81.9 ± 1.55<sup>a</sup> | 79.2 ± 1.98<sup>a</sup> | 82.8 ± 1.12<sup>a</sup> |
| **Moisture %**      | 0.11% ± 0.03<sup>a</sup> | 0.16% ± 0.00<sup>b</sup> | 0.06% ± 0.02<sup>c</sup> |
| **Water Activity**  | 0.34 ± 0.01<sup>a</sup> | 0.44 ± 0.01<sup>b</sup> | 0.37 ± 0.03<sup>a</sup> |
| **a<sub>w</sub>**   |                      |                     |             |

Results are presented as average values (of three replicates) and ± indicates standard deviation. Different letters indicate significant difference (P<0.05) by row.
Table 2. Major fatty acid composition (percent of total fatty acids by weight) of rendered animal fats.

|                    | Beef Tallow     | Choice White Grease | Chicken Fat   |
|--------------------|-----------------|---------------------|---------------|
| Myristic acid (14:0) | 0.58 ± 0.10<sup>a</sup> | 1.63 ± 0.09<sup>b</sup> | 0.63 ± 0.05<sup>a</sup> |
| Palmitic acid (16:0) | 25.6 ± 0.63<sup>a</sup> | 23.5 ± 0.10<sup>b</sup> | 27.2 ± 0.36<sup>c</sup> |
| Palmitoleic acid (16:1) | 6.64 ± 0.26<sup>a</sup> | 2.84 ± 0.03<sup>b</sup> | 6.58 ± 0.05<sup>a</sup> |
| Stearic acid (18:0)  | 6.35 ± 0.17<sup>a</sup> | 8.76 ± 0.20<sup>b</sup> | 6.14 ± 0.09<sup>a</sup> |
| Oleic acid (18:1)   | 38.6 ± 0.70<sup>a</sup> | 43.8 ± 0.05<sup>b</sup> | 36.8 ± 0.26<sup>c</sup> |
| Linoleic acid (18:2) | 20.3 ± 0.28<sup>a</sup> | 17.0 ± 0.04<sup>b</sup> | 21.1 ± 0.18<sup>c</sup> |
| Alpha-linoleic acid (18:3:3) | 0.97 ± 0.07<sup>a</sup> | 0.62 ± 0.01<sup>b</sup> | 0.86 ± 0.05<sup>a</sup> |
| Eicosenoic acid (20:1) | 0.25 ± 0.05<sup>a</sup> | 0.82 ± 0.02<sup>b</sup> | 0.25 ± 0.01<sup>a</sup> |
| Eicosadienoic acid (20:2) | 0.11 ± 0.00<sup>a</sup> | 0.69 ± 0.01<sup>b</sup> | 0.11 ± 0.01<sup>a</sup> |
| Arachidonic acid (20:4) | 0.69 ± 0.08<sup>a</sup> | 0.43 ± 0.00<sup>b</sup> | 0.31 ± 0.02<sup>b</sup> |

Results are presented as average values (of three replicates) and ± indicates standard deviation. Different letters (a, b, c) indicate significant difference (P<0.05) by row.
Table 3. Water activity values when fat samples were stored at 76ºC at 0 hour and 48 hours.

|                | Beef Tallow | Beef Tallow | Beef Tallow | Beef Tallow | Choice White Grease | Choice White Grease | Choice White Grease | Choice White Grease | Chicken Fat |
|----------------|-------------|-------------|-------------|-------------|--------------------|---------------------|--------------------|--------------------|-------------|
|                | 0%          | 0.5%        | 1.0%        | 3.0%        | 0%                 | 0.5%                | 1.0%                | 3.0%                | 0%          |
| 0 hour         | 0.29±0.07a  | 0.44±0.18a  | 0.51±0.24a  | 0.63±0.19a  | 0.38±0.09a         | 0.63±0.20a          | 0.64±0.11a          | 0.87±0.03a          | 0.57±0.18a   |
| 48 hours       | 0.18±0.03b  | 0.20±0.04a  | 0.19±0.02a  | 0.45±0.23a  | 0.19±0.05a         | 0.19±0.03a          | 0.18±0.02a          | 0.28±0.12b          | 0.20±0.05a   |

Results are presented as average values (of three replicates) and ± indicates standard deviation.

Different letters (a, b, c) indicate significant difference ($P<0.05$) by row.
Table 4. Selected *Salmonella* cocktail survival kinetics in white grease and beef tallow challenged at 48 °C with different moisture levels.

| Inoculum condition | Fat type | Moisture level | Kinetics parameters |
|--------------------|----------|----------------|---------------------|
|                    |          |                | $\alpha$ (day) | $\beta$          |
| High               | White grease | 0.0%          | 0.47±0.07  | 1.91±0.18  |
|                    |          | 0.5%          | 0.37±0.14  | 1.62±0.35  |
|                    |          | 1.0%          | 0.24±0.03  | 1.13±0.07  |
|                    |          | 3.0%          | 0.18±0.07  | 1.12±0.16  |
|                    | Beef tallow | 0.0%          | 0.84±0.23  | 3.30±0.91  |
|                    |          | 0.5%          | 0.89±0.06  | 3.41±0.29  |
|                    |          | 1.0%          | 0.87±0.04  | 3.26±0.12  |
|                    |          | 3.0%          | 0.59±0.17  | 2.27±0.56  |
| Low                | White grease | 0%            | 1.96±0.07  | 5.08±0.28  |
|                    |          | 0.5%          | 0.50±0.14  | 1.17±0.17  |
|                    |          | 1.0%          | 0.52±0.25  | 1.17±0.27  |
|                    |          | 3.0%          | 0.98±0.63  | 1.90±0.95  |
|                    | Beef tallow | 0.0%          | 0.76±0.28  | 2.62±0.14  |
|                    |          | 0.5%          | 0.56±0.14  | 2.51±0.398 |
|                    |          | 1.0%          | 0.90±0.39  | 2.65±0.25  |
|                    |          | 3.0%          | 0.87±0.36  | 2.70±0.21  |
Figure 1

A

B

C

D

![Graphs showing data over time](image-url)