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Salmonella Inactivation During Extrusion of an Oat Flour Model Food

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Little research exists on *Salmonella* inactivation during extrusion processing, yet many outbreaks associated with low water activity foods since 2006 were linked to extruded foods. The aim of this research was to study *Salmonella* inactivation during extrusion of a model cereal product. Oat flour was inoculated with *Salmonella enterica* serovar Agona, an outbreak strain isolated from puffed cereals, and processed using a single-screw extruder at a feed rate of 75 kg/h and a screw speed of 500 rpm. Extrudate samples were collected from the barrel outlet in sterile bags and immediately cooled in an ice–water bath. Populations were determined using standard plate count methods or a modified most probable number when populations were low. Reductions in population were determined and analyzed using a general linear model. The regression model obtained for the response surface tested was \( \log (N_f/N_0) = 20.50 + 0.82T - 141.16a_w - 0.0039T^2 + 87.91a_w^2 \) (\( R^2 = 0.69 \)). The model showed significant (\( p < 0.05 \)) linear and quadratic effects of \( a_w \) and temperature and enabled an assessment of critical control parameters. Reductions of \( 0.67 \pm 0.14 \) to \( 7.34 \pm 0.02 \log \text{CFU}/g \) were observed over ranges of \( a_w \) (0.72 to 0.96) and temperature (65 to 100 °C) tested. Processing conditions above 82 °C and 0.89 \( a_w \) achieved on average greater than a 5-log reduction of *Salmonella*. Results indicate that extrusion is an effective means for reducing *Salmonella* as most processes commonly employed to produce cereals and other low water activity foods exceed these parameters. Thus, contamination of an extruded food product would most likely occur postprocessing as a result of environmental contamination or through the addition of coatings and flavorings.

**Keywords:** cereal, extrusion, flour, low water activity, *Salmonella*, thermal inactivation

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**Introduction**

Among the outbreaks of salmonellosis associated with low water activity \( (a_w) \) foods in the United States in recent years, several were associated with dry dog food and cereal products (CDC 2016). Extruded pet foods, snacks, and treats remain among the most frequently recalled low \( a_w \) products due to contamination with microbial pathogens (FDA 2016). These foods are produced by extrusion processing, a continuous high-temperature, short-time process (HTST) that performs several unit operations including mixing, shearing, cooking, and forming (Grasso and others 2014). The food and feed industry uses extrusion extensively to produce snack foods, cereals, pet foods, and pet treats. Although extrusion processing provides thermal treatment to the product, it was designed mainly to achieve quality outcomes, not necessarily safety outcomes with respect to the destruction of foodborne pathogens. However, it is now being implemented to achieve food safety outcomes as a preventive control to meet new food safety regulations for human and animal food (FDA 2015a, b). Yet, little research exists on microbial inactivation during this operation. Under typical circumstances, where product moisture content (14% to 28%) and extrusion temperatures are high (>100 °C), *Salmonella* would not be expected to survive the thermal extrusion process. However, the possibility that *Salmonella* may survive the extrusion process, as evidenced by both outbreaks and recalls, is troubling. Since thermal resistance of *Salmonella* in foods increases with decreasing product \( a_w \), much longer times and higher temperatures may be required during thermal extrusion in order to significantly reduce or eliminate pathogens (Podolak and others 2010).

Residence time and temperature of the extrusion process may be controlled by altering feed rates, screw speeds, and barrel temperature during processing. These physical parameters are often explored, but extrusion studies describing microbial inactivation reported in the literature often lack comprehensive data with respect to varying moisture content (4% to 18%) of extruded foods and feed examined (Quéguiner and others 1989; Likimani and others 1990; Ukuku and others 2012). In the few studies that did examine both the effect of moisture content and temperature...
on microbial reduction, intermediate to high moisture products (21% to 31%) were used (Okeo and others 2006; Bianchini and others 2012). As Salmonella has greater thermal resistance at low $a_w$, inactivation of Salmonella by thermal treatment may become difficult as moisture content decreases (Goepfert and others 1970; Quéguiner and others 1989). Thus, it would be beneficial to explore the efficacy of extrusion at lower $a_w$ levels.

Therefore, the goals of this research were to evaluate the efficacy of thermal extrusion as an inactivation step for Salmonella in a model low $a_w$ food over a wide range of moisture contents and extrusion temperatures, and to identify fail-safe conditions for Salmonella inactivation during thermal extrusion processing.

Materials and Methods

Bacterial culture preparation
Salmonella enterica serovar Agona (strain 447967; originally isolated from puffed rice cereal) was obtained from the FDA Arkansas Regional Laboratory (Jefferson, Alaska, U.S.A.) and stored as a stock culture at refrigeration temperatures on trypticase soy agar supplemented with 0.6% yeast extract (TSAYE) (BD Difco, Sparks, Md., U.S.A.). The culture was transferred to a fresh TSAYE plate on a monthly basis. The $D_{90_{PC}}$ value for this isolate is 29.7 min (Hildebrandt and others 2016). For each inoculum, a single isolated colony was transferred from the stock plate to 10 mL of tryptic soy broth supplemented with 0.6% yeast extract (TSBYE) (BD Difco) and incubated for 24 h at 37 °C. The culture (0.5 mL) was transferred to TSAYE plates, spread using a sterile L-shaped spreader, and then incubated for 24 h at 37 °C. The cells were harvested by pipetting 3 mL of buffered peptone water (BPW) (Remel, Lenexa, Kans., U.S.A.) onto the plate, which was then scraped gently using a sterile L-shaped spreader. The resultant slurry was then pipetted into a sterile Falcon tube (BD, Franklin Lakes, N.J., U.S.A.). Each set of 5 plates harvested yielded approximately 11 mL of cells at approximately 11 log CFU/mL. The harvested cells were serially diluted in BPW, plated on TSAYE, and incubated for 24 h at 37 °C for enumeration.

Preparation of inoculated flour
Oat flour (ConAgra Foods, Omaha, Nebr., U.S.A.), which has an indigenous fat content of approximately 8.5%, was selected for use as a simple model food system that fills a gap in the literature with respect to extrusion of low $a_w$ foods. Oat flour (1 kg) was aseptically transferred to a sterile mixing bowl that was placed in a mixer (Model N50A, Hobart, Troy, Ohio, U.S.A.) inside of a biosafety level 2 (BSL-2) cabinet (SterilGard III Advance SG603, The Baker Co., Sanford, Maine, U.S.A.). Prepared inoculum (10 mL) was loaded into a sterile syringe (Cole Parmer Instrument Co., Vernon Hills, Ill., U.S.A.) that was attached using sterile silicone tubing to a liquid atomizer (Model CV24, Sonic and Materials, Inc., Newtown, Conn., U.S.A.). The inoculum was atomized (660 Hz) onto the flour, which was then mixed for 30 min to homogeneously distribute the cells. The inoculated flour was stored in airtight buckets for 3 d at 25 °C for moisture equilibration. From each batch of inoculated flour, ten 1-g samples were aseptically taken from random locations to evaluate homogeneity. Each 1-g sample was weighed on a balance (Model E1B120, Ohaus Corp., Parsippany, N.J.) and was mixed with 99 mL BPW and stomached for 30 s. Samples were enumerated on TSAYE as indicated previously. Sampling was reduced to five 1-g samples per week after the stability of the batches and the consistency of the inoculation procedure were established.

Each 1-kg batch of inoculated oat flour was divided into two 500-g batches and each portion was added to 5.5 kg of uninoculated flour. Approximately 1.5 g of Red 40 pellets (Sensient Technologies, Milwaukee, Wisc., U.S.A.) were added to each 6-kg batch to aid in detection of the inoculated flour during processing. The 6-kg batch was mixed in a large mixer (Model HL200, Hobart) inside of a walk-in BSL-2 cabinet (Model S125.636, Nuaire, Plymouth, Minn., U.S.A.) for 30 min and stored for 7 d in an airtight plastic bucket at ambient temperature (23 ± 2 °C) until utilized for experimentation. On the day of experimentation (day 7), in a BSL-3 pilot plant (Inst. for Food Safety and Health/Illinois Inst. of Technology, Bedford Park, Ill.), the two 6-kg batches produced from 1 inoculum were combined as a single 12-kg batch in a plastic bucket, lidded and tumbled to combine. To evaluate homogeneity in the final inoculated batches, five 1-g samples were removed from random locations and weighed into sterile Whirl-Pak bags. Each 1-g sample was enumerated as described earlier. Sampling was reduced to three 1-g samples per batch each week after the stability of the batches and the consistency of the method was established.

Experimental design

Worst-case process criteria such as low temperature (<100 °C), low $a_w$, low pressure, low shear, and a single-screw extruder were targeted in thermal extrusion processing experiments. A central-composite response surface design (CCCRSD) was used to determine 9 target extrusion treatment conditions to be tested over target ranges for water activity ($a_w$, 0.72 to 0.96; 14% to 28% moisture content) and temperature (60 to 100 °C). The response surface design was developed based on previously published values of thermal resistance of Salmonella at similar $a_w$ levels. As shown in Figure 1, 2 replications of the factorial points (open circles), 2 replications of the axial points along the coordinate axes of the factor levels (gray circles), and 5 replications of the central point of the 2-level factorial design (solid black circle) were planned to improve the precision of the experiment. Three treatment conditions of similar temperature (for example, the axial and center points along the horizontal centerline) were on any given day of extrusion trials, evaluated in decreasing order of $a_w$ to minimize carryover effects. Replications of the treatment conditions were conducted on different days with different inoculum.

![Figure 1–Central composite response surface experimental design for temperature and water activity treatment conditions targeted during extrusion trials. Open circles (○) indicate factorial points, shaded gray circles (●) represent axial points, and the solid black circle (●) represents the central point.](image-url)
Extruder setup

A single-screw extruder (Model X85, Wenger Manufacturing, Inc., Sebeka, Kans., U.S.A.) was installed in the BSL-3 pilot plant mentioned previously (Figure 2). The system utilized was comprised of several components. A live bin feed hopper metered the oat flour into the preconditioner at a set weight-based rate of 75 kg/h, and a variable speed peristaltic pump delivered ambient temperature water to the preconditioner near the inlet. The preconditioner served as a continuous mixer and operated at ambient temperature, that is, no heat, hot water, or steam was added. The preconditioner fed the moistened oat flour into the inlet of the extruder barrel. The extruder had a screw diameter of 85 mm (3.3 inch), a capacity of 75 to 800 kg/h with a variable screw speed up to 600 rpm. The extruder barrel was divided into 5 sections, each of which had a linear groove pattern parallel to the direction of product flow. In the first 4 sections, a single-flight conveying screw was used; the barrel jacket was not heated and no steam or water was added. In the last section before the outlet, a double-flight screw with a uniform shaft diameter was used and the barrel steam jacket was used to preheat the extruder and to maintain the target temperature. A conical-shaped die head assembly (Figure 2) was fitted to the extruder outlet. A manual screw was used to move the die head in or out, which resulted in extrudate of thickness that varied from 3 to 40 mm, to achieve the desired target extrudate temperature. The die head was also equipped with a resistive thermal device (RTD) probe (Model RBF2853MB38Z-00-9HP23-F3J012-9, Pyromation, Fort Wayne, Ind., U.S.A.) to measure temperature of the extrudate at the die face. The feed rate and extruder speed were held constant at 75 kg/h and 500 rpm, respectively, and monitored for all trials. At the beginning of each trial, appropriate steady-state conditions were first established by running un inoculated flour through the extruder. From each collected sample, 10 g was removed and added to a Whirl-Pak bag with 90 mL of BPW. When high populations of Salmonella were anticipated, that is, above background microflora levels (2.63 ± 0.58 log CFU/g), appropriate serial dilutions were made with BPW and 0.1 mL was spread plated in duplicate onto both TSAYE and xylose lysine deoxycholate agar (XLD, BD Difco). Plates were incubated for 24 h at 37 °C. When total colony counts on TSAYE and XLD did not coincide, which indicated the presence of either injured cells or other microorganisms, colonies on TSAYE were picked and cultured on XLD for confirmation. Plates showing growth of black colonies typical of Salmonella on XLD were considered positive for Salmonella. Representative colonies from XLD were later confirmed as Salmonella using a Salmonella O Antiserum Poly A test (BD Difco).

When low populations of Salmonella were anticipated, a modified 5-tube most probable number (MPN) method (Blodgett 2010) was used to determine Salmonella populations. Serial dilutions (n ≥ 3) were inoculated into test tubes containing 10 mL TSAYE (limit of detection 0.018 MPN/g). Tubes were incubated for 24 h at 37 °C. Following incubation, tubes showing growth were streaked onto XLD agar and incubated at 37 °C for 24 h.

Figure 2–Wenger X85 single screw extruder and adjustable, conical shaped die face.
Typical black colonies on XLD were presumed to be *Salmonella*. MPN tubes showing both growth and subsequently identified as containing *Salmonella* via black colonies on XLD were considered positive and enumerated as MPN/g. Representative colonies from XLD were later confirmed as *Salmonella* using a *Salmonella* O Antiserum Poly A test (BD Difco).

### Statistical analysis

A 2nd-order regression model was fitted to the log reduction data to describe the effect of extrusion process variables (\(a_w\) and temperature) on inactivation of *Salmonella*. The model included the linear and quadratic terms of the independent variables. Statistical analysis with a general linear model (GLM) was completed using SAS 9.2 (SAS Inst. Inc., Cary, N.C., U.S.A.).

### Results and Discussion

**Homogeneity and stability of inoculum**

In total, 21 extrusion trials were conducted. A separate 12-kg batch of inoculated flour was prepared for each trial. The background microflora of the oat flour was determined to be 2.63 ± 0.58 log CFU/g. The average *Salmonella* population of the initial cultures (\(n = 21\)) was 10.81 ± 0.19 log CFU/mL. The average *Salmonella* population of the 1-kg inoculated flour batches was 8.44 ± 0.21 log CFU/g. A representative data set collected to evaluate homogeneity and stability of inoculated flour is shown in Figure 3. *Salmonella* populations were considered to be homogeneous if the standard deviation for the *Salmonella* levels was ≤0.3 log CFU/g. Because the extruder was running 75 kg of material per hour, 12 kg of inoculated flour was needed in order to provide adequate run time (approximately 10 min) with inoculated material for obtaining samples. The larger 12-kg batches of inoculated flour had *Salmonella* populations of 7.00 ± 0.16 and 6.48 ± 0.20 log CFU/g at days 7 and 15, respectively. Extrusion trials were carried out on day 15 after preparation of the inoculated flour. An average of a 0.68 log CFU/g drop was observed between the day of preparation of inoculated flour and the day of use. This initial drop and subsequent *Salmonella* survival is consistent with observations made in other low \(a_w\) foods (Keller and others 2013; Blessington and others 2014; Gradl and others 2015).

**Extrusion of inoculated oat flour**

Extrusion treatment conditions, the respective number of replications, and *Salmonella* reductions achieved are given in Table 1. At the beginning of each trial, appropriate steady-state conditions (75 kg/h, 500 rpm, and the target temperature and moisture content) were first established by running uninoculated flour through the system. Target moisture content was determined from the moisture sorption isotherm shown in Figure 4 and per the \(a_w\) level in experimental design. Pressure inside the extruder barrel was observed to be 0 kPa for all trials, which indicated that back pressure in the barrel was minimized. The residence time of the product inside the barrel was 11.3 s. The actual \(a_w\) and temperatures documented during extrusion are given in Table 1. Extrudate temperature and \(a_w\) at the die confirmed that target treatment conditions were achieved. Reaching the highest temperatures (95.6 and 100 °C) proved difficult, and therefore it was only possible to run 1 replicate at these temperatures. When attempting to replicate the trial at \(a_w\) 0.92 and 95.6 °C, the temperature only reached 88.8 °C (see Table 1), but these data were still included in the analysis. During actual extrusion trials, the GLM output from preliminary analysis suggested that additional testing was necessary to better define *Salmonella* inactivation at the extremes of the response surface. Thus, \(a_w\) and temperature combinations of \(a_w\) and temperature.

### Table 1—Product and process parameters recorded during extrusion trials and *Salmonella* population reduction obtained at test conditions.

<table>
<thead>
<tr>
<th>Target (°C)</th>
<th>Measured (°C)</th>
<th>Die opening (mm)</th>
<th>Replicates</th>
<th>Reduction (log CFU/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>65.0 ± 0.72</td>
<td>65.3 ± 0.70</td>
<td>30</td>
<td>1</td>
<td>0.67 ± 0.14</td>
</tr>
<tr>
<td>74.4 ± 0.72</td>
<td>74.6 ± 0.72</td>
<td>28</td>
<td>1</td>
<td>4.20 ± 0.63</td>
</tr>
<tr>
<td>85.0 ± 0.72</td>
<td>84.8 ± 0.68</td>
<td>26</td>
<td>2</td>
<td>6.26 ± 1.15</td>
</tr>
<tr>
<td>74.4 ± 0.75</td>
<td>74.4 ± 0.78</td>
<td>29</td>
<td>2</td>
<td>3.84 ± 0.72</td>
</tr>
<tr>
<td>95.6 ± 0.75</td>
<td>95.4 ± 0.75</td>
<td>12</td>
<td>1</td>
<td>5.00 ± 1.76</td>
</tr>
<tr>
<td>70.0 ± 0.84</td>
<td>69.6 ± 0.84</td>
<td>29</td>
<td>2</td>
<td>1.95 ± 0.75</td>
</tr>
<tr>
<td>85.0 ± 0.84</td>
<td>84.8 ± 0.82</td>
<td>18</td>
<td>5</td>
<td>4.67 ± 1.15</td>
</tr>
<tr>
<td>100.0 ± 0.84</td>
<td>101.1 ± 0.81</td>
<td>6</td>
<td>1</td>
<td>7.24 ± 0.02</td>
</tr>
<tr>
<td>74.4 ± 0.92</td>
<td>74.5 ± 0.91</td>
<td>12</td>
<td>2</td>
<td>3.33 ± 1.21</td>
</tr>
<tr>
<td>95.6 ± 0.92</td>
<td>95.8 ± 0.90</td>
<td>12</td>
<td>1</td>
<td>1.95 ± 0.75</td>
</tr>
<tr>
<td>70.0 ± 0.84</td>
<td>69.6 ± 0.84</td>
<td>29</td>
<td>2</td>
<td>1.95 ± 0.75</td>
</tr>
<tr>
<td>85.0 ± 0.96</td>
<td>84.1 ± 0.95</td>
<td>7</td>
<td>2</td>
<td>7.05 ± 0.36</td>
</tr>
</tbody>
</table>

*Denotes log reduction values plated on TSAYE, nonasterisk values enumerated with MPN.

Figure 3—Homogeneity and stability over time of *Salmonella* Agona inoculated in 1 kg of oat flour. Error bars expressed as standard deviation.

Figure 4—Moisture sorption isotherm for oat flour at 25 °C.
Salmonella inactivation during extrusion...

Table 2–Statistical analysis of extrusion temperature and \( a_w \) parameters.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimate</th>
<th>Standard error</th>
<th>t Value</th>
<th>Pr &gt;</th>
<th>t</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>20.50</td>
<td>14.90</td>
<td>1.38</td>
<td>0.1720</td>
<td></td>
</tr>
<tr>
<td>Temperature</td>
<td>0.82</td>
<td>0.29</td>
<td>4.17</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>( a_w )</td>
<td>−141.16</td>
<td>28.15</td>
<td>−5.02</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Temperature (^2)</td>
<td>−0.004</td>
<td>0.001</td>
<td>−3.38</td>
<td>0.0010</td>
<td></td>
</tr>
<tr>
<td>( a_w ) (^2)</td>
<td>87.92</td>
<td>17.32</td>
<td>5.08</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
</tbody>
</table>

Reductions of 0.67 ± 0.14 log CFU/g at \( a_w \) 0.72, 65 °C and 7.34 ± 0.02 log CFU/g at \( a_w \) 0.96, 100 °C were demonstrated (Table 1). The \( a_w \) and temperature treatment combinations that resulted in greater than 5-log reductions were \( a_w \) 0.72, 85.0 °C; \( a_w \) 0.75, 95.6 °C; \( a_w \) 0.84, 100 °C; \( a_w \) 0.92, 88.8 °C; \( a_w \) 0.92, 95.6 °C; and \( a_w \) 0.96, 85.0 °C. With the exception of the treatment conditions of \( a_w \) 0.72, 65 °C and \( a_w \) 0.84, 70 °C, plate counts for all other treatment conditions were below the limit of detection (50 CFU/g) even on nonelective media (TSAS). Furthermore, when treatment conditions allowed the use of plate media, populations determined on XLD were always much lower than those determined on TSAS. Subsequent testing of colonies on TSAS suggested that the Salmonella that failed to grow on XLD, but grew on TSAS, were injured cells. Therefore, the log reduction values for all the treatment conditions except the aforementioned 2 conditions were determined by the modified MPN, which involved the use of TSBYE as enrichment and general growth, followed by identification of that growth as Salmonella on XLD.

Statistical analysis

The regression model consisted of linear and quadratic terms of the 2 factors (Eq. (1))

\[
\log \left( \frac{N_0}{N_f} \right) = 20.50 + 0.82T - 141.16a_w - 0.004T^2 + 87.92a_w^2
\]

where \( N_0 \) represents the initial population, \( N_f \) corresponds to the population after extrusion, \( T \) is the temperature and \( a_w \) is the water activity. The response surface curve of the reduction data is given in Figure 5. Though the model showed significant \((p < 0.05)\) linear and quadratic effects of \( a_w \) and temperature (Table 2). Figure 5 shows that temperature has a pronounced effect on inactivation of Salmonella during extrusion. For example, at \( a_w \) 0.84, the average reductions obtained at 70, 85, and 100 °C were 1.95, 4.67, and 7.24 log CFU/g, respectively; however, at a constant temperature with increasing \( a_w \), a decreasing trend in Salmonella reduction was not observed. At 85 °C, the average reductions achieved at \( a_w \) 0.72, 0.84, and 0.96 were 6.26, 4.67, and 7.05 log CFU/g, respectively.

Figure 5–Response surface curve for survival of Salmonella Agona during extrusion as a function of temperature and \( a_w \).

Conclusion

The current study examined inactivation of Salmonella in a wide range of initial product moisture levels (14% to 28%, 0.72 to 0.96...
Salmonella inactivation during extrusion...