Salmonella survival in low \(a_w\) environment

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ABSTRACT

Since the early 1970’s a number of large Salmonella outbreaks have been associated with low \(a_w\) food products, including chocolate, cereal, peanut butter, spices, and powdered infant formula. This research is design to help the food industry to determine Salmonella survival in the production environment and thus understand the potential risk of cross contamination to the product. The studies sought to assess the survival of 9 isolates of Salmonella on stainless steel surfaces, the influence of different serotypes on survival and the comparison of the survival rate within the same serotype.

The following isolates were used for the experiments: S. Agona, S. Napoli, S. Enteritidis, S. Tennessee, S. Muenchen, S. Typhimurium (whey powder), S. Typhimurium DT104, S. Typhimurium NCTC 12023, S. Typhimurium, NCTC (PHLS), NC000 74-17. The stainless steel surfaces were inoculated with 10\(^3\) cfu of each Salmonella isolate and placed in a controlled environment: relative humidity 33% and temperature 23°C. The number of microorganisms surviving on the stainless steel surfaces was assessed at various time points from 1 hour to 30 days. Differential survival was observed for different serotypes of Salmonella. The highest survival rate was associated with S. Agona, S. Enteritidis, and S. Typhimurium DT104 where, after 30 days, the number of microorganisms on the surfaces ranged from 4.0 logs cfu to 4.5 logs cfu. There was 1 log cfu survival of S. Typhimurium NCTC (PHLS), NC000 74-17 after 10 days in experimental conditions, therefore differences within the same serotype were observed. The average number of Salmonella surviving after 30 days ranged from 2.5 logs cfu to 3.5 logs cfu.

The results of the studies could provide insight into potential growth and survival mechanisms for Salmonella in dry processing environments. These results will be incorporated into a Campden BRI guidance document on the survival of Salmonella in dry process environments with relation to strain, temperature, humidity, water and soiling, which would help the industry to control Salmonella endurance in the environment and thus in the final product.

Keywords: Salmonella; survival; water activity; dry; environment, surfaces

INTRODUCTION

For many years, low moisture foods, such as chocolate, were regarded as microbiologically safe due to their inherent product characteristics. The first outbreak of Salmonella that could be traced back to low moisture products was registered in the 1970’s and since this time it has been the main pathogen of concern for these foods. Currently, there is growing concern for the potential presence and survival of Salmonella in low \(a_w\) products. The problem within the food industry is widespread with reports from the UK Food Standards Agency and Rapid Alert System for Foods and Feeds (RASFF) concerning such foods and ingredients as: whole egg powder, coriander, soy bean meal, Tahini, minced dehydrated white onions, sesame seeds, dried sage, peanut butter, black pepper, dried mushrooms, oat cereals, low \(a_w\) sausage, chocolate and flour being able to harbour Salmonella. In addition, the same Salmonella type has been isolated from products produced over a number of years (e.g. 10 years for Malt-O-Meal in the USA; [11]) from the same plant. The assumption has been that this particular Salmonella strain had survived in the plant for this time period and may have recontaminated products.

All of those facts underline the importance of understanding the mechanisms of Salmonella survival in low moisture environments, establishing the environmental factors likely to favour their long term survival and determining ways to control them.

Whilst there is some information on the survival of Salmonella in dried products, there is little information on the survival of Salmonella in the environment, both in terms of the length of survival and the possible factors contributing to such survival. It is also well known that as the water activity of foods becomes lower and the available moisture is decreased, the probability of growth is reduced; however the ability of organisms to survive is greatly increased.
The ability of *Salmonella* to survive for long periods in low $a_w$ products e.g. halva [16] and peanut butter [5] has been documented. Uesugi *et al.* demonstrated potential for long-term environmental persistence of *Salmonella* in almond orchards. In his studies, 53 *Salmonella* isolates collected from the selected orchard during 5 years were identified as the same serovar: *Salmonella* Enteritidis PT 30 [24]. The approach of this study is to establish a survival model for *Salmonella* strains and compare the differences between each serotype and the same serotypes isolated from different products. The environmental conditions which were used (RH=33% and T=23°C) reflect the environment of low $a_w$ food factories.

**MATERIALS & METHODS**

*Salmonella* strains. All *Salmonella* isolates were obtained from Campden BRI culture collection. *S. Agona* was isolated from cotton seeds, *S. Napoli* from chocolate confectionary (1986 outbreak, Italy), *S. Enteritidis* was obtained from cereals, *S. Tennessee* from sesame seeds and *S. Muenchen* from cocoa bean environment. Four isolates of *S. Typhimurium* were used: multi-drug resistant *S. Typhimurium DT104*, *S. Typhimurium NCTC 12023* (high heat resistant; HR), *S. Typhimurium, NCTC (PHLS), NC000 74-17* (used as a standard strain for disinfectant testing; DS) and *S. Typhimurium* isolated from whey powder. To ensure that the results would be as useful and applicable to the industry as possible, the same *Salmonella* strains, from factories manufacturing the same low $a_w$ products as from the foods which caused recent *Salmonella* outbreaks, were used (Table 1).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Food</th>
<th>Year</th>
<th>Location</th>
<th>Number affected</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. Agona</em></td>
<td>Cereal</td>
<td>2008</td>
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<td>28</td>
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<td>S. Agona</td>
<td></td>
<td>1998</td>
<td>USA</td>
<td>209</td>
<td>[7]</td>
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<tr>
<td><em>S. Napoli</em></td>
<td>Chocolate</td>
<td>1982</td>
<td>UK</td>
<td>245</td>
<td>[13]</td>
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<td><em>S. Enteritidis</em></td>
<td>Almonds</td>
<td>2000</td>
<td>USA, Canada</td>
<td>168</td>
<td>[14]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2005</td>
<td>Sweden</td>
<td>15</td>
<td>[18]</td>
</tr>
<tr>
<td><em>S. Tennessee</em></td>
<td>Peanut butter</td>
<td>1996</td>
<td>USA</td>
<td>628</td>
<td>[8]</td>
</tr>
<tr>
<td></td>
<td>Powdered infant</td>
<td>1993</td>
<td>Canada, USA</td>
<td>3</td>
<td>[6]</td>
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<tr>
<td><em>S. Typhimurium</em></td>
<td>Chocolate</td>
<td>1987</td>
<td>Norway, Finland</td>
<td>361</td>
<td>[15]</td>
</tr>
<tr>
<td></td>
<td>Peanut butter</td>
<td>2008</td>
<td>USA, Canada</td>
<td>684</td>
<td>[10]</td>
</tr>
</tbody>
</table>

Table 1. Outbreaks of foodborne *Salmonella* infections associated with consumption of contaminated dry foods

**Surfaces.** Stainless steel discs (2 cm diameter, Grade 2 B 1.4301 (EN 10088-1), EN 10 088-2) were used. They were soaked in hot water with detergent for 60 minutes. Each disc was then cleaned with a sponge and rinsed with Sterile Distilled Water. They were then left to dry, wiped with alcohol wipes, and sterilised in the autoclave.

**Test suspensions.** The cultures were maintained at -80°C in cryo vials containing glycerol. Working cultures of each serotype were sequentially prepared by adding a cryo bead to 150 ml Nutrient Broth (Oxoid) which was incubated for 24 h in a shaking incubator at 37°C and 100 rpm. The broth was then centrifuged at 3000 g for 10 minutes. Pellets were resuspended in a 500 ml of Maximum Recovery Diluent (LabM; MRD) which formed $10^7$ cfu/ml suspensions.

**Preparation of controlled RH chamber.** In this experiment, an RH of 33% ± 1% was established and maintained by the presence of saturated magnesium chloride salt. The sealed boxes were held at a temperature of 23±2°C. For the measurement of temperature and RH, a calibrated, digital thermohygrometer was used (Rotronic; Art no Hygrolog-D).

**Survival of Salmonella on stainless steel surfaces.** 50µl of each suspension was deposited onto stainless steel discs to produce an inoculum of approximately $5 \times 10^6$ – $2 \times 10^7$ cfu/surface. Discs were placed in uncovered Petri Dishes and transferred to an incubator to dry at 30°C for 1h 20 min. After drying, Petri Dishes were covered and placed in air tight transparent plastic boxes (32cm x 11cm x 21cm). The number of microorganisms surviving on the stainless steel surfaces was assessed at the following time points after drying: 0 h, 1 h, 3 h, 6 h, 24 h, 30 h, 2 days, 3 days, 7 days, 10 days, 15 days and 30 days.
Determination of viable counts of drying bacteria. The discs were aseptically transferred into sterile plastic universal containers (diameter 4-5cm) containing 10 ml MRD. The containers were vortexed for 1 minute to recover the remaining bacteria into suspension. Each sample was then serially diluted in MRD to 10^3 and plated out in duplicate using Tryptone Soya Agar (Oxoid; TSA).

To validate the bacterial recovery process, each disc was recovered from its container and then placed onto a prepoured TSA plate. A 0.1ml volume of sterile, distilled water was pipetted onto the disc and rubbed over the top and the bottom surface with a pipette tip for 30 seconds. The discs were then overpoured with TSA. All plates were incubated at 37°C for 48 hours. Plates were then enumerated and the colony forming units (cfu) per test surface calculated.

RESULTS & DISCUSSION

The serial dilution method used in this experiment was not sensitive enough to detect a viable cell concentration of less than 5 cfu/ml (0.7 log cfu). However, the agar incorporation method was capable of detecting any culturable colonies left on the surface after recovery. The initial bacterial level on the stainless steel discs ranged from 6.5 to 7.5 log cfu/surface. Strains had different sensitivity to drying which resulted with different starting levels when placing the discs in the boxes with controlled humidity. S. Typhimurium and S. Typhimurium DS were found to be most sensitive to drying (1.8 and 2.1 log cfu reduction respectively). S. Tennessee and S. Enteritidis were most resistant to drying (0.7 log cfu reduction). On the basis of the response to desiccation, the strains of Salmonella tested could be divided into two different groups: desiccation sensitive and desiccation resistant. The desiccation sensitive group included S. Typhimurium (isolated from whey powder) and S. Typhimurium DS. The rest of the strains belonged to desiccation resistant group: S. Agona, S. Typhimurium DT104, S. Enteritidis, S. Tennessee, S. Muenchen, S. Typhimurium and S. Napoli. Figure 1 shows representative survival curves for the 9 different isolates of Salmonella.

![Graph showing survival of different strains of Salmonella](image_url)

**Figure 1.** Survival of different strains of Salmonella suspended in MRD, inoculated onto stainless steel discs, dried and kept at 33% RH and 23°C.

There was a relatively high rate of bacterial death at the beginning of the survival curves for all isolates and a decrease in the death rate after 100 hours. For this reason, curves were split into two time periods: early survival (all results obtained from times: 0 h, 1 h, 3 h, 6 h, 24 h, 30 h, 2 days and 3 days) and later survival (all results obtained from times: 7 days, 10 days, 15 days and 30 days ). Straight lines were fitted to each time period for each strain. Slopes were measured between time 0 h and 3 days and showed strong evidence of a reduction with time (p > 0.001) for each organism except S. Muenchen (p = 0.09). For the later sampling points (7 days and more), the slopes for the majority of strains showed no statistically significant evidence of decline, except for S. Napoli (strong, p = 0.004) and S. Typhimurium (weak, p=0.049). Most of the strains, after reaching a certain reduction level, showed no further, significant viability loss for 27 days (Figure 2.)
Figure 2. Survival rate of 9 Salmonella strains suspended in MRD, inoculated into stainless steel discs, dried and kept at 33\% RH and 23\°C, time 0 h to 72 h (left) and time 168 h to 720 h (right).

The mean number of survivors for each Salmonella strain, after 30 days under the defined desiccating conditions, is shown in Figure 3. The highest log reduction was associated with S. Typhimurium and S. Typhimurium DS, where the level of bacteria dropped from 7.3 and 7.2 log cfu (inoculum) to 5.5 and 5.1 log cfu after drying and to 1.3 and 1 log cfu after 30 days, respectively.

Figure 3. Overall reduction of Salmonella strains suspended in MRD, inoculated onto stainless steel discs, dried and kept at 33\% RH and 23\°C for 30 days.

The highest survival rate was associated with S. Agona, S. Typhimurium DT104 and S. Enteritidis, where the microorganism level dropped from 7.2, 7.1 and 7.0 log cfu to 6.4, 5.9 and 6.4 log cfu after drying and 4.5, 4.3 and 4.3 log cfu respectively, after 30 days on stainless steel surfaces. Differences in survival within the same serotype were noted, with S. Typhimurium DT104 and S. Typhimurium HR surviving significantly better than S. Typhimurium and S. Typhimurium DS.

Kusumaningarum et al. reported already that S. Enteritidis survived for more than 96 h on stainless steel surfaces at 20 – 25\°C and 40 – 45\% RH which was confirmed in these findings. Their experiments also showed that food residues (saline solution, milk, chicken fillet) improved the survival of the pathogen, probably by forming a layer that might protect the cells on surfaces [17].
It is not clear why some of the *Salmonella* strains survive better than others. The way by which *Salmonella* survives the inimical conditions of the environment may be via formation of filaments. It has been reported that when cells undergo stress conditions that are bacteriostatic, they elongate and produce filamentous cells. This has been demonstrated in low water activity conditions, extreme pH and refrigerated temperatures [19, 22]. However, based on the available literature, it is not clear whether *Salmonella* cells form filaments at an $a_w$ less than 0.85. Anriany et al. showed that S. Typhimurium DT104 displays a rugose phenotype which has a unique function in allowing the cells to survive in the presence of acid and hydrogen peroxide and possibly other adverse conditions [4].

A number of researches have also investigated other mechanisms that may enhance *Salmonella* survival including accumulation of betaine via specific transporters [2], accumulation of proline [21] modification of the outer membrane [23] and the function of $\sigma^E$ and $\sigma^A$ regulated genes [20, 3]. Evidence of *Salmonella* survival has been found in plant processing environments. An outbreak of *Salmonella* Agona associated with toasted oats cereal showed wide-spread low levels of the organism in the plant environment, including samples taken from the floor, production equipment and the exhaust system in the plant [12]. In a study involving contaminated chocolate by Craven et al. [11], investigators found that the cause of the contamination was un-controlled airborne spread of dust. Understanding the ability of *Salmonella* strains to survive in the environment is necessary to control this pathogen and prevent contamination of food being produced.

### CONCLUSION

Some of the *Salmonella* strains studied survived at least 30 days and potentially could survive much longer. Strains also have different responses to desiccation. The results of this study will give an insight into potential growth and survival mechanisms for *Salmonella* in dry processing environments by providing strains that can be used to investigate physical and molecular changes which accrue within the cells under low $a_w$ conditions. The relationship between *Salmonella* survival and environment is very complex. It is not easy to explain this relationship based only on one environmental factor which can be manipulated in the laboratory. Bacterial survival is affected by a complex of stresses in the environment, not by a single stress. For this reason, the strains which have the best survival level will be selected to perform further studies using a range of humidity levels, temperatures, variations in food debris components, water access or inoculation levels. All these conditions will reflect the genuine factory environment. Systematic studies will be directed to provide solutions for controlling *Salmonella* in dry foods and will help to determine equipment design, environmental conditions for reduced survival and novel methods/programs for plant decontamination. These results will be incorporated into a Campden BRI guidance document on the survival of *Salmonella* in dry process environments with relation to strain, temperature, humidity, water and soiling, which would help the industry to control *Salmonella* endurance in the environment and thus in the final product.

### REFERENCES


