Improving Recovery of *Salmonella enterica* Serovar Typhimurium DT104 Cells Injured by Heating at Different Water Activity Values

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ABSTRACT

This study describes the evaluation of potentially more sensitive methods for the recovery of *Salmonella* cells injured by heating (54 to 60°C) at different water activity values (0.65 to 0.90, reduced using equal portions of glucose and fructose). These methods included gradual rehydration, the use of diluting media with added solutes or blood, the addition of blood to plating agar, and the use of different incubation temperatures and times. Gradual rehydration of cells that had been challenged at low water activity (0.65 and 0.70) and high temperature markedly improved recovery, measured as a >50% increase in the time to obtain a 3-log₁₀ reduction in cell numbers, compared to dilution into media with a high water activity. Adding sucrose, glycerol, or blood to the diluting media (maximal recovery diluent) did not improve recovery, but a plating agar containing blood recovered approximately 38% more cells than nutrient agar. Prolonged incubation of agar plates allowed recovery of injured *Salmonella* cells that presumably had extended lag periods, with significantly higher recovery rates after 48 h incubation at 37°C than after 24 h (P = 0.05). This work highlights that by recovering *Salmonella* using a method specific to the nature of the injury, a better prediction of food safety and the success of food processing can be made.

*Salmonella* is of great social and economic importance due to the large number of associated food-poisoning cases worldwide (3, 4, 10, 29). The two most prevalent serovars of *Salmonella* currently isolated from human cases in the United States and the United Kingdom are *Salmonella enterica* serovar Enteritidis and *S. enterica* serovar Typhimurium (3) (PHLS Communicable Disease Surveillance Centre, UK, data for 1987 to 1999).

The safety of food production and processing partly relies upon good detection methods to determine if viable foodborne pathogens, such as *Salmonella*, are present in a finished product. Detection usually involves the culture of microorganisms with selective and nonselective media. While it is relatively straightforward to recover uninjured bacterial cells, it can be more difficult with those that are sublethally injured (25), as will often be the case with populations surviving food processing. Injured cells may remain infectious or be able to recover and grow in the food product, and cases of food poisoning could result.

Desiccation or increasing the humectant content results in a reduced water activity (aₜ, measured by relative vapor pressure, rvp) that limits microbial growth and is commonly used in food preservation (5). Some bacteria including *Salmonella* spp., however, often show an increased heat tolerance when in a low aₜ environment (7), and this may have contributed to food poisoning outbreaks associated with heat-processed low aₜ foods such as chocolate (9, 12).

Sensitive recovery methods for injured *Salmonella* cells are required to ensure the safety of food products that undergo heat processing and those with a low aₜ value, including cakes and biscuits, are no exception. In addition, the ingestion of *Salmonella* in a low aₜ food has been associated with a low infectious dose (10 to 100 cells) (12, 24), although fat content may equally play a role. These observations increase the importance of detecting low numbers of cells in the finished product.

There is enhanced recovery of *Salmonella* and other bacteria injured by heating or exposure to low aₜ when measures to eliminate oxygen are taken (8, 16, 32). Following exposure to low aₜ, control of rehydration may also be important, because the influx of water into an injured cell may cause lysis (1, 22, 27). For example, studies by van Schothorst et al. (27) and Andrews (1) demonstrated that slow rehydration improved the recovery of *Salmonella* from dried milk. Recovery of low aₜ and heat-injured *Salmonella* may also be improved when glycerol or sucrose is added to the diluting media (7, 11, 13). There are few published data, however, on the development of enumeration methods that incorporate controlled rehydration, the use of prolonged incubation, or the use of blood in the recovery media to counteract reactive oxygen species.

In this study, improvements in the recovery methodology for heat- and low aₜ-injured *Salmonella* cells were sought, in an effort to give more accurate indications of the number of viable cells present during laboratory investigations or in finished food products. To date, data analyses often assume first-order death kinetics, but in this study we accurately describe curvature of the inactivation data. It is
hoped that making such improvements will contribute to reducing the number of cases related to *Salmonella* in low *a*<sub>0</sub> foods.

**MATERIALS AND METHODS**

Preparation and enumeration of heat- and low *a*<sub>0</sub>-injured *Salmonella* cells. Equal portions of AnalAR grades of glucose and fructose (BDH, Leicestershire, UK) were used as the humectants to produce defined *a*<sub>0</sub> tryptone soy broth (TSB; Oxoid, Hampshire, UK; rvp 0.65 to 0.95 ± 0.003, pH 6.5 ± 0.2) as described previously (18). Values for rvp were confirmed using an Aqualab CX-3T (Labcell, Hampshire, UK) *a*<sub>0</sub> meter at 25°C. An rvp of 0.65 and 0.95 (achieved using equal portions of glucose and fructose) is equivalent to approximately 80 and 32% wt/wt total sugar, respectively. Note that values for rvp and *a*<sub>0</sub> are identical in very dilute solutions but the term *a*<sub>0</sub> is defined in terms of ideal equilibrium solutions and the high solute broths used here are too concentrated to approach ideal behavior.

*Salmonella* Typhimurium DT104 strain 30 (30), a sucrose-negative, was recovered from storage at −20°C, and stationary-phase cultures were prepared as described previously (18). One hundred fifty microliters of the *Salmonella* culture was inoculated into 15 ml defined TSB to give an initial cell density of approximately 10<sup>8</sup> CFU ml<sup>−1</sup>. The cells were immediately heated at 54 or 60°C for 56 or 80 min, respectively, as previously described (18), using a submerged heating coil (6). CFUs on agar were determined at nine predetermined time intervals using the following standard method unless otherwise stated. Aliquots of 0.2 ml culture were ejected from the heating coil and an immediate 10-fold dilution was made in 1.8 ml maximal diluent (MRD; Oxoid; containing 1 g liter<sup>−1</sup> peptone and 8.5 g liter<sup>−1</sup> NaCl, pH 7.0) to reduce the broth temperature and minimize further cell death. Further 10-fold dilutions of the cells to extinction were prepared in buffered peptone water-containing Oxyrase, and 200-µl aliquots were distributed aseptically into each well of a 96-well microtiter plate, using one plate per dilution and at least three dilutions per sample (i.e., equivalent to a 96-well MPN). The microtiter plates were incubated in a humid atmosphere at 37°C, with plate reading at 72 h. Turbid wells were confirmed as *Salmonella* spp. on xylose lysine desoxycholate agar, using a multipoint replicator (Mast Diagnostics, Merseyside, UK).

The proportion of turbid wells to total wells at each dilution was entered into a computer program (26) that calculated a viable count based on statistical distribution.

Effect of plating media and duration and temperature of incubation postheating on measured death rates. In one series of experiments, *Salmonella* cells were challenged at 54 or 60°C and an rvp of 0.95 or 0.65 (achieved using equal portions of glucose and fructose) for up to 80 min. At nine intervals, identical samples were removed, diluted using MRD, and plated onto blood and nutrient agars, with incubation at 37°C for 48 h. The ratio of colony counts on blood agar to those on nutrient agar was calculated for each time interval, and an average was calculated to indicate overall benefit. The influence of incubation temperature used and the effects of prolonged incubation of the plates were also studied. Cells were challenged at rvp 0.95 (achieved using equal portions of glucose and fructose) and 54°C and plated on duplicate blood agar plates that were incubated at 30 or 37°C. Colonies were counted after 24, 48, and 72 h.

Data analysis. For each variation of recovery methodology, experiments were carried out in triplicate. Data analysis was performed in Microsoft Excel 97. Curves were fitted using the Weibull model (20) by the equation Log S<sub>b</sub> = −b × ×<sup>n</sup> and Microsoft Excel. In this equation, b represents the slope of the inactivation and n represents the degree of curvature (n = 1 gives a straight line, n < 1 shows upward concavity [tailing], and n > 1 shows downward concavity [shoulder]). Times to obtain a 3-log<sub>10</sub> reduction were calculated by rearrangement of this equation.

**RESULTS**

Recovery of injured DT104 cells in diluting media containing blood and solutes. The addition of blood to MRD did not significantly improve the recovery of injured *Salmonella* cells, as measured as the time to obtain a 3-log<sub>10</sub> reduction in viable cells, following any of the challenge conditions tested (Table 1). The use of reduced *a*<sub>0</sub> diluting media gave no consistent improvement in the recovery of injured *Salmonella* cells (Table 2). In fact, the addition of glycerol to the diluting media was not beneficial under any of the conditions tested (Table 2) and gave significantly poorer recovery than when sucrose was added for three of the four conditions tested (P = 0.019, 0.001, 0.333, 0.001).
TABLE 1. *Time in minutes* to obtain a 3-log$_{10}$ reduction in *Salmonella* cell numbers during heating at an $a_w$ reduced using equal portions of glucose and fructose, when the recovery protocol involves dilution in MRD with or without blood and plating onto blood agar

<table>
<thead>
<tr>
<th>Heating conditions</th>
<th>Recovery conditions (time to obtain a 3-log$_{10}$ reduction in cell numbers [min])</th>
<th>MRD</th>
<th>MRD + blood</th>
<th>MRD − blood</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$a_w$ (rvp)</td>
<td>T (°C)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.95</td>
<td>54</td>
<td>133 (42)</td>
<td>106 (11)</td>
<td>0.57</td>
<td></td>
</tr>
<tr>
<td>0.95</td>
<td>60</td>
<td>30 (1)</td>
<td>26 (2)</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td>0.65</td>
<td>54</td>
<td>52 (9)</td>
<td>45 (8)</td>
<td>0.60</td>
<td></td>
</tr>
<tr>
<td>0.65</td>
<td>60</td>
<td>30 (5)</td>
<td>32 (8)</td>
<td>0.91</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Standard error is given in parentheses.

Enumeration following gradual rehydration. Stepwise rehydration improved recovery compared with rapid rehydration, with a $>50\%$ increase in the time to obtain a 3-log$_{10}$ reduction in cell numbers when challenged at rvp 0.65 or 0.70 but only a small increase at rvp 0.85 (Table 3). The $n$ values (describing the curvature of the inactivation) when the recovery method involved gradual rehydration were always closer to 1 than when injured cells were inoculated directly into high $a_w$ media (Table 3). This indicates that more linear inactivation with less pronounced tailing is observed when the recovery protocol involves gradual rehydration.

Effect of plating media and duration and temperature of incubation. For cells challenged at rvp 0.95 and 54°C, rvp 0.95 and 60°C, rvp 0.65 and 54°C, or rvp 0.65 and 60°C, the ratio of colonies of blood agar to nutrient agar was 1.2 (standard error [SE] = 0.1), 1.5 (SE = 0.2), 1.4 (SE = 0.1), or 1.4 (SE = 0.1), respectively. The ratio of CFU on blood agar to those on nutrient agar (calculated from nine data points) was always $>1$, indicating superior recovery on blood agar, and on average 38% more cells were recovered when this medium was used.

TABLE 2. *Time in minutes* to obtain a 3-log$_{10}$ reduction in *Salmonella* cell numbers during heating at an $a_w$ reduced using equal portions of glucose and fructose, when the recovery protocol involves dilution in MRD with or without sucrose or glycerol and plating onto blood agar

<table>
<thead>
<tr>
<th>Heating conditions</th>
<th>Recovery conditions: MRD ± added sucrose or glycerol (time to obtain a 3-log$_{10}$ reduction in cell numbers [min])</th>
<th>None</th>
<th>% sucrose</th>
<th>% glycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td>$a_w$ (rvp)</td>
<td>T (°C)</td>
<td>0% (rvp 0.99)</td>
<td>20% (rvp 0.98)</td>
<td>30% (rvp 0.98)</td>
</tr>
<tr>
<td>0.95</td>
<td>54</td>
<td>62</td>
<td>75</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(9)</td>
<td>(16)</td>
<td>(7)</td>
</tr>
<tr>
<td>0.95</td>
<td>60</td>
<td>19</td>
<td>23</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(2)</td>
<td>(3)</td>
<td>(3)</td>
</tr>
<tr>
<td>0.65</td>
<td>54</td>
<td>58</td>
<td>59</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(2)</td>
<td>(2)</td>
<td>(4)</td>
</tr>
<tr>
<td>0.65</td>
<td>60</td>
<td>36</td>
<td>41</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(5)</td>
<td>(6)</td>
<td>(7)</td>
</tr>
</tbody>
</table>

$^a$ Standard error is given in parentheses.

The duration of incubation was found to have a marked effect on measured death rates and after challenge at rvp 0.95 and 54°C for 80 min the log$_{10}$ CFU ml$^{-1}$ was 4.8 when the plates were counted after 24 h but 5.5 after 48 h ($P = 0.05$). No differences were seen when uninjured cells were enumerated (data not shown). If incubation was continued for 72 h, however, some of the colonies were so large that they merged together. In contrast, the incubation temperature used for the agar plates had no significant effect. When blood agar plates were incubated at 30°C for 48 h, the time for the heated population of *Salmonella* cells to be reduced by 3 log$_{10}$ was 58 min (SE = 6). If the plates were incubated at 37°C the equivalent time was 53 min (SE = 13) ($P = 0.76$).

**DISCUSSION**

In this study, ways to improve the recovery of heat and low $a_w$-injured *Salmonella* cells were investigated. Although foods often harbor mixed populations, studies of pure cultures of bacteria are important, for example to develop kinetic inactivation models that are used as predictive...
tools for food process design and risk assessment. It is also important that a sensitive method for the recovery of sublethally injured organisms is used for this, in order to produce fail-safe models. Such studies can indirectly benefit routine food analysis or outbreak investigations (where Salmonella must be recovered from mixed culture) by using the data on bacterial injury and their requirements for recovery to aid the development of sensitive, yet selective, isolation methods.

The optimal growth of Salmonella is at rvp 0.99. When Salmonella is inoculated into TSB at rvp 0.95 (achieved using equal portions of glucose and fructose), there is no significant change in either cell numbers or broth pH for more than 100 h at 21°C (18) and at rvp < 0.95, death occurs. Therefore, bacterial metabolism of the solutes used to reduce the aw of the broth is unlikely to have occurred at the values used in these studies (rvp ≤ 0.95), and the observations can be attributed to osmotic effect.

Cells subjected to low aw (high solute concentrations) undergo rapid water loss, followed by the uptake or synthesis of compatible solutes in order to maintain cellular viability under these conditions. During exposure to low aw, cells may sustain injury and lose their ability to grow on selective media, for example. If cells that are adapted to low aw are subsequently rehydrated, water will rapidly enter because the cytoplasm is now more concentrated than the surrounding environment. Lysis may result upon rehydration, particularly if the cellular membranes are damaged or if rehydration spans a large increase in aw. Therefore, while exposure to low aw can result in bacterial injury, gradual rehydration may promote cell recovery by reducing the demands on cell homeostasis.

The reduced aw diluting media investigated in our study covered the rvp range 0.84 to 0.99. Glycerol and sucrose were chosen to reduce aw as the addition of either solute to diluting media has previously been found beneficial by other researchers (7, 11, 13). In this study, adding these solutes to the diluting media did not improve recovery, and three possible reasons for the apparent discrepancy between our findings and those of other researchers have been identified. The aw range of the diluting media differed between studies. For example, Hsieh et al. (13) investigated adding glycerol to 0.1% peptone water. This was beneficial only at concentrations of >50% wt/wt glycerol, corresponding to aw below the range tested in this present study. In addition, some researchers have added solutes to the diluting media without presenting or referring to comprehensive data to indicate a benefit. For example, Goepfert et al. (11) incorporated 45% sucrose into peptone water to recover Salmonella cells challenged at 57°C and rvp < 0.96, but no supporting data were presented. Finally, if adding these solutes to peptone water actually did improve recovery (although few supporting quantitative data are presented), then the apparent discrepancy may be due to the use of isotonic MRD in this study, as opposed to peptone water used previously. The osmolarity of bacterial cells and MRD is reported to be 300 mosmol kg⁻¹ H₂O but 0.1% peptone water is 5 mosmol kg⁻¹ H₂O (15, 28); thus, dilution in peptone water is likely to cause significantly more osmotic stress than when MRD is used.

Despite the lack of observed benefit of low aw diluting media (rvp 0.84 to 0.99), there is evidence that slow rehydration from much drier conditions (e.g., dried milk powder) may improve the recovery of dehydrated cells. For example, van Schothorst et al. reported enhanced recovery of Salmonella from dried condensed milk by reconstituting samples in buffered peptone water at a 1:2 sample:broth ratio, with dilution to a final 1:9 sample:broth ratio 30 min later (27). Andrews et al. compared the recovery of Salmonella from nonfat dried milk reconstituted by a rapid and a slow procedure (the soak method) for sample rehydration. In both studies, gradual rehydration improved the recovery of Salmonella from these food types. Wilson et al., however, found that the soak method did not improve the recovery of Salmonella from dried yeast, onion powder, or soy flour (31). To date, such methods have only enabled presence or absence enrichment culture (2, 23). In this study, an enumeration method involving gradual rehydration of cells following heating at rvp 0.65 to 0.85 was investigated, and this was of clear benefit, presumably due to the holding stages at rvp < 0.84 (between the challenge and recovery rvp's) or the increased overall duration of rehydration. This also demonstrates the benefit of gradual rehydration in a medium other than dried milk.

Enumeration involving gradual rehydration would be useful, for example to accurately determine the infectious dose of Salmonella cells associated with a low aw food in an outbreak situation. The benefit of gradual rehydration indicates that too large or too fast an increase in aw can result in the death of some of the population of injured cells. During studies into the rehydration of air-dried cells of Saccharomyces cerevisiae, an rvp range of 0.12 to 0.46 had to be crossed slowly in order to maintain viability (21), and there may be a similar critical range for Salmonella. The absolute value of the critical range may relate to the rvp that the cells are adapted to. Tailing may reflect the rapid death of sensitive individual cells, with more resistant cells surviving longer. The less pronounced tailing observed when using gradual rehydration indicates that this recovery method may give more benefit to the sensitive cell population than the resistant cells.

The length of incubation of the blood agar plates had a marked effect on viable counts, presumably due to the increased and highly variable lag time of injured cells that can exceed 20 h (17, 26). It is also reported that prolonged incubation of acid injured cells of E. coli O157 for >24 h promotes the recovery of those cells with increased lag times (15). Blood agar recovered more cells than nutrient agar, indicating that the presence of blood in the recovery agar is important, probably as an agent protecting against reactive oxygen species. The addition of blood to agar was also beneficial for freeze- or heat-damaged Campylobacter jejuni (14), but this was perhaps less surprising as these bacteria are microaerophilic.

This study indicates that an isosmotic diluting media, gradual rehydration, prolonged incubation, and agents to protect against reactive oxygen species will all improve the
recovery of bacterial cells injured by heating at low aw. We recommend that these factors be incorporated into existing protocols, where applicable.

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