Standardized Laboratory-Scale Preparation of Mayonnaise Containing Low Levels of *Salmonella enterica* Serovar Enteritidis

RENATA G. K. LEUSCHNER* AND MARTIN P. BOUGHTFLOWER

Central Science Laboratory, Sand Hutton, York YO41 1LZ, UK

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ABSTRACT

*Salmonella enterica* serovar Enteritidis PT4 and PT6 are associated with food poisoning outbreaks and are often found in food only in low concentrations. In this study a reproducible laboratory-scale procedure for preparation of mayonnaise is presented. The mayonnaise that simulates a naturally low-level contaminated product can be used for validation of new methods and is also suitable to study the behavior of low numbers of food pathogenic spoilage microorganisms in a food environment.

During processing, liquid egg was artificially contaminated with low levels of *S. enterica* serovar Enteritidis that resulted in levels of 1 to 3 log_{10} CFU/g in the final mayonnaise. Cells of *S. enterica* serovar Enteritidis had increased stability in the mayonnaise when they were subjected to low pH in two stages, first to pH 5.8 and afterward to pH 4.5 before addition to the mayonnaise. The pH of the mayonnaise was between 4.2 to 4.5 and remained stable over the storage period. Low-level *S. enterica* serovar Enteritidis remained stable in artificially contaminated mayonnaise for 4 weeks at 4°C.

**MATERIALS AND METHODS**

*Strains.* Two strains of *S. enterica* serovar Enteritidis were used during the study. Both strains were kindly provided by Prof.

*Author for correspondence. Tel: 0044-1904-46228; Fax: 0044-1904-462111; E-mail: r.leuschner@csl.gov.uk.*
### TABLE 1. Ingredients for mayonnaise production

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>% (wt/wt)</th>
<th>Example (2-liter batch)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sunflower oil</td>
<td>79.3</td>
<td>1,586 ml</td>
</tr>
<tr>
<td>Pasteurized whole egg</td>
<td>10</td>
<td>200 ml</td>
</tr>
<tr>
<td>Water</td>
<td>6.7</td>
<td>134 ml</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>2</td>
<td>40 g</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0.8</td>
<td>16 g</td>
</tr>
<tr>
<td>Citric acid</td>
<td>0.7</td>
<td>14 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.5</td>
<td>10 g</td>
</tr>
</tbody>
</table>

T. Humphrey (Public Health Laboratory Service, Exeter, UK). *S. enterica* serovar Enteritidis PT4 2Y was isolated from naturally infected free range egg (Yorkshire), and *S. enterica* serovar Enteritidis PT4 74S was isolated from egg. *S. enterica* serovar Enteritidis PT6 was isolated from chocolate mousse made with fresh egg that was associated with a large outbreak of *Salmonella* poisoning (4).

**Culture conditions.** Cultures of *Salmonella* used for artificial contamination were resuscitated by inoculating nutrient broth (NB) or brain heart infusion broth (BHIB) (Oxoid, Hampshire, UK) and incubating aerobically at 37°C for 72 ± 4 h.

Acid tolerance response (ATR) was induced by culturing test strains in BHIB aerobically at 37°C for 72 ± 4 h. The procedure was carried out as described for ATR of *S. enterica* serovar Enteritidis (13). The culture was transferred to BHIB with a reduced pH of 5.8 and was incubated at 42°C for 24 ± 2 h to produce a stationary-phase culture with a partially induced ATR. The post-acid shock phase to induce full ATR was completed in the mayonnaise that had a pH of 4.4.

Confirmation of the test strains was carried out using polyvalent agglutinating sera (O and H) for *Salmonella* spp. (Murex Biotech Ltd., Kent, UK) using a slide agglutination technique.

**Preparation of mayonnaise.** The production of mayonnaise comprised three successive stages: Blending of all ingredients except oil, addition of oil under controlled conditions during continuous blending, and storage of mayonnaise aliquots for up to 4 weeks at 4°C.

The ingredients for mayonnaise production are listed in Table 1: retail sunflower oil (Trex, Princes Ltd., Liverpool, UK), pasteurized liquid egg (Daylay, Newnark, UK), acetic acid (wine vinegar 6% acidity) (Dutras, Nestlé, York, UK), sucrose and NaCl (BDH, Poole, UK), and citric acid (concentrated lemon juice, Coop, CWS Ltd., Manchester, UK).

The following equipment was used as shown in Figure 1: a blender motor unit with a maximum of 18,000 rpm, a 4-litre stainless steel blender receptacle, a peristaltic pump, tubing (autoclavable) with a 6.4-mm inner diameter bore suitable for use with the peristaltic pump, sterile measuring cylinder(s), sterile pipettes, 60 ml sterile straight-sided screwcap sample pots (Fisher, Loughborough, UK).

**Production protocol for mayonnaise.** The sterilized stainless-steel jug of the blender was cooled by storing it at -20°C for at least 5 min before use. The peristaltic pump has to be set up to allow the required volume of oil to be added to the Waring blender (Waring, New Hartford, Conn.) (Fig. 1). The pump was calibrated for each mayonnaise production to ensure reproducible results.

All ingredients (Table 1) except the oil were placed into the cooled base of the Waring blender and processed for 5 s at 18,000 rpm. For small volumes of mayonnaise a 1-litre, two-speed and for production of larger batches a 4-litre, three-speed blender (Waring) was used.

For preparation of mayonnaise the critical point was the blending speed and the rate of addition of oil during blending controlled by the use of a peristaltic pump. The required volume of oil was added to the inside edge area of the Waring blender (see diagram 1) via the peristaltic pump. The oil was added through one 6.4-mm bore tubing over a period of 100 s if 500-ml mayonnaise batches were prepared and through a 6.4-mm dual tube for 2-litre batches. The rate of oil addition was 480 ml/min under continuous blending at a speed of 18,000 rpm. The mayonnaise was aseptically weighed in 25-g aliquots and stored in 60-ml sterile straight-sided screwcap pots at +4°C. The final mayonnaise was creamy white, opaque, and of a smooth-spooning or slow-pouring consistency. After a few hours at refrigeration temperature, the mayonnaise viscosity increased slightly but remained without any noticeable changes at this consistency for 4 weeks.

**Addition of *Salmonella* strains to mayonnaise.** Although microbial specifications were supplied by the ingredient manufacturer, the microbiological quality of five subsamples of the first batch of liquid egg was investigated for the presence of total *Enterobacteriaceae*, *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* spp., and total viable counts. Pasteurized liquid egg was artificially contaminated with a previously enumerated culture of *S. enterica* serovar Enteritidis. Numbers of *Salmonella* in the artificially contaminated egg were enumerated 30 min after inoculation, using xylose lysine desoxycholate (XLD) spread plates. A second enumeration was carried out after mixing of the artificially contaminated egg with other ingredients (except oil) to ensure that no bactericidal effects reduce the viable cell counts. All results represent a mean of three replicate counts for each artificial contamination level at each day.

**Determination of low artificial contamination level of *S. enterica* serovar Enteritidis in mayonnaise.** Prior to preparation of each mayonnaise batch, the pasteurized egg was inoculated with the test strain that was diluted to three different inoculum levels. The actual levels were determined using a most probable number (MPN) modification of the *E. coli* detection method that comprised pre-enrichment in buffered peptone water, selective enrichment in Rappaport Vassiliadis broth, and isolation on xylose XLD agar (BS5763 (2) and BSEN12824 (3)). All media were obtained from Oxoid. Direct plating from buffered peptone water onto XLD agar was also carried out to enumerate at the higher level. Each batch of artificially contaminated mayonnaise was divided into 25-g aliquots and stored at +4°C until use. The levels of *Salmonella* in mayonnaise were monitored at 1- to 7-day in-
tervals over a 28-day period using the MPN technique. The pH of the mayonnaise was also monitored over the entire storage period.

The total viable counts for each batch of mayonnaise were determined at intervals of 1 to 7 days for up to 28 days by serially diluting cultures in maximum recovery diluent and preparing spread plates on plate count agar incubated at 30°C for 72 h.

To produce a stable validation sample and to determine a realistically low artificially contaminated mayonnaise preparation, the experiment was repeated using a high (approximately 3.7 log_{10} CFU/g) and a medium (approximately 3.4 log_{10} CFU/g) level of \textit{S. enterica} serovar Enteritidis. The culture was grown in BHIB broth to see if this increased the stability of the \textit{Salmonella} strains during storage.

\textit{S. enterica} serovar Enteritidis PT4 with induced ATR also were added to the liquid egg used for mayonnaise preparation to assess their cell stability in mayonnaise. The effects of the mayonnaise production procedure on \textit{S. enterica} serovar Enteritidis PT6 was also investigated by subjecting them to the liquid egg.

After stable low inoculum levels were established for \textit{S. enterica} serovar Enteritidis PT4 and PT6 in experiments described above the mayonnaise production procedure was repeated several times to ensure the reproducibility of the protocol.

\textbf{Statistics.} All results where \textit{S. enterica} serovar Enteritidis was isolated from food matrices represent a mean of three replicate counts for each artificial contamination level at each day. Experiments were carried out on at least two independent occasions. Linear regression was used to interpolate survival of \textit{S. enterica} serovar Enteritidis in mayonnaise.

\section*{RESULTS}

\textbf{Growth characteristic of \textit{S. enterica} serovar Enteritidis PT4.} Two different strains of \textit{S. enterica} serovar Enteritidis PT4 (2Y and 74S) were grown in NB and BHIB. The growth characteristic demonstrated that both media were suitable for the strain. The use of BHIB resulted in slightly higher viable cell concentrations in the stationary growth phase for both strains than for NB that were 9 log_{10} CFU/g and 8.7 log_{10} CFU/g, respectively. Further studies were carried out using \textit{S. enterica} serovar Enteritidis PT4 2Y that was an isolate from a naturally infected free range egg.

\textbf{Viable counts in liquid egg and artificial contamination with \textit{Salmonella}.} The examination of the pasteurized liquid egg that was used for mayonnaise production was free of \textit{Escherichia coli}, \textit{Enterobacteriaceae}, \textit{Staphylococcus aureus}, and \textit{Salmonella} spp. Typical total viable counts of 2.21 log_{10} CFU/g were found.

A potential loss of viability expressed as difference between added and recovered viable cells of \textit{S. enterica} serovar Enteritidis PT4 2Y by bactericidal effects of the egg on the culture was investigated. A loss of viability of around 0.28 log_{10} CFU/g during a 30-min contact phase was observed (Table 2). A further average reduction in bacterial counts was observed after mixing with additional ingredients except the oil (Table 3). The inoculum grew to 8.91 log_{10} CFU/g after 24 h.

\begin{table}[h]
  \centering
  \begin{tabular}{|c|c|c|c|}
    \hline
    Inoculum level & Inoculum \ (log_{10} CFU/g) & Viable cells recovered \ (log_{10} CFU/g) & Difference \ (log_{10} CFU/g) \\
    \hline
    High & 4.91 & 4.64 & 0.27 \\
    Medium & 3.91 & 3.62 & 0.29 \\
    Low & 2.91 & 2.62 & 0.29 \\
    \hline
  \end{tabular}
  \caption{The effect of egg on viability of \textit{S. enterica} serovar Enteritidis PT4 directly after addition as determined by direct plating on XLD agar.}
\end{table}

\textbf{Mayonnaise production with \textit{S. enterica} serovar Enteritidis.} Results of artificially contaminated mayonnaise with \textit{S. enterica} serovar Enteritidis PT4 2Y grown in nutrient broth are shown in Figure 2. Poor stability was observed during the storage period at 4°C for different inoculation levels. The numbers of cells had declined over a period of 10 to 12 days to a level below the limit of detection by MPN (<0.3 CFU/g). Direct plating on XLD yielded inconsistent data, and direct enumeration was not possible for the low bacteria levels.

From the results for the inoculation of mayonnaise with two different levels of \textit{S. enterica} serovar Enteritidis PT4 2Y cultured in BHIB, it can be seen that these samples were more stable than those observed from cultures that were grown in NB (Fig. 3).

Most of the background flora present in the original pasteurized egg did not survive the mayonnaise production process and thus the dominant flora of the final sample was \textit{Salmonella}. The morphology of \textit{S. enterica} serovar Enteritidis isolated from mayonnaise samples was very distinctive on plate count agar when incubated at 30°C for 72 h and could be used as an indicator for the presence of the target microorganism.

\textbf{Artificial contamination of mayonnaise with ATR cells of \textit{S. enterica} serovar Enteritidis.} Induction of an ATR was carried out in two stages: a preacid shock and a postacid shock. Preacid shock is induced at around pH 5.8 and enables the organism to maintain a neutral pH at low external pH. Postacidic shock is induced at approximately pH 4.5 and involves the synthesis of new proteins needed to resist extreme acidity. Results for the inoculation of mayonnaise test materials with three different levels of \textit{S. enterica} serovar Enteritidis PT4 2Y cultured in BHIB to give an induced ATR are shown in Figure 4. The pH value of mayonnaise remained around 4.25 ± 0.29 during the storage period (Table 4).
FIGURE 2. Viability of cells of *S. enterica* serovar Enteritidis PT4 2Y in laboratory mayonnaise after growth in nutrient broth as determined by the MPN method. The mayonnaise was artificially inoculated with three different levels: high (♦), medium (■), and low (▲), whereby results represent a mean of three replicate counts for each spiking level at each day.

FIGURE 3. Viability of cells of *S. enterica* serovar Enteritidis PT4 2Y in laboratory mayonnaise after growth in BHIB as determined by the MPN method. The mayonnaise was artificially inoculated with two different levels: high (♦) and medium (■), whereby results represent a mean of three replicate counts for each spiking level at each day.
Reproducibility of the mayonnaise-spiking protocol.

Results obtained for the second strain used in this study, *S. enterica* serovar Enteritidis PT6, demonstrated that the production protocol was applicable to different strains of *Salmonella*. The reproducibility of the production protocol using BHIB cultured cells of *S. enterica* serovar Enteritidis PT4 2Y and PT6 with induced ATR is shown in Figure 5. The validation sample can be reproducibly made, are reasonably stable, and fit for purpose. The laboratory-scale mayonnaise production could be applied in independent laboratories to produce low levels of viable *Salmonella* cells in artificially contaminated mayonnaise.

**TABLE 4.** The pH of laboratory-made mayonnaise during a storage period at 4°C

<table>
<thead>
<tr>
<th>Test day</th>
<th>Low-level spike</th>
<th>Medium-level spike</th>
<th>High-level spike</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4.24</td>
<td>4.32</td>
<td>4.11</td>
</tr>
<tr>
<td>2</td>
<td>4.22</td>
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</tr>
<tr>
<td>30</td>
<td>4.34</td>
<td>4.27</td>
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</tr>
</tbody>
</table>

DISCUSSION

The laboratory-based protocol reported here for the production of a traditional mayonnaise containing low levels of *S. enterica* serovar Enteritidis PT4, PT6, and low levels of background flora was successful and reproducible. Although the samples were reasonably stable over a period of 1 month, the variation in cell number between triplicate mayonnaise samples at any one sampling point throughout the storage period has been greater than desired. However, this is primarily due to the need to use MPN technique to enumerate the *Salmonella*, and this method can only be considered as an estimate.

The higher level contained numbers of *Salmonella* that were too high to be of use as a validation sample for the evaluation of the sensitivity of new methods, and the testing of these samples was discontinued. The samples inoculated at the medium and low level were tested at intervals over a 30-day period and were reasonably stable with detectable levels of the target microorganism throughout the duration of the experiment. The low level was considered to be the most fit for the purpose. The production of naturally contaminated mayonnaise is also a suitable ecosystem to study the ecology of *Salmonella* spp. Low contamination levels represent a situation that needs to be anticipated when food is analyzed for the presence or absence of *Salmonella* spp. Further parameters such as abusive handling practices of the product could be investigated with regard to their effects on the concentration of spoilage microorganisms and the natural background flora. Our investigations revealed that the initial background flora disappeared during may-
FIGURE 5. Repeatability of preparation of two independent mayonnaise batches containing low levels of S. enterica serovar Enteritidis PT4 with induced ATR (△) y = −0.0395x + 1.2005, R² = 0.8327; (▲) y = −0.0529x + 1.7568, R² = 0.8922; and S. enteridis PT6 (○) y = −0.0518x + 1.649, R² = 0.9284; (●) y = −0.0616x + 1.7539, R² = 0.9437.

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onaise storage and that Salmonella was the remaining bacterium.

Incubation of S. enterica serovar Enteritidis PT4 in media at pH values between 3.0 and 6.0 resulted in a marked and rapid increase in acid resistance, manifested when cells were subsequently challenged at pH 2.5 to 2.9 (10). The induction of ATR will be necessary to ensure that the stability of mayonnaise validation samples containing Salmonella spp. is maximized. This treatment may not be necessary for the production of a less traditional mayonnaise with a higher pH value. The ATR of S. enterica serovar Typhimurium involves two stages: preacid shock, induced at a pH of about 5.8, and postacid shock at a pH around 4.5 (13). Preacid shock makes the organisms able to maintain a neutral internal pH at low external pH (7). Postacid shock involves synthesis of new proteins that are needed to resist extreme acidity (6). These proteins may protect cells from acid damage or aid repair. In the present study, ATR of S. enterica serovar Enteritidis resulted in more acid-resistant cells and a similar mechanism seems to apply as described for S. enterica serovar Typhimurium. Survival of S. enterica serovar Enteritidis in reduced-calorie and in cholesterol-free, reduced-calorie mayonnaise was not observed after 1 week, whereby the mayonnaise had a pH of 4.3 to 3.9 adjusted with acetic acid (8). In that study, Salmonella had an initial inoculum of was 6 log_{10} CFU/ml, and the cells were not induced with ATR and displayed a significant lower survival rate than in our study. S. enterica serovar Enteritidis PT4 grown in NB grew to slightly lower viable counts in the stationary phase than during growth in BHIB. The cultures used for artificially contaminating the mayonnaise were more stable after growth in BHIB than in NB.

The growth conditions affected the stability and acid resistance of S. enterica serovar Enteritidis in mayonnaise.

This study may encourage the development of other defined food model systems to study Salmonella in other relevant matrices such as egg, whereby this would imply an investigation of other injury mechanisms. In the present study, we mixed Salmonella with the egg prior to processing of mayonnaise and observed a loss in viability. This is only a limited investigation and storage effects in eggs caused by lysozyme (cell wall damage) and ovotransferrin (iron deprivation) over a prolonged time period on the viability of Salmonella would be an important aspect.

The developed naturally contaminated mayonnaise with low, medium, and high levels of S. enterica serovar Enteritidis provides a useful tool for a wide range of applications such as method validation, evaluation of bacterial survival during different production conditions, or changes of product formulations.

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REFERENCES


