Kinetics of growth and inactivation of *Salmonella enterica* serotype Typhimurium DT104 in pasteurised liquid egg products

Olivia J. McQuestin [a,*], Michael T. Musgrove [b], Mark L. Tamplin [a]

[a] *Tasmanian Institute of Agricultural Research, School of Agricultural Science, University of Tasmania, Private Bag 54, Hobart, Tasmania 7001, Australia*
[b] *Egg Safety and Quality Research Unit, Agricultural Research Service, United States Department of Agriculture, 950 College Station Road, Athens, GA 30604, USA*

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ABSTRACT

The potential impact of post-pasteurisation contamination of liquid egg products with the multi-antibiotic resistant pathogen *Salmonella enterica* serotype Typhimurium definitive type 104 (DT104) was assessed by determining the viability of this bacterium in whole egg, albumen and 10% w/w sugared and salted yolk incubated at 4–42 °C. Results indicated that populations of *S.* Typhimurium DT104 were slowly inactivated in all four products when stored at 4 °C. However, based on the typical shelf-lives of cold-stored liquid egg, less than 0.6 log-kill would be achieved in those products prior to their use. Incubation at temperatures pertaining to abuse situations (10, 15, 20 and 25 °C) revealed an increasing potential for growth of *S.* Typhimurium DT104 in whole egg, albumen and sugared yolk, as indicated by trends in growth rate, lag duration and maximum population density. At even higher temperatures (30, 37 and 42 °C), growth rates of *S.* Typhimurium DT104 in whole egg and sugared yolk continued to increase. The same was true for *S.* Typhimurium DT104 in albumen except that growth was not observed at 42 °C and instead populations were inactivated within 30 h. At no temperature tested was *S.* Typhimurium DT104 able to grow in salted yolk. The influence of these growth and inactivation patterns on the risk of salmonellosis in relation to product type and storage temperature is discussed.

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1. Introduction

The widespread use of antibiotics in agriculture and human medicine has raised concern about the emergence of antibiotic resistant bacteria and their impact on public health. One study conducted in the US indicated that the prevalence of strains of *Salmonella enterica* serotype Typhimurium resistant to five antibiotics increased from 0.6% in 1979/1980–34% in 1996 (Glynn et al., 1998). The growing incidence of multi-antibiotic resistances in *S.* Typhimurium appears to be a worldwide phenomenon (Evans and Davies, 1996; Hollingsworth and Kaplan, 1997; Kam, 1996; Mackie et al., 1996; Martel et al., 1995; Ramos et al., 1996). Of particular concern is a multi-antibiotic resistant strain of *S.* Typhimurium known as definitive phage type 104 (DT104) that is an increasing cause of salmonellosis infection worldwide and is associated with a greater risk of hospitalisation, invasive illness and death (Helms et al., 2005; Varma et al., 2005).

S. Typhimurium DT104 is typically resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline.

Molecular studies have indicated that the genes encoding those resistances are contained on the bacterial chromosome (Ridley and Threlfall, 1998; Threlfall et al., 1994) and, therefore, it is unlikely that removal of selective pressure by antibiotics will rid this bacterium of its resistant properties compared to plasmid carriage. The identification of *S.* Typhimurium DT104 isolates that are also resistant to trimethoprim and ciprofloxacin (Threlfall et al., 1996) further limits the means by which these infections can be treated.

Humans acquire *S.* Typhimurium DT104 infection primarily through consumption of animal food products. Outbreaks have been linked to poultry, meat and meat products and unpasteurised dairy products (Cody et al., 1999; Dechet et al., 2006; Ethelberg et al., 2007; Grein et al., 1999; Villar et al., 1999). Contamination of these foods is typically *via* direct contact with faecal matter of infected animals, for example during hide removal and gut evisceration following slaughter. Currently, the control of foodborne *S.* Typhimurium DT104 infection at food processing and preparation levels relies upon well-established strategies that are typical of those for other salmonellae (Hogue et al., 1997), including adherence to good manufacturing practices, heat-treating foods during pasteurisation or cooking, avoiding cross-contamination of other foods and storage at cool temperatures. However, because infectious agents may be intentionally or unintentionally added to foods

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* Corresponding author. Tel.: +613 6226 6385; fax: +613 6226 7450.
 E-mail address: Olivia.McQuestin@utas.edu.au (O.J. McQuestin).

post-production, it is also useful to identify the potential for pathogens to survive in foods after processing, based on the inherent food environment (e.g. the presence of antimicrobial molecules or competitive microflora) and extrinsic factors (e.g. storage time and temperature, and in-house cooking).

The current project was conducted to assess the ability of S. Typhimurium DT104 to survive and grow in four liquid egg products including whole egg, albumen (egg white) and 10% sugared and salted yolk at a range of temperatures relevant to the manufacture and storage of these products. Parameters of viability curves were determined and can be incorporated into modelling programs that, in conjunction with risk assessment, may be used to determine the likelihood of associated foodborne disease outbreaks occurring.

2. Materials and methods

2.1. Strains used and preparation of inocula

S. Typhimurium DT104 strains #10 TX and #7470C-1, isolated from poultry and swine, respectively, were used in this study. The strains were maintained in nutrient broth (CM0001, Oxoid, Adelaide, Australia) supplemented with 30% glycerol (Sigma, Melbourne, Australia) at −80 °C. Experimental inocula were cultured from frozen stocks and grown on brilliant green agar (CM0263, Oxoid) supplemented with 0.8 g/l sulfadiazine ("BGS agar"; Sigma) and the antibiotics ampicillin, chloramphenicol, streptomycin and tetracycline (Sigma) at 25 μg/ml each with incubation at 37 °C for 18 (±2) h. Cells were inoculated into peptone buffered saline (PBS; BR0014, Oxoid) and absorbance was determined to estimate population density. A two-strain cocktail was prepared with equal amounts of each strain in PBS to achieve a concentration of approximately 2.5×10^6 CFU/ml.

2.2. Liquid egg products used and assessment of background microflora

Four frozen and pasteurised, liquid egg products including whole egg, albumen, 10% w/w sugar yolk and 10% w/w salted yolk were obtained from an Australian egg supplier, transported under cold-storage and stored at −20 °C until use. Aliquots were thawed by incubation at 4 °C (for yolk samples) and 10 °C (for whole egg and albumen samples) for 16 (±2) h prior to use. Product specifications from the supplier indicated low numbers of aerobes (1×10^4 CFU/g), coliforms (<10 CFU/g), yeasts and moulds (<20 CFU/g) and Salmonella (not detected in 1 g) in the liquid egg products and this was corroborated in the current study prior to inoculation with S. Typhimurium DT104. Specifically, undiluted amounts (4×250 μl) of each liquid egg product were plated to plate count agar (CM0463, Oxoid; for an aerobic plate count), violet red bile glucose agar (CM0485, Oxoid; to detect Enterobacteriaceae), dichloran rose-bengal chloramphenicol agar (CM0727 and SR0078, Oxoid; for the enumeration of yeasts and moulds) and BGS agar (for the enumeration of Salmonella spp.). Plates were incubated at 37 °C for 24 h, except for dichloran rose-bengal chloramphenicol agar, which were incubated at 25 °C for 5 days, and CFU were enumerated.

2.3. Preparation of egg samples and inoculation with S. Typhimurium DT104

Experimental samples were prepared by weighing 50 (±0.5) g of each liquid egg product to a sterile 50 ml centrifuge tube. The tubes were incubated at the appropriate temperature (4, 10, 15, 20, 25, 30, 37 or 42 °C) for 1 h to equilibrate to temperature. In triplicate, 100 μl of the two-strain cocktail containing S. Typhimurium DT104 #10 TX

and #7470C-1 was added to 50 g volumes of each liquid egg product, thus providing an initial cell density of approximately 5×10^3 CFU/g. The inoculated egg samples were mixed thoroughly by manual inversion and incubation on a roller deck (Luckham, Burgess Hill, UK) for 10 min. Inoculated egg samples were incubated at the appropriate temperature, which was monitored at 10 s intervals using a temperature data logger (TinyTag Ultra 2, Hastings Data Loggers, Port Macquarie, Australia). Temperatures were within ±0.7 °C of the required temperature for the duration of each trial.

2.4. Enumeration of survivors and determination of growth or inactivation rates

At appropriate intervals aliquots were withdrawn and, where necessary, serially diluted in PBS. Diluted samples were surface-plated using a spiral plater (Autoplate 4000, Spiral Biotech, Bethesda, USA) onto BGS agar with antibiotics. When cell viability was expected to be low, undiluted aliquots (0.5–0.15 g) were manually plated. Plates were incubated at 37 °C for 24 (±1) h and typical red-coloured colonies were counted. Viability was determined over a period that ranged from 24 h, when liquid eggs were incubated at 42 °C, to 3076 h (i.e. 128 d), when stored at 4 °C.

Viability curves were constructed by plotting viable numbers ($\log_{10}$ CFU/g) against time. For each product type and temperature combination, the viability of S. Typhimurium DT104 was determined in triplicate, providing 96 growth or, in some instances, inactivation curves. When viable cells could not be detected in a 0.5 g amount of an undiluted sample (i.e. < 1.30 $\log_{10}$ CFU/g) the data were not used to estimate the inactivation rate. When appropriate, the inactivation rate was determined by linear regression fitted to the data using Microsoft® Excel. Where growth occurred, the viability data were fitted using the Baranyi D model (Baranyi and Roberts, 1994) in DMFit Version 2.1 software kindly provided by the Institute of Food Research, Norwich, UK. This primary model was used to obtain estimates of growth rate (maximum potential rate), lag period and maximum population density (MPD). The effect of temperature on those parameters was tested using various regression models starting with a separate slopes model for each liquid egg product using SAS.

3. Results

The viability of S. Typhimurium DT104 in commercially-sourced whole egg, albumen, sugared yolk and salted yolk stored at specific temperatures ranging from 4 to 42 °C was determined (see Fig. 1 for examples of the typical growth or inactivation curves obtained). The ability of S. Typhimurium DT104 to survive and grow in liquid egg, as indicated by inactivation or growth rate, lag period and MPD, is shown in Table 1. At 15–37 °C, the growth of S. Typhimurium DT104 in albumen occurred at two distinct rates. That is, a faster rate of growth preceded a slower rate that commenced when the cell density was between 10^6 and 10^7 $\log_{10}$ CFU/g. When this occurred, the viability curve was fitted with two log-linear models, providing a growth rate for the first and second phases of growth.

Results indicated that there was no growth of S. Typhimurium DT104 in any of the liquid egg products stored at 4 °C. Instead, viability decreased by 2.25 $\log_{10}$ CFU/g to below detectable limits (i.e. 1.30 $\log_{10}$ CFU/g) in all three replicates within approximately 2400 h (i.e. 100 d) in albumen and salted yolk products and within 3000 h (i.e. 125 d) in whole egg and sugared yolk (data not shown).

As shown in Table 1, when eggs were incubated at 10–37 °C, growth was observed in whole egg, sugared yolk and albumen, although a relatively slower rate of growth was observed in the albumen at each temperature. In salted yolk incubated at 10–37 °C, the S. Typhimurium DT104 population was inactivated and the