



Effects of environmental conditions on growth and survival of *Salmonella* in pasteurized whole egg



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ABSTRACT

This study investigated the influence of three parameters (time, temperature and NaCl concentration) on survival and four parameters (temperature, NaCl and lysozyme concentrations and pH) on growth of *Salmonella enterica* serovar Enteritidis (*S. Enteritidis*) in pasteurized whole egg (PWE). Doehlert uniform shell design was employed to choose conditions for trials and data was fitted to polynomial models and were presented as estimated response surfaces. A model for prediction of reduction of *S. Enteritidis* in PWE within temperatures between 50 and 58 °C, NaCl concentrations of 0–12%, and heating times between 30 and 210 s and a model for prediction of growth rate of *S. Enteritidis* in PWE in the temperature range of 1–25 °C, NaCl concentration of 0–12%, pH between 5 and 9, and lysozyme concentrations of 107–1007 U/mg proteins were developed. The maximum reduction condition was 58 °C, 0% of NaCl at a fixed heating time of 120 s, while maximum growth rate was estimated at 25 °C and 0% of NaCl. pH and lysozyme concentration were shown not to influence growth performance significantly in the range of values studied. Results inform industry of the optimal pasteurization and storage parameters for liquid whole egg.

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1. Introduction

Salmonella enterica serovar Enteritidis (*S. Enteritidis*) is an important food borne pathogenic bacterium. Most outbreaks caused by this bacterium have been associated to eggs and egg products (Anon., 2013). Pasteurization of liquid egg products is widely applied in the industry to reduce this risk, however several *Salmonella* outbreaks associated with consumption of pasteurized egg products indicate that *Salmonella* may still be present in the final product (HPA, 2007, 2012).

Hurdle effects might be used to prevent regrowth of *Salmonella* in food products (Leistner, 2000). Addition of NaCl is often used as a preservative in food, however, already several decades ago Garibaldi et al. (1969) observed that addition of both salt and sucrose to egg yolk increased thermal resistance of *Salmonella*. Likewise, a number of studies have concluded that heat tolerance of *Salmonella* increases when a_w decreases both in broth or buffer systems (Goepfert et al., 1970), and in liquid egg products (Garibaldi et al., 1969; Ng et al., 1979; Palumbo et al., 1995). This has however been contradicted in studies by Mattick et al. (2001) who observed the opposite effect when the temperature was lower than 65 °C, and Aljarallah and Adams (2007) reported a switch from protective to sensitizing effect of salt at even lower temperatures

(53 °C–55 °C). There is therefore a need to revisit this problem, if the *Salmonella* risk from liquid egg products shall be reduced by the use of hurdle effects.

Predictive models have been used to estimate the influence of multiple parameters on growth of *Salmonella* in growth media (Park et al., 2007; Thayer et al., 1987), but so far nobody have used such multiple parameter models to describe *Salmonella* growth in liquid egg. In the current investigation we used predictive modeling to determine individual and combined effects of three environmental conditions: time, temperature and NaCl concentration on survival of *S. Enteritidis* in pasteurized whole egg (PWE) and to investigate growth of *S. Enteritidis* as a function of four parameters: NaCl and lysozyme concentrations, temperature and pH in the same product.

2. Materials and methods

2.1. Bacterial strains, media and egg products

S. Enteritidis strain E2187 (Olsen et al., 1994) was used for survival experiments. It was plated on Xylose-Lysine-Deoxycholate Agar (XLD, Oxoid, Basingstoke, United Kingdom) and incubated overnight at 37 °C and colonies were harvested in physiological saline (0.9% NaCl, Merck, Darmstadt, Germany) to an OD-value corresponding to a concentration of approximately 10^9 CFU/ml. PWE (Danæg, Roskilde,

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Denmark) was spiked to a final concentration of approximately 10^7 CFU/ml. Actual concentrations were determined by plating appropriate decimal dilutions in saline on Luria-Bertani (LB) agar (Becton, Dickinson and Company, Sparks, MD, USA) plates.

S. Enteritidis strain MJG01 (previously isolated from egg product) was used for growth experiments. It was plated on a Plate Count Agar (PCA, AES, Bruz, France) and incubated at 37 °C for 24 h. One colony was then transferred to a Brain Heart Infusion broth (AES, Bruz, France) and incubated at 37 °C for 24 h. This stock solution was used to inoculate the PWE (ADRO Ouest, France) after appropriate dilutions in a Tryptone Salt (Biomérieux, Craaponne, France) broth to reach the final concentration as shown below in the description of the growth experiment. PWE used for both survival and growth experiments was prepared by industry by pasteurization to ensure sterility.

2.2. Doehlert uniform shell design

Both growth and survival experiments were carried out according to a Doehlert experimental design (Doehlert, 1970). For survival experiments, two experimental matrices 1 and 2 were designed with low (50, 52, 54 °C) and high (54, 56, 58 °C) temperatures, respectively (Table 1). Each experimental matrix contained 13 trials with variation in three variables (NaCl concentration (% w/w), time (s) and temperature (°C)). Three repeats were carried out of experiment with conditions at the center to ensure reproducibility (total number of trials 16). Results were presented as $\log_{10}$ reduction of *Salmonella* in PWE.

For growth experiments 21 trials were performed with variation in four variables (pH, temperature (°C), lysozyme concentration (U/mg proteins) and NaCl concentration (% w/w)) (Table 2). To ensure reproducibility, two repeats of experiment were carried out with conditions at the center (total number of trials 23). For each trial a growth curve was determined based on CFU and the growth rate was calculated according to the primary model of Rosso (1995) based on 10 to 15 enumerations for each growth curve.

2.3. Survival experiments

Solid NaCl was added at the defined concentrations to PWE maximum 24 h before the experiment and samples of 90 µl of salted PWE in 1.5 ml Eppendorf tubes were pre-heated to different temperatures (+/− 1 °C) listed in Table 1 with an Eppendorf thermomixer with shaking at 1000 rpm. Temperatures were measured with a penetration thermometer Ebro TDC 200. 10 µl of spiked PWE without NaCl was added to reach final concentrations of NaCl and a final concentration of approximately 10^6 CFU/ml. Samples were incubated for different times as indicated in Table 1 followed by placing samples on ice and addition of 900 µl ice cold saline to stop the heating. Further decimal dilutions were prepared in saline and plated on LB agar plates. Two independent experiments were performed for each matrix with two replicates for each trial. Numbers of bacteria were converted to $\log_{10}$ CFU/ml and subtracted from initial concentration to obtain reductions.

2.4. Growth experiments

PWE samples were inoculated with 10^3 CFU/ml of *S. Enteritidis*, where after growth kinetics within the given range of temperature, pH, salt and lysozyme concentrations (Table 2) were determined.

Table 1
Range of parameters for survival experiments according to Doehlert uniform shell design.

Factors	No. of levels	Values
Temperature (°C)	3	50–52–54 (matrix 1) 54–56–58 (matrix 2)
NaCl (% w/w)	5	0–3–6–9–12
Time (s)	7	30–60–90–120–150–180–210

Table 2
Range of parameters for growth experiments according to Doehlert shell design.

Factors	No. of levels	Values
pH	3	5–7–9
Temperature (°C)	5	1–7–13–19–25
Lysozyme (U/mg proteins)	7	107–257–407–557–707–857–1007
NaCl (% w/w)	7	0–2–4–6–8–10–12

Before the experiments the defined pH values were obtained by adding acid (HCl) or base (NaOH) at a suitable titer in order to avoid introducing an excessive dilution factor into the PWE. Solid NaCl was added at the defined concentrations after a moderate heat treatment and ionization to ensure the sterility before adding it to the PWE. Numbers of bacteria were determined by CFU counts on PCA.

2.5. Statistical analysis

For survival experiments Grubb's test of outliers with $P < 0.05$ (<http://graphpad.com/quickcalcs/Grubbs1.cfm>) identified one CFU count out of four in three samples as outliers. These were eliminated before further analysis. Data from matrices 1 and 2 were merged and used to build one model for survival experiment. Statistical analysis of data was performed using Statgraphics© software. Results explained the individual effect of each parameter and their interactions. Polynomial models were built for prediction of *S. Enteritidis* behavior using the same software. Moreover, the results were illustrated as estimated response surfaces. Lack-of-fit and R^2 were used to determine whether the selected model is adequate to describe the observed data, or whether a more complicated model should be used. Lack-of-fit test was performed by comparing the variability of the current model residuals to the variability between observations at replicate settings of the factors.

3. Results and discussion

3.1. Effects of environmental conditions on survival of *Salmonella* in PWE

Three environmental conditions (NaCl concentration (% w/w), time (s) and temperature (°C)) were investigated in relation to survival of *S. Enteritidis* in PWE. Data presented in Table 3 show the estimated individual and combined effects of all parameters on *S. Enteritidis* $\log_{10}$ reduction and their significance in both matrices. Time and temperature had significant positive effect on $\log_{10}$ reduction, while increasing NaCl concentration protected against killing. Fig. 1 shows estimated response surface of $\log_{10}$ reduction as a function of temperature and NaCl concentration with time fixed at the central value of 120 s. In both matrices the effect of NaCl on heat killing of *S. Enteritidis* was protective. This corresponds well with results of Garibaldi et al. (1969) and a protective effect of NaCl was also observed by others (Ng et al., 1979; Palumbo et al., 1995), however the challenge temperatures in their

Table 3
Estimated effects of factors individually and their interactions for reduction of *S. Enteritidis* in PWE in the temperature range of 50° to 58 °C.

Factors	Estimated effects on $\log_{10}$ reduction	SD	P value ^a
A: NaCl conc.	−1.14	0.165366	0.0005
B: Time	2.095	0.143211	0.0000
C: Temperature	2.56636	0.141024	0.0000
AA	2.28772	0.273981	0.0002
AB	−0.779082	0.316945	0.0492
AC	−0.106122	0.298819	0.7346
BB	0.12839	0.205485	0.5551
BC	0.947347	0.258784	0.0106
CC	0.587489	0.252847	0.0592
Lack-of-fit			0.0742
R^2			0.921892

^a Significant values are indicated in bold.

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