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Influence of the Initial Cell Number on the Growth Fitness of *Salmonella* Enteritidis in Raw and Pasteurized Liquid Whole Egg †

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Abstract: *Salmonella* growth and survival in egg and egg products has been widely studied. Nevertheless, there are still some aspects that are still not fully known. Therefore, the objective of this work was to study the influence of the initial cell number on the growth fitness of *Salmonella* in raw and pasteurized liquid whole egg. Growth curves of *Salmonella* Enteritidis in raw and pasteurized liquid whole egg and starting from different initial numbers were obtained and fitted to the Baranyi and Roberts model. Results obtained revealed that in raw whole egg lower initial numbers led to longer Lag phases (λ) and lower maximum specific growth rates (μ_{max}). However, the application of thermal treatments of increasing intensity to the liquid egg -before inoculation with *Salmonella* cells- led to a progressive reduction of this effect. Supplementation assays with lysozyme and ovotransferrin revealed that these proteins would not be the responsible for the decreased growth fitness of *Salmonella* cells when inoculated in raw egg at low concentrations. By contrast, addition of iron (0.020 g/L) to raw egg increased the growth fitness of *Salmonella* cells inoculated at a low initial dose but not a high one. Thus, under these conditions the growth fitness of *Salmonella* was similar regardless of the initial dose and thermal history of the whole liquid egg, indicating the major role of iron bioavailability on the phenomenon observed.

Keywords: *Salmonella*; egg products; inoculum dose; thermal treatments; foodborne pathogen

1. Introduction

The microorganisms of the genus *Salmonella* are the second most commonly reported causative agent of foodborne outbreaks in the European Union [1] and constitute one of the major public health challenges worldwide. One of the most important sources of *Salmonella* contamination is eggs and egg products. Thus, raw and undercooked eggs are still the most frequently identified products as responsible for foodborne *Salmonella* infections in the European Union (45.6% of *Salmonella* outbreaks in Europe in 2018) [1]. *Salmonella* growth and survival in egg and egg products has been widely studied, in particular, that of the serovar Enteritidis, because it is the predominant serovar in foodborne diseases associated to the consumption of these products [2–4]. Growth models have been developed in order to predict the growth of *Salmonella* in egg products in an effort to establish the optimal temperature and time for its conservation and distribution [5–7]. Nevertheless, there are still some aspects, such as the influence that the initial cell number and/or the physiological state of cells have on *Salmonella* growth fitness and resistance to stress, that are still not fully known. Therefore, the aim of this work was to study the influence of the initial cell number on the growth fitness of *S.*

Enteritidis in raw and pasteurized liquid whole egg. In addition, the potential mechanisms underlying the results obtained were explored.

2. Materials and Methods

2.1. Bacterial Strains and Obtention of Bacterial Suspensions

Salmonella Enteritidis STCC 4300 was used in this study. Cultures were grown in tryptic soy broth (Oxoid, Basingstoke, UK) supplemented with 0.6% *w/v* yeast extract (Oxoid, TSB-YE) in 96 wells microtiter plates and incubated at 37 °C under static conditions as described in Guillén et al., 2020 [8].

2.2. Growth Curves

Growth experiments were carried out in raw liquid whole egg obtained from medium-sized raw eggs (53–63 g) purchased from a local supermarket and in commercial ultra-pasteurized liquid whole egg (Pascual, Aranda de Duero, Spain). For some experiments (see below) the raw liquid whole egg was exposed to heat treatments simulating pasteurization conditions (60 °C/3.5 min).

Growth media were inoculated with 10² (low dose) and 10⁶ (high dose) CFU/mL *S. Enteritidis* and incubated at 37 °C. Samples were taken at preset time intervals, from 0 to 30 h, adequately diluted in buffered peptone water (Oxoid, BPW) and plated in the recovery medium, Xylose Lysine Deoxycholate agar (Oxoid, XLD). XLD plates were incubated for 48 h at 37 °C, and the number of colony forming units (CFU) per plate was counted.

2.3. Thermal Treatments

Thermal treatments (pasteurization of raw whole liquid egg) were carried out in a specially designed thermoresistometer implemented with a compatible control thermostat that allowed the performance of heating ramps at different rates [9]. The thermoresistometer was programmed to perform a linear temperature profile from 25 to 60 ± 0.1 °C at a rate of 2 °C/min and held at that temperature for 3.5 min. After treatments, pasteurized whole liquid egg was cooled and stored at 4 °C.

2.4. Supplementation Assays

Supplementation assays were carried out by adding different concentrations of the following proteins/compounds to the growth media (liquid whole egg): lysozyme (Sigma, St. Louis, MO, USA) up to a concentration of 1.93 mg/mL, ovotransferrin (Sigma) up to 8.8 mg/mL and ferric citrate (Sigma-Aldrich) at a concentration of 0.02 mg/L. The concentrations of these compounds were selected based on their amount in egg white [10].

2.5. Growth Curve Fitting and Statistical Analysis

Growth curves were constructed by plotting the logarithm of the number of *S. Enteritidis* versus time at the different conditions assays. Each point of the growth curve corresponds to the average value of all the samples analyzed (at least three replicates). The curves obtained were fitted with the Baranyi and Roberts model [11]:

$$Y_t = Y_0 + \mu_{max} \cdot A_t - \frac{Y_{max} - Y_0}{M} \cdot \ln \left[1 - e^{-M} + \left(e^{-M} \cdot \frac{Y_{max} - Y_0 - \mu_{max} \cdot A_t}{Y_{max} - Y_0} \right) \right] \quad (1)$$

$$A_t = t - \lambda \cdot \left[1 - \frac{1}{h_0} \cdot \ln \left(1 - e^{-h_0 \cdot \frac{t}{\lambda}} + e^{-h_0 \cdot \left(\frac{t}{\lambda} - 1 \right)} \right) \right] \quad (2)$$

where Y_t is the Log of cell concentration at time t (CFU/mL); Y_0 is the Log of the initial cell concentration (CFU/mL); Y_{max} is the Log of maximum cell concentration (CFU/mL); μ_{max} is the maximum growth rate (h⁻¹); λ is the Lag phase (h); and M and h_0 are curvature parameters, taking

them as constant values, set for both a value of 10. The growth curves were then analyzed to obtain the parameters with GraphPad PRISM® statistical software (GraphPad Prism version 8.00 for Windows, GraphPad Software, San Diego, CA, USA). Goodness of the fits of model were estimated through R² and RMSE calculated with GraphPad software. Student's *t* tests were carried out using the same program and differences were considered significant for *p* ≤ 0.05.

3. Results

3.1. Effect of Initial Concentration on the Growth Fitness of *Salmonella* in Raw and Pasteurized Liquid Whole Egg

The influence of the initial contamination dose on *Salmonella* Enteritidis growth fitness was analyzed in raw and commercial ultra-pasteurized liquid whole egg by obtaining growth curves starting at 10² (low dose) or 10⁶ CFU/mL (high dose) in both media. As can be observed in Figure 1, the initial inoculum dose significantly affected the growth parameters calculated in raw liquid whole egg but not in commercial ultra-pasteurized liquid egg. Thus, the maximum growth rate in raw liquid whole egg determined for growth curves starting at a concentration of 10² CFU/mL (0.663 ± 0.015 h⁻¹) was significantly (*p* < 0.05) lower than that in curved starting at a concentration of 10⁶ CFU/mL curves (0.845 ± 0.038 h⁻¹). Similarly, also significant differences were found among the *Lag* values calculated in raw liquid whole egg (6.315 ± 0.549 h vs. 1.966 ± 0.206 h, for the curves starting at 10² and 10⁶ CFU/mL, respectively). By contrast, no significant differences were found among the growth parameters calculated in commercial ultra-pasteurized liquid whole egg regardless of the inoculum dose. In addition, it should be noted that the growth parameters calculated for *S. Enteritidis* in raw liquid whole egg inoculated at the high dose were also comparable (no significantly different) to those calculated in ultra-pasteurized liquid whole egg.

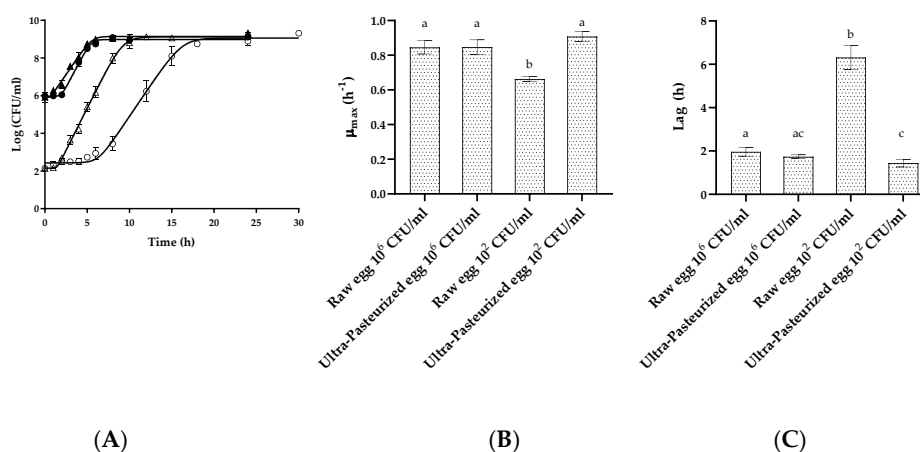


Figure 1. Effect of the inoculum dose, 10² or 10⁶ CFU/mL, on the growth fitness of *Salmonella* in raw and ultra-pasteurized liquid whole egg: (A) Growth curves of *S. Enteritidis* STCC 4300: Raw egg 10⁶ CFU/mL ●; Ultra-pasteurized egg 10⁶ CFU/mL ▲; Raw egg 10² CFU/mL ○; Ultra-pasteurized egg 10² CFU/mL △. Lines correspond to the fit to the Baranyi Model.; (B) μ_{max} values (h⁻¹) and (C) *Lag* phases values (h) calculated after the fit of the growth curves to the Baranyi Model. Error bars represent the standard deviation and letters indicate statistically significant differences.

3.2. Effect of the Intensity of the Whole Liquid Egg Pasteurization Treatment on the Growth Fitness of *Salmonella* Enteritidis Cells

In order to better understand the differences in *Salmonella* growth fitness observed when comparing raw and ultra-pasteurized commercial raw egg additional growth curves were obtained using whole liquid egg exposed to a conventional pasteurization treatment (60 °C/3.5 min) (Figure 2). As can be seen in Figure 2B, after the application of this conventional pasteurization treatment (60

°C/3.5 min) the maximum growth rate of *S. Enteritidis* cells (when inoculated at a low dose) increased as compared to those calculated in raw egg. However, this value did not reach the value obtained when cells were grown in ultra-pasteurized commercial liquid whole egg. Similarly, the *Lag* values, also decreased as a function of the intensity of the pasteurization treatment (Figure 2C). In addition, and as previously observed, no differences in the growth parameters were found regardless of the thermal history of the whole liquid egg when growth curves were started at the high inoculum dose (10^6 CFU/mL) (data not shown).

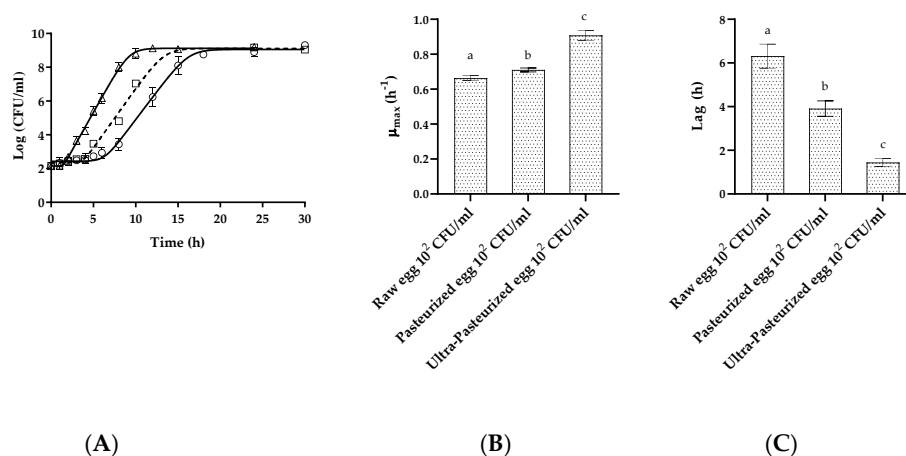


Figure 2. Influence of the intensity of the pasteurization treatment on the growth fitness of *Salmonella* Enteritidis cells inoculated at 10^2 CFU/mL: (A) Growth curves of *S. Enteritidis* 4300: Raw whole egg 10^2 CFU/mL o; Commercial ultra-pasteurized whole egg 10^2 CFU/mL □; Pasteurized whole egg 10^2 CFU/mL Δ. Lines correspond to the fit to the Baranyi Model.; (B) μ_{max} (h^{-1}) and (C) *Lag* (h) values obtained after the fit of the growth curves to the Baranyi Model. Error bars represent the standard deviation and letters indicate statistically significant differences.

3.3. Effect of the Supplementation with Egg White Proteins with Known Antimicrobial Properties

Commercial ultra-pasteurized egg was supplemented with antimicrobial egg white proteins (lysozyme and ovotransferrin) and growth curves of *Salmonella* Enteritidis cells were obtained. The addition of these proteins to the ultra-pasteurized egg, at the low dose, did not result in either a decrease in growth rate or an increase in the *Lag* phase (Figure 3) of *Salmonella* Enteritidis cells as compared to those determined in ultra-pasteurized liquid whole egg without supplementation.

3.4. Effect of Iron Supplementation

Supplementation of raw egg with 0.02 g/L of ferric citrate (approximately the amount of iron naturally present in raw egg) resulted in an increase in growth rate and a decrease of the *Lag* phase when *Salmonella* cells were inoculated at low dose (Figure 4). However, no effect was observed when iron was supplemented to either in the ultra-pasteurized egg (regardless of the inoculation dose) or in raw egg when inoculated at the high dose (data not shown).

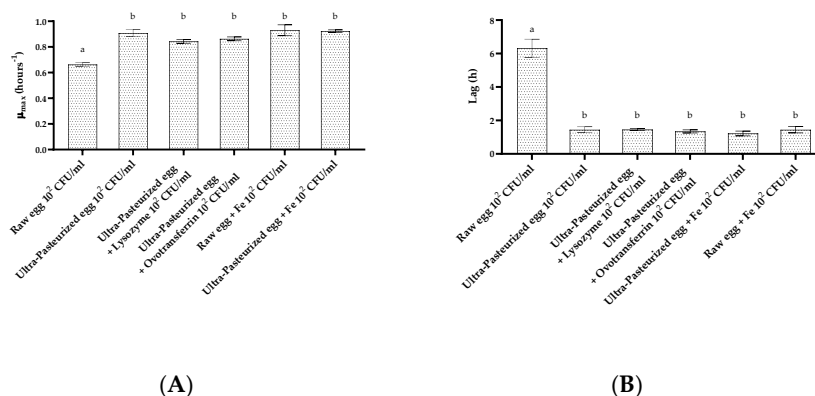


Figure 3. Effect of supplementation of antimicrobial egg white proteins and/or ferric citrate on the growth fitness of *Salmonella* in raw and pasteurized liquid whole egg inoculated at 10^2 CFU/mL: (A) μ_{max} values (h^{-1}) and (B) Lag phases values (h) values obtained after the fit of the growth curves to the Baranyi Model. Error bars represent the standard deviation and letters indicate statistically significant differences.

4. Discussion

Our results demonstrate that the growth rate of *Salmonella* Enteritidis cells in whole liquid eggs depends on both the thermal history of the whole liquid egg and, in the case of raw egg, also on the initial concentration of *Salmonella* cells. Thus, *Salmonella* cells grew faster (when inoculated at low doses) the higher the heat treatment previously applied to the whole liquid eggs and the higher the initial dose when *Salmonella* cells were inoculated in raw egg. Zaher and Fujikawa (2001) also observed a similar dose-effect phenomenon on *Salmonella* growth and attributed their results to the competition between *Salmonella* and natural microbiota in raw ground chicken [12] but this would not be the case of raw egg since given the extremely low concentrations of microorganisms in this latter product. On the other hand, pasteurization treatments, 1–10 min at 60–72 °C, applied in the industry are limited because of the sensitivity of the egg white proteins to heat treatments, what might lead to egg coagulation. These treatments can also denature egg white proteins with antimicrobial properties such as ovotransferrin and lysozyme [10]. Nevertheless, supplementation assays of pasteurized egg with of ovotransferrin or lysozyme that were designed to determine if thermal denaturation of these proteins was the cause for the higher growth fitness of *Salmonella* cells in pasteurized whole egg showed that no reduction in growth rate nor an increase in the length of the Lag phase was observed at the minimum inoculum dose. On the contrary, supplementation of iron to raw egg, resulted in an increase in *Salmonella* growth fitness, although only when cells were inoculated at a low dose. In any case, this would mean that the bioavailability of iron in the raw egg would depend on the thermal history of the egg, being higher after more intense pasteurization treatments. Further work will be required to fully elucidate all the components of the egg (white) with antimicrobial activity, including those limiting iron bioavailability. In this sense, it should be noted that recent studies suggest that low-mass components, <10 kDa, of egg white are largely responsible for the bactericidal activity of egg white at high temperature [13].

In summary, our results demonstrate that the initial dose and thermal history of the whole liquid egg can determine the maximum rate of *Salmonella* cells. They also indicate that lysozyme and ovotransferrin would not be responsible for the differences in growth rate found between raw and pasteurized whole liquid egg and that iron bioavailability would be playing a major role in this phenomenon.

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review and editing, M.M., I.Á., P.M. and G.C.; supervision, G.C.; project administration, G.C.; funding acquisition, I.Á., P.M. and G.C. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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