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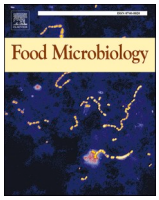

Relationship between iron bioavailability and *Salmonella* Typhimurium fitness in raw and pasteurized liquid whole egg

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ABSTRACT

Salmonella: Enteritidis growth rates in liquid whole egg have been shown to be dependent on the initial inoculum dose and on the egg product's thermal history. This study's objective is to obtain further insight into the mechanisms underlying both phenomena. First we verified that *Salmonella* Typhimurium ATCC 14028s cells displayed the behavior already described for *S.* Enteritidis cells. Then, we carried out supplementation assays by adding different concentrations of egg-white antimicrobial proteins, iron, or siderophores to the egg samples (raw or pasteurized liquid whole egg, depending on the assay). These experiments revealed that addition of lysozyme (at the concentration at which it is present in liquid whole egg) did not affect *Salmonella* growth in pasteurized liquid whole egg, but that ovotransferrin as well as Ex-FABP caused a significant ($p < 0.05$) reduction in *Salmonella* growth rates in whole egg pasteurized at 70 °C for 1.5 min. Furthermore, we observed that the inactivation of ovotransferrin was dependent on treatment intensity within the range studied. On the other hand, addition of iron or siderophores to raw or low temperature (60 °C/3.5 min) pasteurized liquid whole egg increased the growth rate of *Salmonella* cells inoculated at a low initial dose. The concentration of these supplements required to reach the growth rate of cells inoculated at a high dose was lower for pasteurized than for raw egg. Finally, growth of a set of deletion mutants in genes coding for proteins related to different iron uptake systems, along with supplementation assays using spent medium revealed the key role of salmochelin in growth of *S.* Typhimurium in raw whole egg. In summary, our results strongly suggest that iron bioavailability determines the fitness (growth rates) of *Salmonella* cells in liquid whole egg. Thus, the higher the intensity of the thermal treatment applied to liquid egg, the more iron would be available, a phenomenon that would be linked to the denaturation of iron and/or siderophore binding egg proteins. Further work is still required to fully elucidate why lower *Salmonella* initial doses lead to lower growth rates, but it can be hypothesized that this might be related to a lower amount of siderophores being released to the medium (especially salmochelin), which would also limit iron bioavailability.

1. Introduction

Foodborne diseases are an acknowledged public health challenge worldwide. In the European Union, salmonellosis is the second most commonly reported gastrointestinal infection in humans after campylobacteriosis (EFSA, 2021). The microorganisms of the genus *Salmonella* were the most frequently detected causative agent in foodborne outbreaks (FBOs) in 2019, accounting for 17.9% of total FBOs. One of the most important sources of *Salmonella* contamination are eggs and egg products, principally raw and undercooked eggs (37.0% of *Salmonella* outbreaks in Europe in 2019) (EFSA, 2021). Eggs are contaminated by *Salmonella* through two main routes: vertical transmission, i.e., before oviposition by infection of the reproductive organs, and horizontal transmission, i.e., during or after oviposition upon penetration of the eggshell (De Reu et al., 2006; Gantois et al., 2009; Keller et al., 1995; Messens et al., 2005; Timoney et al., 1989). Apart from presenting several physical barriers (eggshell, cuticle, testaceous membranes), egg, and more particularly egg albumen, contains a potent complex of antimicrobial molecules that limit bacterial growth and migration into the egg yolk (Baron et al., 2016). The antimicrobial activity of egg albumen is ensured, on the one hand, by its harsh physicochemical properties, including its alkaline pH as well as its high viscosity, which impair

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bacterial mobility and accessibility to nutrients (Baron et al., 2016, 2017). However, it should be noted that this activity decreases as the pH of the albumen increases and the quality of the egg declines, depending on the time and temperature of the egg during storage (Guyot et al., 2016). Egg albumen likewise contains a series of compounds (proteins) with specific antimicrobial properties, including lysozyme (a cell wall hydrolase), proteinase inhibitors, and ovotransferrin (an iron chelator that limits iron bioavailability), among others (Baron et al., 1999; Chart and Rowe, 1993). Although these well-known and widely characterized proteins are naturally abundant in egg albumen, high-throughput approaches have recently revealed that less abundant proteins (such as the siderophore-sequestering "lipocalin" protein Ex-FABP) and peptides might also play a role in the defense against bacterial contamination (Baron et al., 2016; Julien et al., 2020).

The particular composition and physicochemical characteristics of egg albumen make *Salmonella* growth ability in egg and egg products highly dependent on the egg fraction (yolk *vs* white *vs* whole) (Guillén et al., 2021; Kim et al., 2018; Messens et al., 2004; Moon et al., 2016). However, egg fraction is not the only factor determining *Salmonella* growth ability/rate in egg products. Incubation/storage temperature and time, as well as the supplementation of additives such as NaCl or sucrose to control water activity, also determine *Salmonella* growth fitness in egg and egg products (Gurtler and Conner, 2009; Jakočiūnė et al., 2014; Ng et al., 1979).

Recent studies have demonstrated the influence of the initial cell number and of thermal history (raw *vs* pasteurized) on the growth fitness of *Salmonella* in egg albumen and liquid whole egg (Guillén et al., 2020a, 2021; Kang et al., 2021); these authors hypothesized that the lower growth rates observed for *Salmonella* cells in raw products inoculated at low initial doses could be related to the antimicrobial activity of egg albumen proteins, such as ovotransferrin and lysozyme (Baron et al., 2016; Lechevalier et al., 2017). Further research is necessary to verify this hypothesis. Therefore, the aim of the present study is to obtain further insight into the mechanisms responsible for the initial-dose-dependence and thermal-history-dependence of *Salmonella* growth in liquid whole egg.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The strains used in this study are listed in Table 1. They were obtained from the Single-Gene Deletion Mutant Library of *Salmonella enterica* subsp. *enterica*, Strain 14028s (Serovar Typhimurium) through BEI Resources (http://www.beiresources.org/); the parental strain was provided by the Spanish Type Culture Collection (STCC). Cultures were grown in tryptic soy broth (Oxoid, Basingstoke, UK) supplemented with 0.6% w/v yeast extract (Oxoid, TSB-YE) in 96-well microtiter plates, and incubated at 37 °C under static conditions as described in Guillén et al. (2020b).

Table 1
Strains used in this study.

Strain	Source
S. Typhimurium ATCC 14028s	STCC (strain 4594)
S. Typhimurium ATCC 14028s *ΔentC:Kan*	BEI Resources (NR-29399)
S. Typhimurium ATCC 14028s *Δfes:Kan*	BEI Resources (NR-29399)
S. Typhimurium ATCC 14028s *ΔfeoB:Kan*	BEI Resources (NR-29399)
S. Typhimurium ATCC 14028s *ΔfhuC:Kan*	BEI Resources (NR-29399)
S. Typhimurium ATCC 14028s *ΔiroB:Kan*	BEI Resources (NR-29399)
S. Typhimurium ATCC 14028s *ΔiroN:Kan*	BEI Resources (NR-29399)
S. Typhimurium ATCC 14028s *ΔmntH:Kan*	BEI Resources (NR-29399)
S. Typhimurium ATCC 14028s *ΔsitC:Kan*	BEI Resources (NR-29399)
S. Typhimurium ATCC 14028s *ΔzupT:Kan*	BEI Resources (NR-29399)

2.2. Growth curves

Growth experiments were carried out in raw liquid whole egg obtained from medium-sized raw eggs (53–63 g, 5–12 days old) purchased from a local supermarket either before (raw) or after the application of a pasteurization treatment (see below, section 2.3). Eggs were stored at 4 °C until use. For some experiments, commercial pasteurized liquid whole egg (Pascual, Aranda de Duero, Spain) was also used. The pH of pasteurized and raw liquid whole egg was measured using a pHmeter BASIC 20 (Crison Instrument, Barcelona, Spain) and was 7.6 ± 0.2.

Prior to use, the eggshells were thoroughly washed with 70% ethanol, allowed to air dry and the eggs were gently broken, the contents placed in a sterile container and the eggshells discarded. The contents of at least three eggs, depending on the experiment, were shaken vigorously and stored until use at 4 °C, not to exceed 48 h. The egg products were inoculated with different initial *Salmonella* doses, 10^2 (low dose) and 10^6 (high dose) CFU/mL, and were then incubated at 37 °C. Samples were taken at preset time intervals, from 0 to 30 h, unless otherwise noted, adequately diluted in buffered peptone water (Oxoid, BPW), and plated on Xylose Lysine Deoxycholate agar (Oxoid, XLD), the recovery medium. 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 liquid whole egg) were carried out in a specially designed thermoresistometer implemented with a compatible control thermostat that allowed for the performance of heating ramps at different rates (Conesa et al., 2003). Briefly, this instrument consists in a 400 mL vessel provided with an electrical heater for thermostation, a cooling system, an agitation device to ensure distribution and temperature homogeneity, and ports for the injection of microbial suspension as well as for the extraction of samples. The thermoresistometer was programmed to perform a linear temperature profile from 25 °C to the target temperature at a rate of 1 °C/min, and then to hold at that temperature (±0.1 °C). After treatments, pasteurized liquid whole egg was cooled and stored at 4 °C. Raw liquid whole egg was exposed to two different treatment conditions simulating different pasteurization conditions: 60 °C 3.5 min and 70 °C 1.5 min.

For some experiments (application of thermal treatments to ovotransferrin and to Ex-FABP in liquid whole egg, along with "re-pasteurization" treatments), a T100 thermal cycler (Bio-Rad, CA, USA) was used. This thermal cycler was programmed with the same heating ramps and used to apply the same treatment conditions as described for the thermoresistometer. We verified that the liquid whole egg temperature attained after the heating ramp was correct (data not shown).

2.4. Supplementation assays

Supplementation assays were carried out by adding different concentrations of proteins (section 2.4.1. Proteins), iron, or siderophores (section 2.4.2. Iron and siderophores) to the egg samples (liquid whole egg). In another set of assays, supplementation with spent media (section 2.4.3. Spent medium) obtained from different strains was also studied. Then, liquid whole egg (raw or pasteurized, depending on the assay) supplemented with those molecules or extracts was inoculated with different concentrations of *Salmonella* cells and incubated as described above in order to determine its effect on *Salmonella* growth fitness. Table S1 summarizes the thermal treatments and conditions tested in this work.

2.4.1. Proteins

Lysozyme (Sigma-Aldrich, St. Louis, USA) up to a concentration of 2.57 mg/mL and ovotransferrin (Sigma-Aldrich) up to 8.80 mg/mL were added to 1 mL of the egg product (liquid whole egg) as powder. Extracellular fatty-acid-binding protein (Ex-FABP), a recombinant egg white

lipocalin from *Coturnix japonica* (CSB-EP878006DXJ; Cusabio Biotech Co. Ltd, Wuhan, China) up to 0.22 mg/mL were added to the egg product. Concentrations of those compounds were selected based on their quantity proportion in egg albumen (Baron et al., 2016; Julien et al., 2020), and assuming a 2:1 ratio between egg albumen and egg yolk in liquid whole egg. The commercial protein Ex-FABP was desalted before supplementation using an Amicon ultra-0.5 mL centrifugal filter (MWCO = 3 kDa; Millipore, Billerica, MA, USA).

2.4.2. Iron and siderophores

A suspension of ferric citrate (Sigma-Aldrich) was added to 1 mL of egg product up to a concentration of 0.1 mg/mL. Enterobactin (ENT) and salmochelin S4 (SAL), two siderophores in iron-free form, were purchased from EMC Microcollections (Tuebingen, Germany) and supplemented at concentrations up to 5.00 μg/mL in 1 mL of egg product.

2.4.3. Spent medium

Cells of *S.* Typhimurium ATCC 14028s (wild type) and the *ΔiroB* and *ΔentC* mutants were inoculated (approx. 10 CFU/mL) in 10 mL of M9-broth (Sigma-Aldrich, M9) supplemented with magnesium sulfate (Panreac-AppliChem, Darmstadt, Germany) and glucose (Panreac-AppliChem), as indicated by the manufacturer, and incubated at 37 °C 24 h until cultures reached 10^2 and 10^6 CFU/mL. Bacterial suspensions were then centrifuged at 12,000 g for 20 min at 4 °C and the supernatant, the spent medium, was first filtrated with a 0.22 μm sterile cellulose filter and then through an Amicon ultra-15 mL centrifugal filter (MWCO = 3 kDa; Millipore). Approximately 9 mL of spent medium was obtained from a 10 mL culture. The filtrate was then dyalized using a previously washed cellulose ester (CE) dialysis membrane with a nominal cutoff of 100–500 Da (Spectra/Por®, Biotech CS, US). The sealed membrane was immersed in sterile distilled water (1:1000 ratio) and dialyzed for 9 h at 4 °C with gentle stirring (water was changed every 3 h). Before supplementation, the filtered and dialyzed spent medium was concentrated in a centrifuge vacuum dryer (GeneVac, Ltd, United Kingdom) at 30 °C until liquid was completely evaporated. The obtained pellet was resuspended in sterile distilled water to the desired concentration (1/100 of the initial volume), and was added to the egg samples (liquid whole egg). For certain experiments, non-filtered/dialyzed spent M9 medium or liquid whole egg spent filtered and dialyzed medium (following the same protocol) was used.

2.5. Growth curve fit and statistical analysis

All the determinations were carried out by triplicate in different working days. Growth curves were constructed by plotting the decimal logarithm of the number of *Salmonella* versus time under the different conditions assayed. Each point in the growth curve corresponds to the average value of all samples analyzed (at least three replicates). The curves obtained were fitted with the Baranyi and Roberts model (Baranyi and Roberts, 2000):

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

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

where Y_t is the Log_{10} of cell concentration at time t (CFU/mL); Y_0 is the Log_{10} of the initial cell concentration (CFU/mL); Y_{max} is the Log_{10} of maximum cell concentration (CFU/mL); μ_{max} is the maximum growth rate (Log_{10}/h); λ is the lag phase (h); and M and h_0 are curvature parameters, taking them as constant values, and with both set at a value of 10. For this purpose, we used GraphPad PRISM® statistical software (GraphPad Prism version 8.00 for Windows, GraphPad Software, San Diego, California, USA). The same software was used to calculate the goodness of fit parameters (R^2 and RMSE) and to carry out the statistical analysis (Student's t tests and ANOVA). Differences were considered significant for $p \leq 0.05$.

3. Results

3.1. Effect of initial concentration on the growth rates of Salmonella in liquid whole egg

The influence of the initial contamination dose of *Salmonella* Typhimurium ATCC 14028s (*S.* Typhimurium) on its growth rates in raw and pasteurized (60 °C/3.5 min or 70 °C/1.5 min) liquid whole egg was analyzed by obtaining growth curves starting at 10^2 (low dose) or 10^6 CFU/mL (high dose) in the three media (Fig. 1). The growth parameters calculated after fitting the curves with the Baranyi model are shown in Fig. 2. As can be observed in Fig. 2A and B, the initial inoculum dose significantly ($p < 0.05$) affected the growth parameters λ and μ_{max} calculated in raw liquid whole egg and in egg pasteurized at 60 °C for 3.5 min, but not in egg pasteurized at 70 °C for 1.5 min. Thus, the maximum growth rate in raw liquid whole egg determined for growth curves starting at a concentration of 10^6 CFU/mL (0.743 ± 0.022 log/h) was significantly ($p < 0.05$) higher than for curves starting at a concentration of 10^2 CFU/mL (0.605 ± 0.024 log/h). Significant differences ($p < 0.05$) were also found among the lag values calculated in raw liquid whole egg (4.62 ± 0.228 h *vs* 1.53 ± 0.123 h, for the curves starting at 10^2 and 10^6 CFU/mL, respectively). By contrast, no significant differences were found among the growth parameters λ and μ_{max} calculated in egg pasteurized at 70 °C for 1.5 min, regardless of the inoculum dose. The results obtained also indicate that increasing the intensity of the heat treatment applied to liquid whole egg led to a progressive decrease in the lag values and an increase in the μ_{max} for curves starting at 10^2 CFU/mL. Thus, when *S.* Typhimurium ATCC 14028s cells were inoculated (10^2 CFU/mL) into whole egg exposed to 60 °C for 3.5 min, they displayed intermediate values (0.689 ± 0.010 log/h and 2.23 ± 0.099 h for the μ_{max} and λ parameters, respectively) lying between those calculated in raw whole egg and egg pasteurized at 70 °C for 1.5 min (significantly different from both of them; $p < 0.05$). However, when inoculated with high doses (10^6 CFU/mL), no significant differences were found in the growth parameters determined for *S.* Typhimurium ATCC 14028s cells, regardless of the heat treatment applied to the liquid whole egg. Neither were significant differences found among the Y_{max}

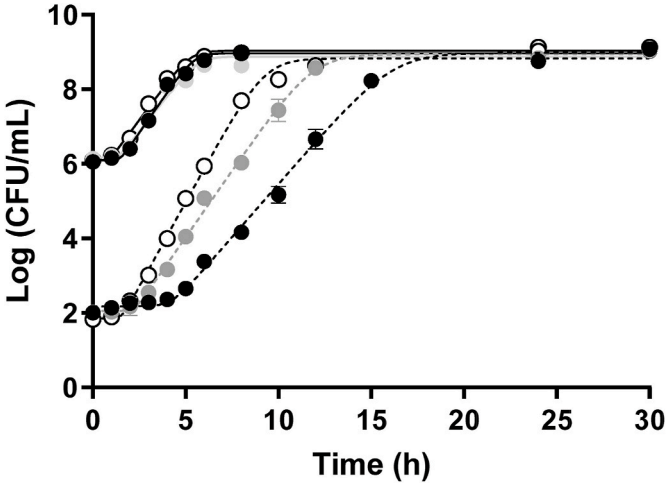

Fig. 1. Influence of the intensity of whole liquid egg pasteurization treatment and of the inoculum dose (10^2 and 10^6 CFU/mL) on the growth fitness of *S.* Typhimurium cells. Growth curves obtained in raw liquid whole egg (●), pasteurized whole egg at 60 °C 3.5 min (●), and pasteurized whole egg at 70 °C 1.5 min (o). Lines correspond to the fit of the Baranyi model to the experimental data. Error bars represent the standard deviation.

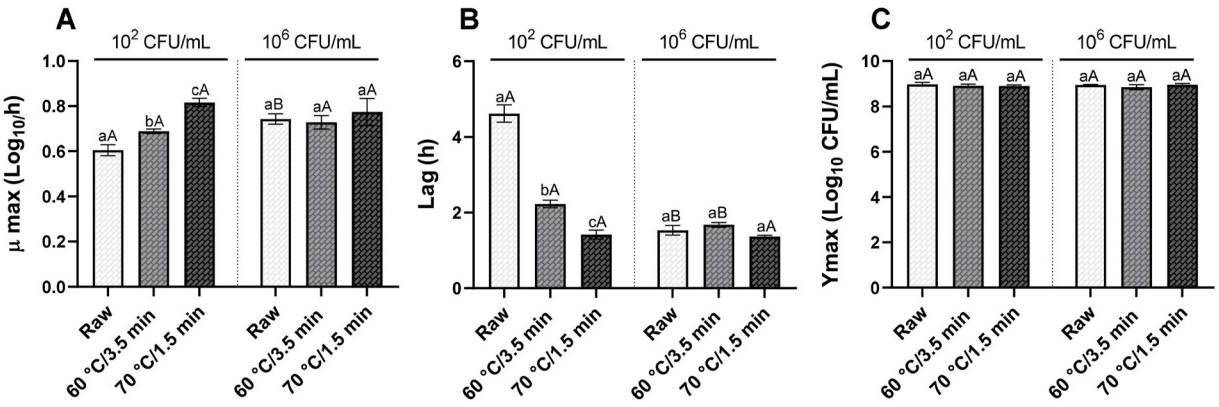

Fig. 2. Growth parameters of *S.* Typhimurium cells in raw and pasteurized (60 °C/3.5 and 70 °C/1.5 min) liquid whole egg when inoculated at 10^2 and 10^6 CFU/mL **(a)** μ_{max} (Log_{10}/h); (**b**) lag (h); (**c**) Y_{max} Log_{10} (CFU/mL) values calculated with the Baranyi model. Differences in the lower-case letters indicate statistically significant differences ($p < 0.05$) among the parameters determined in each growth medium (raw or pasteurized at 60 °C/3.5 or 70 °C/1.5 min). Differences in the upper-case letters indicate statistically significant differences ($p < 0.05$) among the parameters determined in each growth medium as a function of the starting dose (10^2 vs 10^6 CFU/mL).

values, regardless of the type of whole egg studied (raw *vs* pasteurized), and regardless of the inoculation dose (Fig. 2C). Results obtained in commercial pasteurized liquid whole egg were similar to those obtained in egg pasteurized at 70 °C for 1.5 min (Supplementary Material, Figure S1).

These results indicate that increasing the intensity of the heat treatment applied to liquid whole egg leads to a progressive disappearance of the initial dose dependence of *S.* Typhimurium ATCC 14028s growth fitness observed in the raw product.

3.2. *Effect of the supplementation of commercial pasteurized liquid whole egg with egg albumen proteins with known antimicrobial properties*

Since, as suggested in the introduction, the thermal inactivation of egg albumen proteins with antimicrobial activity might be the cause for the increased growth fitness of *Salmonella* cells in pasteurized liquid whole egg (as compared to raw egg), we designed a series of experiments to verify this hypothesis.

Raw and liquid whole egg pasteurized at different temperatures (60 °C/3.5 min and 70 °C 1.5 min) was supplemented after pasteurization with different antimicrobial egg albumen proteins (lysozyme, ovotransferrin, and Ex-FABP) at the concentrations at which they are usually present in liquid whole egg (see Material and Methods). The fitness of *S.* Typhimurium ATCC 14028s cells was subsequently determined and compared with that of a raw and pasteurized liquid whole egg without added proteins. Fig. 3 shows the effect of supplementation of those proteins in pasteurized liquid whole egg, measured as the increase in the number (in Log_{10}) of cells after 6 h of incubation at 37 °C ($LogN_6$-$LogN_0$). This length of time was selected because it was the one in which the difference in *Salmonella* counts between raw and pasteurized liquid whole egg (70 °C/1.5 min) was the greatest. Addition of lysozyme to pasteurized liquid whole egg (regardless of thermal treatment) did not cause a significant effect ($p > 0.05$) on the growth fitness of *Salmonella* cells (Supplementary Material, Figure S2). By contrast, the addition of ovotransferrin or Ex-FABP to pasteurized liquid whole egg resulted in a significantly lower increase ($p < 0.05$) compared to control (no protein added) in the number of *S.* Typhimurium ATCC 14028s cells after 6 h of incubation at 37 °C when eggs were inoculated at the lower dose (10^2 CFU/mL). Thus, for instance, in samples of liquid whole egg pasteurized at 70 °C for 1.5 min with added ovotransferrin or

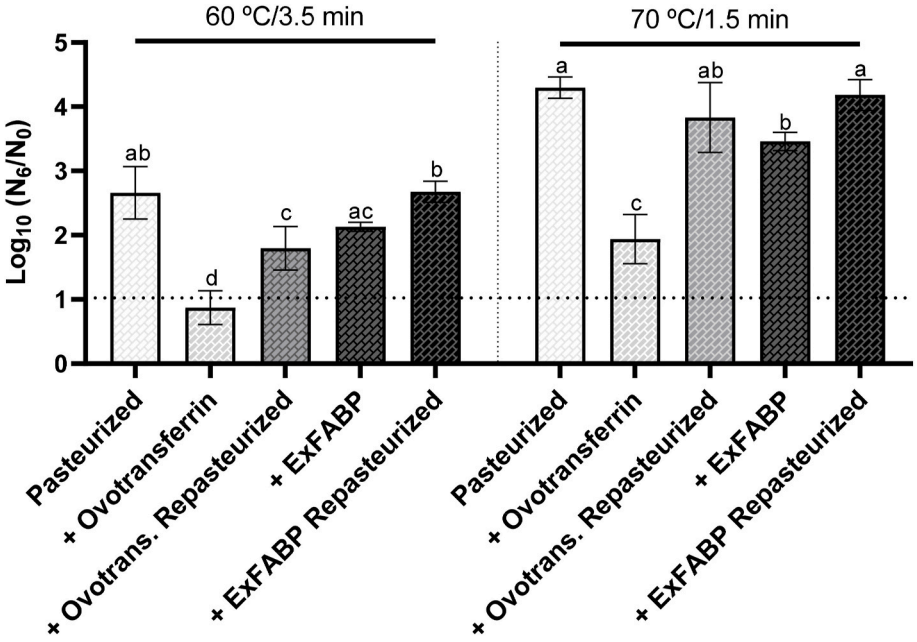

Fig. 3. Effect of the supplementation of ovotransferrin (8.80 mg/mL) or Ex-FABP (0.022 mg/mL) to whole liquid egg pasteurized under different conditions (60 °C/3.5 min and 70 °C/1.5 min) inoculated with 10^2 CFU/mL on the growth fitness of *S.* Typhimurium. Values correspond to the number of decimal log cycles of growth after 6 h at 37 °C (Log_{10} N_6/N_0). Figure also includes the values for raw whole liquid egg (1.02 Log_{10} N_6/N_0; Dashed line). Error bars represent the standard deviation, and different letters indicate statistically significant differences ($p < 0.05$) among the different conditions assayed for each type of pasteurized whole egg (60 °C/3.5 min and 70 °C/1.5 min).

Ex-FABP, the increase in the number of cells after 6 h of incubation at 37 °C was 2.36 and 0.84 log cycles, respectively, lower than the increase in the same medium without the addition of one of those proteins. Similar results were obtained in egg pasteurized at 60 °C for 3.5 min, although in this case the effect of Ex-FABP was not statistically significant ($p = 0.1$). Results nevertheless also indicated that *Salmonella* growth in raw liquid whole egg was still more restricted than in liquid whole egg pasteurized at 70 °C for 1.5 min supplemented with ovotransferrin or Ex-FABP. We therefore carried out a series of assays in which those antimicrobial proteins were supplemented together (ovotransferrin + lysozyme, ovotransferrin + Ex-FABP and ovotransferrin + EX-FABP + lysozyme), but results were not significantly different ($p > 0.05$) from those obtained when only ovotransferrin was added (Supplementary Material, Figure S2). Finally, it should be noted that supplementation of any of these proteins did not have any significant effect ($p > 0.05$) on *Salmonella* growth fitness when inoculated at the high dose of 10^6 CFU/mL (data not shown).

In order to ascertain the impact of thermal treatments on these proteins' antimicrobial activities, we also evaluated the growth ability of *S.* Typhimurium ATCC 14028s in pasteurized liquid whole egg (at 60 °C/3.5 min and at 70 °C/1.5 min) under each of the assayed conditions, supplemented with the same concentrations of proteins, and subsequently re-pasteurized (applying the same conditions). These additional assays were included because they might help to quantify the impact of thermal treatments of different intensity – and applied in the matrix itself – on the antimicrobial activity of ovotransferrin and Ex-FABP. Under most of the conditions assayed, the antimicrobial effect of both proteins was annulled when they were exposed to the pasteurization treatment, as can be observed in Fig. 3. This was true for both proteins treated at 70 °C/1.5 min, and also for Ex-FABP exposed to treatments at 60 °C/3.5 min, but not for ovotransferrin under the latter conditions. Thus, although a heat treatment at 60 °C for 3.5 min did reduce the antimicrobial activity of ovotransferrin (*Salmonella* growth rate increased), growth rate was still lower than in non-supplemented pasteurized egg at the same temperature ($p < 0.05$). Altogether, these results indicate that pasteurization treatments at 70 °C/1.5 min would inactivate ovotransferrin as well as Ex-FABP in liquid whole egg (at least to a high extent), but treatments at 60 °C/1.5 min would not. This strongly suggests that the observed differences in *Salmonella* fitness depending on the intensity of the thermal treatments applied to liquid whole egg might be related, at least to some extent, to the different effect that these thermal treatments would have on those two proteins: mainly, as will be discussed later, on ovotransferrin. Results obtained after carrying out the same experiments in commercial liquid whole egg (re-pasteurization conditions: 70 °C/1.5 min) were similar to those described for whole egg pasteurized at 70 °C for 1.5 (Supplementary Material, Figure S3).

In summary, these supplementation assays indicate that, at the concentrations found in liquid whole egg, ovotransferrin and Ex-FABP, but not lysozyme, would limit *S.* Typhimurium growth rates in liquid whole egg, and that this effect would only be observed when *Salmonella* cells are inoculated at a low dose. In addition, these results strongly suggest that the differences in *Salmonella* growth rates observed when it grows in raw or in pasteurized liquid whole egg might be linked, at least to some extent, to the thermal inactivation of ovotransferrin and Ex-FABP and consequently, to the existence of differences in iron bioavailability between raw and pasteurized egg. Although it cannot be ruled out that other physicochemical changes induced by pasteurization treatments might also be contributing to these differences in growth rates it should be noted that no significant differences ($p > 0.01$) were found among the pH values of liquid whole egg before and after the thermal treatments (Supplementary Material, Table S2).

3.3. Effect of iron and siderophore supplementation on liquid whole egg

In view of the previous results, we studied the influence of the addition of iron to raw and pasteurized liquid whole egg. Supplementation of raw liquid whole egg and liquid whole egg pasteurized at 60 °C for 3.5 min with increasing concentrations of ferric citrate resulted in an increase in the growth rate of *Salmonella* cells when inoculated at low dose (10^2 CFU/mL; Fig. 4). Nevertheless, results obtained also indicate that a lower amount of ferric citrate was required for *Salmonella* cells to match their growth rate in egg treated for 1.5 min at 70 °C when inoculated in liquid whole egg pasteurized at 60 °C for 3.5 min (approx. 0.0125 mg/mL) than when inoculated in raw liquid whole egg (>0.025 mg/mL).

On the other hand, no significant change in growth rates ($p > 0.05$) was observed when *Salmonella* cells were inoculated at the high dose (10^6 CFU/mL) in any of the three media (Fig. 1), or when ferric citrate was added to liquid whole egg treated for 1.5 min at 70 °C, regardless of the initial dose (Supplementary Material, Figure S4) and even at the highest concentration of ferric citrate tested (0.1 mg/mL; approximately the same amount of iron naturally present in raw whole egg).

These results reinforced the hypothesis that the lower growth rates of *Salmonella* cells in raw liquid whole egg when inoculated at the lower doses would be related to iron bioavailability. Thus, since ovotransferrin and Ex-FABP seemed to be playing a significant role in this phenomenon, we proceeded to study the influence of the addition of different concentrations of two siderophores, enterobactin and salmochelin, to raw and pasteurized liquid whole egg. Fig. 5 shows the effect of supplementation of different concentrations of salmochelin and enterobactin (from 0.05 ng/mL to 5.0 μg/mL) on the growth fitness of *S.* Typhimurium in raw liquid whole egg as well as in liquid whole egg pasteurized at 60 °C for 3.5 min inoculated at 10^2 CFU/mL. For both types of liquid whole egg, increasing the concentration of either enterobactin or salmochelin beyond a certain threshold resulted in an increased growth rate of the *Salmonella* cells. It should be noted that in both cases this threshold was lower for salmochelin than for enterobactin and that, as described for ferric citrate, the siderophore concentration required for *Salmonella* cells to reach a growth rate similar to that which they would display in egg pasteurized at 70 °C for 1.5 min was lower when *S.* Typhimurium ATCC 14028s cells were grown in egg pasteurized at 60 °C for 3.5 min than when grown in raw egg. As likewise described for ferric citrate, no effect was observed when siderophores were supplemented in either raw egg or in egg pasteurized at 70 °C for 1.5 min at the high dose, or in egg pasteurized at 70 °C for 1.5 min at the low dose (Supplementary Material, Figure S4). Similarly, addition of ferric citrate, enterobactin, or salmochelin to commercial

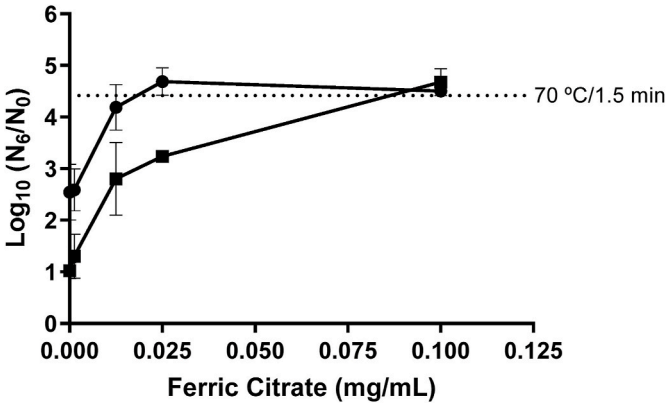

Fig. 4. Effect of the supplementation of different concentrations of ferric citrate (from 0.005 mg/mL to 0.1 mg/mL) on the growth fitness of *S.* Typhimurium in raw (■) and pasteurized (60 °C/3.5 min; ●) liquid whole egg inoculated with 10^2 CFU/mL. Values correspond to the number of decimal log cycles of growth after 6 h at 37 °C (Log_{10} N_6/N_0). Dashed line correspond to the values attained after the same incubation time/conditions in liquid whole egg pasteurized at 70 °C for 1.5 min (no ferric citrate added; 2.54 Log_{10} N_6/N_0). Error bars represent the standard deviation.

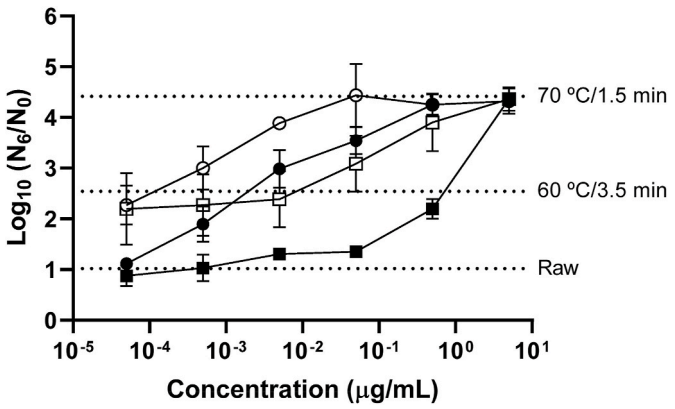

Fig. 5. Effect of the supplementation of different concentrations of salmochelin (circles: ●, ○) and enterobactin (squares: ■,□) (from 0.05 ng/mL to 5.0 μg/mL) on the growth fitness of *S.* Typhimurium in raw (filled symbols; ●, ■) and pasteurized (60 °C/3.5 min; empty symbols; ○*) liquid whole egg inoculated with 10^2 CFU/mL. Values correspond to the number of decimal log cycles of growth after 6 h at 37 °C (Log_{10} N_6/N_0). Dashed lines corresponds to the values attained after the same incubation time/conditions in raw (1.02 Log_{10} N_6/N_0) and pasteurized liquid whole egg (4.42 Log_{10} N_6/N_0 for 60 °C/3.5 min and 2.54 Log_{10} N_6/N_0 for 70 °C/1.5 min) with no salmochelin/enterobactin added. Error bars represent the standard deviation.

liquid egg did not significantly change the observed growth rates, regardless of the initial number of *Salmonella* cells inoculated (data not shown).

3.4. Effects of deletions in iron-uptake-related genes on the fitness (growth rate) of S. Typhimurium in liquid whole egg

In view of the results previously obtained, we selected 8 strains (*ΔentC, Δfes, ΔfeoB, ΔfhuC, ΔiroN, ΔmntH, ΔsitC* and *ΔzupT*) of *S.* Typhimurium ATCC 14028s with deletions in genes coding for proteins involved in different iron uptake systems, determined their growth rate in raw and pasteurized liquid whole egg starting at two different initial doses (10^2 CFU/mL and 10^6 CFU/mL), and compared them to that of the parental (wild type) strain.

The biosynthesis of the siderophore molecule enterobactin, and thus the synthesis of salmochelin molecule, is regulated by six enzymes (EntA-EntF) (Raymond et al., 2003; Rusnak et al., 1991). Therefore, deletion of *entC* makes *Salmonella* cells unable to produce enterobactin or salmochelin. The *iroN* gene encodes the outer membrane receptor of Fe^{3+} bound to salmochelin; if it is deleted, iron uptake by salmochelin will be inhibited, or at least reduced (Hantke et al., 2003). The enterobactin esterase Fes is an enzyme that cleaves iron-free enterobactin, since this mutant uses salmochelin as sole siderophore. In addition to these catecholate siderophores, *Salmonella* has ferric hydroxamate-type siderophores. The protein fhuC forms a complex with the other operon proteins, which, in turn, mediates the translocation of ferrichrome from the periplasm into the cytoplasm (Mademidis and Köster, 1998). Under anaerobic or reducing conditions, Fe^{2+} is the predominant form and is acquired via Fe^{2+} uptake systems (Ratledge and Dover, 2000), such as the FeoABC and MntH transporters. The *feoB* gene encodes an ATP-driven high-affinity transporter that pumps Fe^{2+} across the cytoplasmic membrane (Kammler et al., 1993; Lau et al., 2016); MntH is a divalent cation transport system with high affinity for Mn^{2+} and other divalent metal ions, including ferrous iron (Zaharik et al., 2004). Other complementary systems are the *sitABCD* operon, induced under iron-deficient conditions, which encodes a periplasmic binding protein-dependent ABC transport system that is specific for metal ions (Janakiraman and Slauch, 2000), and the ZupT transporter, a permease that preferentially allows entry of zinc, although the ability to uptake manganese, copper and iron has also been demonstrated (Cerasi et al., 2014).

As can be observed in Fig. 6, the *ΔiroN* and *ΔentC* strains displayed lower growth rates in raw liquid whole egg ($p < 0.05$) than the parental strain in raw liquid whole egg, and also displayed longer lag phases, although in this case only when egg was inoculated with 10^2 CFU/mL. By contrast, no significant differences ($p > 0.05$) among the growth parameters determined in raw egg for the other mutant strains and the parental one were observed (data not shown). It should also be noted that no significant differences were found in the Y_{max} values regardless of strain, initial dose, and/or type of liquid whole egg. On the other hand, no significant differences ($p > 0.05$) between the growth parameters determined for any of the tested strains and the parental strain were observed when grown in egg pasteurized at 70 °C for 1.5 min, regardless of the initial dose (data not shown).

In the light of these results, the fitness of those strains which displayed a reduced growth rate in raw liquid whole egg – i.e., those with deletions in genes related to salmochelin (*ΔiroN*) or salmochelin and enterobactin (*ΔentC)* uptake/synthesis – was also studied in liquid whole egg pasteurized under different conditions, and with or without the addition of Ex-FABP and ovotransferrin. Results obtained indicate that 1) deletion of *iroN* only affected *Salmonella* growth in those conditions in which Ex-FABP was naturally present (raw) or added to the pasteurized egg, and 2) deletion of *entC* caused a decrease in *Salmonella* growth rates under all the conditions assayed, but when grown in egg pasteurized at 60 °C for 3.5 min supplemented with ovotransferrin or in egg pasteurized at 70 °C for 1.5 m.

These results will be discussed in detail below, but they reinforce the hypothesis of the existence of a relationship between iron bioavailability and the dependence of *Salmonella* growth rates in liquid whole egg on its thermal history and the dose. They likewise highlight the relevant role played by egg albumen antimicrobial proteins (ovotransferrin and Ex-FABP), on the one hand, and *Salmonella* siderophores (salmochelin and enterobactin), on the other, in the growth of *Salmonella* in liquid whole egg.

3.5. Effect of supplementation with spent medium on the growth rates of S. Typhimurium in liquid whole egg

In order to obtain further insight into the dependence of *Salmonella* growth rates on initial dose in liquid whole egg, we performed a set of experiments in which different strains (ATCC 14028s and its isogenic mutants *Δfes* and *ΔiroN*) were grown in raw whole egg supplemented with filtered and dialyzed M9 spent medium (prepared as described in Materials and Methods) from parental, *ΔentC,* and *ΔiroB* cultures. Previous experiments showed that the filtered/dialyzed M9 spent medium obtained from *S.* Typhimurium ATCC 14028s increased the growth rates of this same strain inoculated to raw whole egg at a dose of 10^2 CFU/mL to the same extent as the non-filtered/dialyzed one, and that the same was true when the filtered/dialyzed spent medium was obtained from *S.* Typhimurium ATCC 14028s cells grown in raw liquid whole egg (Supplementary Material, Figure S5). Therefore, we carried out all the experiments with filtered/dialyzed M9 spent medium. These experiments also revealed that supplementation with spent medium to samples inoculated at 10^6 CFU/mL did not significantly ($p > 0.05$) modify *S.* Typhimurium ATCC 14028s growth rates.

As can be observed in Fig. 8, supplementation of raw whole egg with M9 spent medium obtained from *S.* Typhimurium ATCC 14028s cultures increased the growth rates of the three strains under study when inoculated at 10^2 CFU/mL. Thus, supplementation with that spent medium resulted, after 6 h, in an increase in 2.2, 2.4 and 1.8 Log_{10} cycles of growth for strain *S.* Typhimurium ATCC 14028s and its isogenic mutants *Δfes* and *ΔiroN*, respectively. This increase was significantly ($p < 0.05$) higher than that observed when the spent medium had been obtained from a *ΔiroB* culture, which cannot synthesize salmochelin, and also when it had been obtained from a *ΔentC* culture, which is unable to synthesize either salmochelin or enterobactin. In addition, no significant

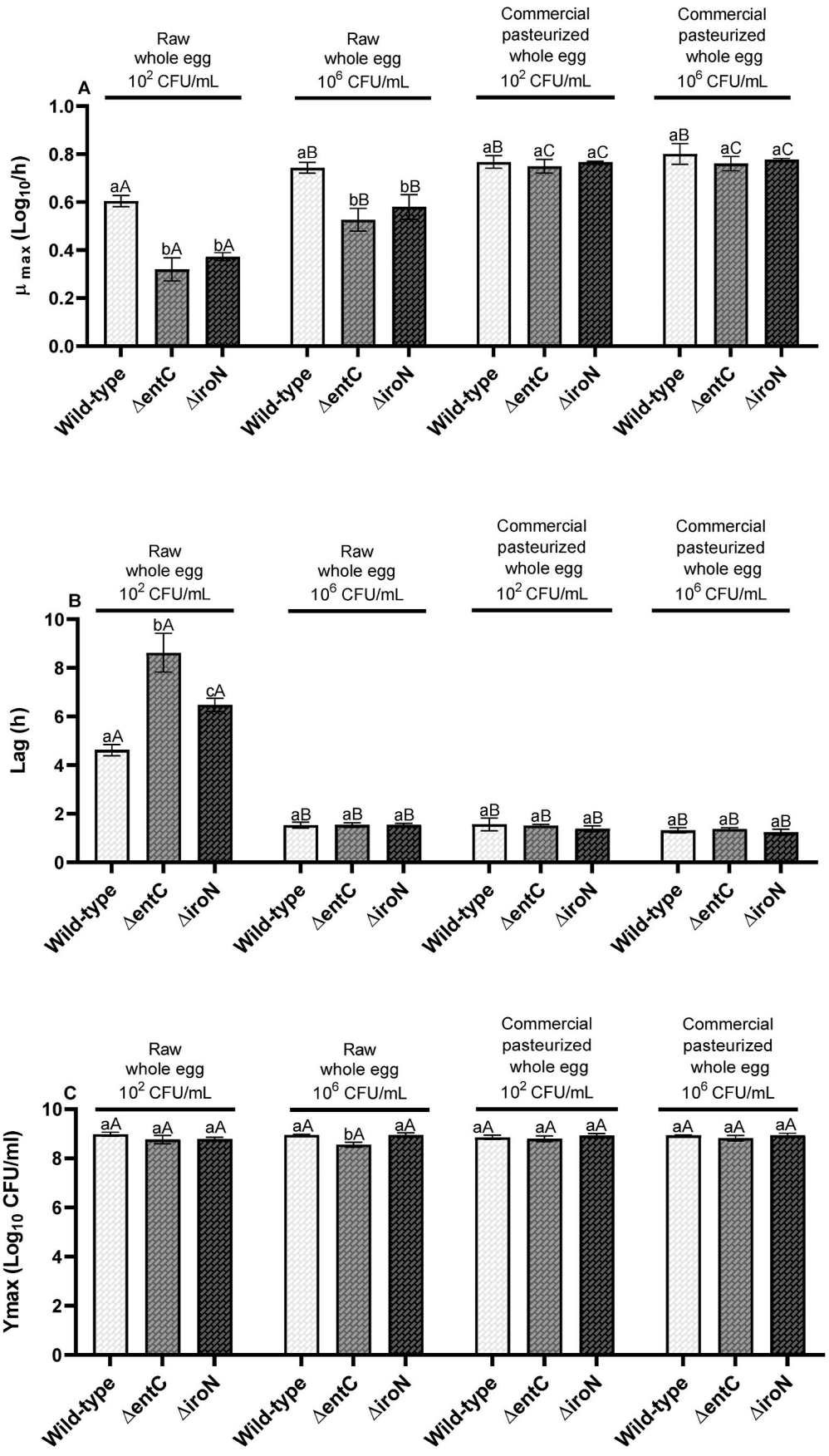

Fig. 6. Growth parameters of *S.* Typhimurium (Wild-type) cells and its isogenic mutants *ΔentC* and *ΔiroN*, in raw and pasteurized liquid whole egg (70 °C/1.5 min) and when inoculated at different doses (10^2 CFU/mL and 10^6 CFU/mL). (A) μ_{max} ($\log_{10}$/h), (B) lag (h) and (C) Y_{max} Log_{10} (CFU/mL) values obtained after the fit of the growth curves to the Baranyi model. Error bars represent the standard deviation. Differences in the lower-case letters indicate statistically significant differences ($p < 0.05$) between strains grown on the same media and conditions (starting dose). Differences in the upper-case letters indicate statistically significant differences ($p < 0.05$) among growth conditions (raw vs. pasteurized and initial dose) for each strain.

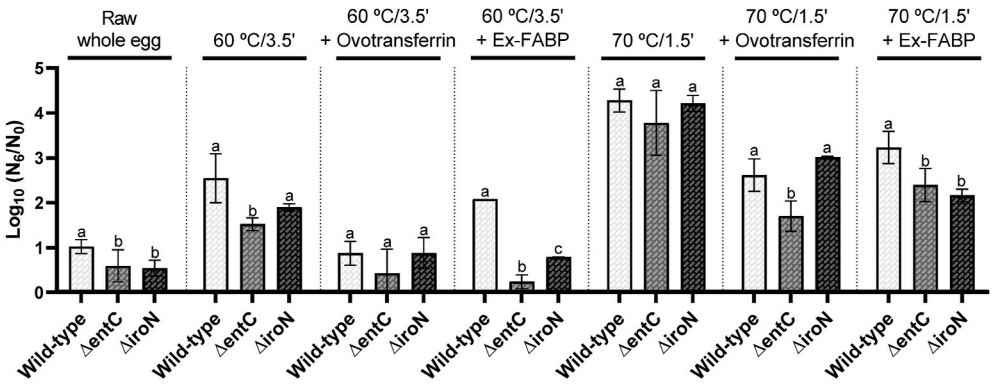

Fig. 7. Effect of the supplementation of ovotransferrin (8.80 mg/mL) or Ex-FABP (0.022 mg/mL) to whole liquid egg pasteurized under different conditions (60 °C/3.5 min and 70 °C/1.5 min) inoculated with 10^2 CFU/mL on the growth fitness *S.* Typhimurium (Wild-type) cells and its isogenic mutants *ΔentC* and *ΔiroN*. Values correspond to the number of decimal log cycles of growth after 6 h at 37 °C (Log_{10} N_6/N_0). Figure also includes the values for raw whole liquid egg. Error bars represent the standard deviation, and different letters indicate statistically significant differences ($p < 0.05$) among the different strains for each growth medium.

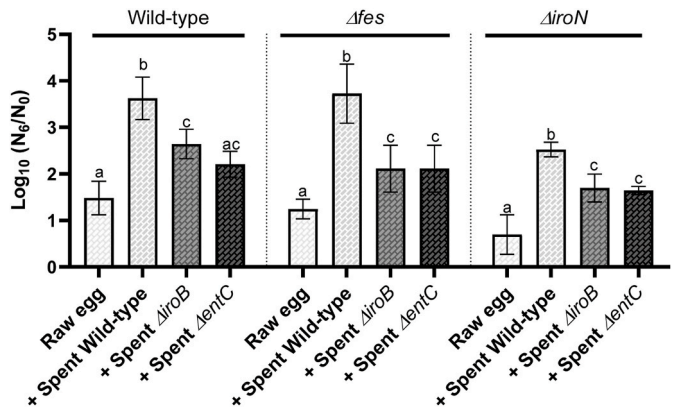

Fig. 8. Effect of the supplementation of filtered and dialyzed M9 spent medium (see text) obtained from *S.* Typhimurium (Wild-type), *ΔiroB* and *ΔentC* mutants to raw whole liquid egg inoculated with 10^2 CFU/mL on the fitness *S.* Typhimurium (wild-type) cells and its isogenic mutants *Δfes* and *ΔiroN*. Values correspond to the number of decimal log cycles of growth after 6 h at 37 °C (Log_{10} N_6/N_0). Figure also includes the values for raw whole liquid egg. Error bars represent the standard deviation, and different letters indicate statistically significant differences ($p < 0.05$) among the different growth conditions for each strain.

differences ($p > 0.05$) were found when strains where supplemented with spent medium only lacking salmochelin (from *ΔiroB* cultures) or also lacking enterobactin (from *ΔentC* cultures). However, the growth of the *Salmonella Δfes* and *ΔiroN* strains in raw whole egg supplemented with *ΔentC* and *ΔiroB* spent media was still significantly higher ($p < 0.05$) than without supplementation.

4. Discussion

Iron restriction has long been considered the major factor limiting bacterial growth in egg (Garibaldi, 1970; Schade and Caroline, 1944). Thus, although the concentration of iron in egg has been estimated to be between 3.6 and 18 μM, it is assumed that there would be no free iron in egg albumen, since it would be chelated by ovotransferrin. Ovotransferrin is a monomeric glycoprotein, a member of the transferrin family. It is composed of two homologous lobes: each lobe has an iron-binding site, the amino-terminal lobe (*N*-lobe) and the carboxyl-terminal lobe (C-lobe). Therefore, 1 mol of ovotransferrin is able to bind 2 mol of iron (Baron et al., 2016; Legros et al., 2021). It is thereby believed that main antimicrobial mechanism of egg against *Salmonella* is this chelator effect of ovotransferrin (Baron et al., 1997). It has been recently demonstrated that one of the three lipocalins identified in egg albumen, the Extracellular Fatty Acid-Binding Protein (Ex-FABP), is able to sequester some siderophores (molecules that bind and transport iron in microorganisms) (Guérin-Dubiard et al., 2006; Julien et al., 2020). Thus, Ex-FABP would be able to sequester enterobactin, but would display no activity against salmochelin (Julien et al., 2020). Therefore, Ex-FABP would also limit iron bioavailability through its ability to bind to siderophores.

Our results are consistent with this assumption, since they indicate that while lysozyme supplementation (at a concentration similar to that present in raw whole egg) did not have any impact on *Salmonella* growth rates in pasteurized liquid whole egg at 70 °C for 1.5 min, supplementation of ovotransferrin or EX-FABP (at their concentrations found in raw egg) to egg pasteurized under the same conditions did slow down *Salmonella* growth, at least when cells were inoculated at the lower dose (10^2 CFU/mL), whereby the effect of ovotransferrin was higher than that of Ex-FABP. In this sense, it can be assumed that none of the components present in liquid whole egg pasteurized at 70 °C for 1.5 min (as well as in the commercial liquid whole egg studied herein) are limiting *Salmonella* growth, since the growth rates reported here are similar (no significant differences; $p > 0.05$) to those obtained in a rich medium such as TSB-YE (Guillén et al., 2021). This would be either because their concentration is too low, or because they are denatured/inactivated. We should take into account that the same medium might still be limiting the growth of other microorganisms and/or other conditions (e.g. at a different temperature), but, as far as this investigation is concerned, the growth rate of *Salmonella* in liquid whole egg pasteurized at 70 °C for 1.5 min would be considered from here on as the maximum growth rate *Salmonella* can achieve under the conditions studied herein, and will be used as a reference. Furthermore, although it can be argued that the antimicrobial effect observed for ovotransferrin and Ex-FABP might be due to the presence of a higher (up to twofold) concentration of both proteins after supplementation (those naturally present + those supplemented), our results demonstrate that the application of a heat treatment of 1.5 min at 70 °C to both proteins and in liquid whole egg does suppress its antimicrobial activity (see below); therefore, our affirmation can be considered valid. Finally, these results also indicate that lysozyme activity would not be the cause of the differences in *Salmonella* growth rates observed between raw and pasteurized liquid whole egg, or between low and high initial doses in raw whole egg.

To overcome this limitation in iron bioavailability which is not exclusive to egg albumen (Dostal et al., 2014), *Salmonella* has developed different systems for the acquisition of iron from the host environment and from host-chelating proteins. These systems have been excellently reviewed in Andrews et al. (2003) and Wellawa et al. (2020). Basically, they can be classified into three groups: ferrous iron transport systems (which include the FeoABC transporter), metal-type ABC transporters (mainly the SitABCD, MntH, and ZupT transporters), and the ferric iron uptake system via siderophores. The main *Salmonella* siderophores are salmochelins, enterobactins and, to a minor extent, ferrichromes, all of which are characterized by their high affinity to iron. Thus, while the affinity constant (with iron) of ovotransferrin is in the range between 10^{14} and 10^{18} M^{-1} (Lin et al., 1994), most of the siderophores secreted

by *Salmonella* have affinity constants between 10^{30} and 10^{52} M^{-1} (Andrews et al., 2003). It has been demonstrated that all *Salmonella* serovars produce the catecholate siderophore enterobactin as well as the C-glucosylated enterobactin derivative salmochelin (Müller et al., 2009; Raymond et al., 2003) and that siderophore production genes are overexpressed in egg albumen (Clavijo et al., 2006; Huang et al., 2019). The relevance of those genes (specifically in egg) is associated with the fact that Ex-FABP can only sequester ferric-enterobactin, and is unable to bind salmochelin (Correnti et al., 2011). Thus, as observed by Julien et al. (2020), the addition of Ex-FABP at the concentration found in egg albumen (5 μM) in M9 medium caused a modest impact on growth; in contrast, the *ΔiroBC* mutant, which relied on enterobactin as the sole siderophore, showed a slower growth rate and yield (Julien et al., 2020). Our results also indicate that salmochelin plays a key role in *Salmonella* growth in egg since, among all the mutant strains tested (each of them with an impaired ability to use one of *Salmonella*'s major iron import systems), only those mutant strains (*ΔiroN* and *ΔentC*) not able to use salmochelin for importing iron displayed reduced growth rates in raw egg. This view is likewise supported by our results obtained from supplementation assays with salmochelin and enterobactin, which demonstrated that the concentration of salmochelin required by *Salmonella* to overcome the iron limitation imposed by egg albumen proteins was lower than that of enterobactin. Nevertheless, those two mutant strains were capable of growing in raw liquid whole egg, and of reaching the same yields, although more slowly. This indicates that other iron import systems can compensate for a lack of salmochelin or salmochelin + enterobactin synthesis/import and sustain *Salmonella* growth in raw whole egg. Conversely, they also indicate that the lack of any other individual iron import system would be entirely compensated (no effect on growth rates in raw whole egg) by *Salmonella* cells through the use of other ones.

Returning to our study's main objective, which was to study the mechanisms underlying the differences in growth rate of *Salmonella* cells in liquid whole egg as a function of the initial inoculum dose (in raw egg) and the egg's thermal history, our results strongly suggest that both phenomena would be linked to differences in iron bioavailability, as shall be discussed below.

Regarding the cause of the growth rate differences in raw whole egg depending on the inoculum dose, our supplementation experiments with spent M9 medium strongly suggest that the amount of siderophores released to the medium would substantially contribute to explain the differences in growth rate as a function of the initial dose in raw egg, since: 1) spent medium lacking siderophores (generated by a *ΔentC* mutant strain) had a significantly lower growth-promoting effect in raw whole egg and, 2) spent medium filtration + dialyzation assays indicate that the molecules responsible for the observed effect would have an approximate molecular weight between 100 and 3000 Da, consistent with the molecular weight of the siderophores salmochelin (1016 Da for S4) and enterobactin (669 Da) (Bister et al., 2004). They also indicate, once more, that salmochelin would play a predominant role, because, in addition to all we have indicated above, supplementation with the spent medium generated by the parental strain to *Salmonella Δfes* cells (unable to use enterobactin-bounded Fe) raised growth rates to the same extent as those of the parental strain, and supplementation of the parental strain with spent medium generated by a *ΔiroB* strain (unable to glycosylate enterobactin, and therefore unable to synthesize salmochelin) only led to a limited increase in *Salmonella* growth rates. Furthermore, supplementation with a spent medium lacking salmochelin as well as enterobactin (generated by a *ΔentC* mutant strain) induced a similar effect than that of a spent medium only containing enterobactin. In any case, our results also seem to indicate that in *Salmonella* spent medium there were molecules other than those two siderophores, which were capable of accelerating *Salmonella* growth in egg. Further work will be required in order to fully elucidate this point.

Therefore, and assuming that siderophore synthesis and uptake would be determining the growth rate of *Salmonella* cells in raw liquid whole egg, the results obtained in this study might be explained on the basis of the theory/model proposed by Scholz and Geenberger (2015). They observed a phenomenon quite similar to the one observed in our study: in their case, the growth of *E. coli* in a low-iron medium supplemented with the iron chelator transferrin was slower when cells were inoculated at low cell densities than at high ones. They also observed that when *Escherichia coli* and an isogenic enterobactin synthesis mutant (*ΔentF*) were inoculated in a low-iron medium at high cell densities, the *ΔentF* mutant could compete equally well with the wild type, but that at low cell densities it could not. This led them to propose a model in which at least a certain amount of enterobactin would remain associated with the cells that produce it, enabling iron acquisition even at very low cell density; whereby enterobactin that was not retained by producing cells at low density would be lost to dilution. Conversely, at high cell densities, cell-free enterobactin could accumulate and be shared by all cells in the group, which would lead to an increase in overall fitness (growth rates). Our scenario is admittedly somewhat more complex, with salmochelin and enterobactin as the siderophores used differently depending on the amount of inoculum, and with ovotransferrin and Ex-FABP limiting iron bioavailability; nevertheless, the fact that the growth-promoting effect of spent medium was observed when it was obtained from M9 cultures with a high amount of cells (10^6 CFU/mL), and not from cultures having achieved a cell density of only 10^2 CFU/mL, seems to indicate that the model proposed by Scholz and Geenberger (2015) would also be valid in explaining the results we obtained for the growth of *S.* Typhimurium ATCC 14028s in raw liquid whole egg. In any case, the *ΔiroN* and *ΔentC* mutants grew even more rapidly in raw whole egg when inoculated at 10^6 CFU/mL than at 10^2 CFU/mL, thereby indicating that other factors/molecules might also be at least partially responsible for the initial dose dependency of *Salmonella* growth in raw liquid whole egg. Further work will be required in other to fully elucidate this point.

On the other hand, and with regard to the influence of pasteurization on *Salmonella* growth rates in liquid whole egg, we wish to point out that the classic pasteurization treatments applied in the industry, 1–10 min at 60–72 °C, are limited due to the sensitivity of egg albumen proteins to heat treatments, which can lead to egg coagulation. Depending on a treatment's intensity, it can denature egg albumen proteins with antimicrobial properties such as ovotransferrin and lysozyme (Baron et al., 2016), as was already suggested as the potential cause of the higher growth fitness of *Salmonella* cells in pasteurized liquid whole egg as compared to raw egg (Guillén et al., 2021). Although we have only estimated the denaturation/inactivation of ovotransferrin and Ex-FABP indirectly through the study of their antimicrobial activity and have not calculated the percentage of denaturation/inactivation, our results indicate that both compounds would be denatured by heat (at least when treated at 70 °C for 1.5 min). This is consistent with previously published data reporting denaturation temperatures for ovotransferrin in the range between 60 and 75 °C (Li-Chan et al., 1995), and the melting temperature predicted for Ex-FABP using the SCooP algorithm (Pucci et al., 2017), which is in the same range (T_m = 67.9 °C).

In any case, our results also indicate that *Salmonella* growth in pasteurized liquid egg supplemented with ovotransferrin or Ex-FABP, or both, was still slightly faster than in raw egg. This suggests that there would be other components with antimicrobial activity that would result affected/denatured upon exposure of liquid whole egg to pasteurization conditions. However, other potential explanations cannot be ruled out, such as the existence of significant differences in antimicrobial activity between recombinant *Coturnix japonica* Ex-FABP (the one used for supplementation assays) and *Gallus* Ex-FABP, or between native and commercially available ovotransferrin. Our results nevertheless clearly demonstrate that the addition of iron or siderophores to raw egg is sufficient for *Salmonella* cells to reach their maximum growth rates in liquid whole egg, indicating that if there are other proteins/components contributing to the differences in *Salmonella* growth rates in raw and pasteurized liquid whole egg, they would also be related to iron

bioavailability. Given the functions of ovotransferrin and Ex-FABP (one as an iron scavenger and the second one as a siderophore sequester), the effect of their simultaneous addition (at the same concentrations as above) to liquid whole egg pasteurized at 70 °C for 1.5 min would be expected to be additive, but we did not observe that effect. Further work will be required to elucidate this point, taking into account that the effect of the addition of Ex-FABP alone was relatively small (even non-significant in egg pasteurized at 60 °C), and that addition of ovotransferrin alone led to *Salmonella* growth rates very similar to those in raw egg, which, together with the limitations of the plate count technique (it is estimated that the standard deviation of three replicates can account for considerably more than 10% of the mean value (Cebrián et al., 2015)) might also explain the results obtained.

Regarding the effect of the different pasteurization treatments on the antimicrobial activity of these proteins (ovotransferrin and Ex-FABP), results obtained for egg pasteurized at 60 °C (3.5 min) should be viewed with care, due to methodological reasons: mainly because of the low difference in growth rates between raw egg and egg pasteurized at 60 °C for 3.5 min (less than 12.27%), and also in view of the high variability of the plate count technique (see above). In any case, our results strongly suggest that a 60 °C/3.5 min treatment would only denature a fraction of ovotransferrin, since the growth rate of *Salmonella* cells in pasteurized egg (60 °C/3.5 min) supplemented with ovotransferrin exposed to the same treatment conditions did not reach the growth rate in non-supplemented pasteurized (60 °C/3.5 min) whole egg, thereby implying that ovotransferrin still retained a certain degree of antimicrobial activity. This is consistent with the results reported by Baron et al. (2003), who observed that the loss of bacteriostatic activity of transferrin depended on the intensity of the treatment, although the ones they studied were more intense (15 days at 67 or 75 °C). This would imply that the amount of active ovotransferrin might be one of the causes, if not the major one, for the observed differences in *Salmonella* growth rates between raw and pasteurized liquid whole egg at different temperatures. On the other hand, results obtained in our study suggest that a heat treatment of 60 °C for 3.5 min would significantly affect Ex-FABP antimicrobial activity, yet they also indicate that the effect of the supplementation of Ex-FABP on egg pasteurized at 60 °C for 3.5 min was non-significant ($p > 0.05$). These contradictory results might be explained on the basis of the methodological limitations discussed above, but the fact that the concentration of salmochelin required by *Salmonella* to overcome the iron limitation imposed by egg albumen proteins in whole egg pasteurized at 60 °C for 3.5 min was lower than that of enterobactin strongly suggests that Ex-FABP was not denatured after treatments at 60 °C for 3.5 min. Alternatively, it can be hypothesized that egg might contain other proteins (still not reported) capable of sequestering enterobactin. Further work will be required to clarify the effect of these low-intensity pasteurization treatments on Ex-FABP activity. Nevertheless, this is, to the best of our knowledge, the first time that the effect of heat treatments on the antimicrobial activity of Ex-FABP has been explored; from our results it can be clearly established that at least pasteurization treatments at 70 °C for 1.5 min do affect its antimicrobial activity against *S.* Typhimurium ATCC 14028s.

Altogether, these results strongly suggest that denaturation of proteins limiting iron bioavailability (including ovotransferrin and Ex-FABP) would be the cause of the faster growth of *Salmonella* cells in pasteurized whole egg, and that the different degree of denaturation they would suffer after pasteurization treatments of different intensity would explain the differences in growth observed in liquid whole egg pasteurized under different conditions, as pointed out above. Regarding the first point, it should be noted that although ovotransferrin has an additional antibacterial activity independent of its iron-restricting activity (Aguilera et al., 2003; Ellison et al., 1988), Baron et al. (2003) already reported that, at least in the range of temperatures they studied (67–75 °C, which is close to ours), the loss of bacteriostatic activity attributable to thermal denaturation of ovotransferrin was due to a reduction of its iron-chelating activity. This provides further support for our hypothesis that the increased growth rate obtained in pasteurized egg would mainly be linked to an increase in iron bioavailability. Regarding the second point, our supplementation assays with iron and siderophores also demonstrated that iron bioavailability progressively increases as the intensity of the treatment is raised, since the maximum growth rates in liquid whole egg were achieved in egg pasteurized at 70 °C for 1.5 min without requiring the addition of any of those compounds, and the concentrations required in egg pasteurized at 60 °C for 3.5 min were lower than in raw egg.

Furthermore, on the basis of all that has been discussed above, the following model would simultaneously explain the all results here reported:

Iron bioavailability would be limited in raw liquid whole egg mainly due to the presence of ovotransferrin and Ex-FAPB. In this medium, *Salmonella* cells require the use of siderophores (especially salmochelin) for iron uptake and in order to reach their maximum growth potential (rate). When *Salmonella* cells are present at low cell density in raw whole egg, those siderophores have a privative effect, and growth would be slow. However, if they are present/inoculated at a sufficiently high density, siderophores are released to the medium; the antimicrobial (iron limiting) systems present in raw liquid whole egg would be overcome, and *Salmonella* would grow at their maximum growth rates (in this case, raw liquid whole egg would not be a growth-limiting medium). Application of heat treatments of increasing intensity would cause a progressive denaturation of those iron-restricting proteins (mainly of ovotransferrin), which would allow *Salmonella* cells to uptake iron by other systems, leading to the disappearance of the initial dose dependence of *Salmonella* growth rates.

5. Conclusions

In summary, our results strongly suggest that iron bioavailability determines the fitness (growth rates) of *Salmonella* cells in liquid whole egg. Thus, the higher the intensity of the thermal treatment applied to the liquid whole egg, the more iron will be available, a phenomenon that would be linked to the denaturation of iron and/or siderophore-binding egg proteins. On the other hand, further work is still required to fully elucidate why lower *Salmonella* initial doses lead to lower growth rates in raw whole egg, but this might be related to the different use (private vs shared) of siderophores on the part of *Salmonella,* depending on the number of cells present in the medium. The present study contributes to a better understanding of the physiology of *Salmonellae,* as well as of the effect of thermal treatments on food products.

Author Contributions

Silvia Guillén; Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, **Guillermo Cebrián**; Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition

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Declaration of competing interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2022.104008.

References

Aguilera, O., Quiros, L.M., Fierro, J.F., 2003. Transferrins selectively cause ion efflux through bacterial and artificial membranes. FEBS Lett. 548, 5–10. https://doi.org/10.1016/s0014-5793(03)00719-1.

Andrews, S.C., Robinson, A.K., Rodríguez-Quiñones, F., 2003. Bacterial iron homeostasis. FEMS Microbiol. Rev. 27, 215–237. https://doi.org/10.1016/S0168-6445(03)00055-X.

Baranyi, J., Roberts, T.A., 2000. Principles and application of predictive modeling of the effects of preservative factors on microorganisms. In: Lund, B., Baird-Parker, T.C., Gould, G.W. (Eds.), Microbiological Safety and Quality of Food. Aspen Publishers, Gaithersburg, Maryland, pp. 342–358.

Baron, F., Bonnassie, S., Alabdeh, M., Cochet, M.-F., Nau, F., Guérin-Dubiard, C., Gautier, M., Andrews, S.C., Jan, S., 2017. Global gene-expression analysis of the response of *Salmonella* Enteritidis to egg white exposure reveals multiple egg white-imposed stress responses. Front. Microbiol. 8 https://doi.org/10.3389/fmicb.2017.00829.

Baron, F., Gautier, M., Brulé, G., 1999. Rapid growth of *Salmonella* Enteritidis in egg white reconstituted from industrial egg white powder. J. Food Protect. 62, 585–591. https://doi.org/10.4315/0362-028x-62.6.585.

Baron, F., Gautier, M., Brule, G., 1997. Factors involved in the inhibition of growth of *Salmonella enteritidis* in liquid egg white. J. Food Protect. 60, 1318–1323. https://doi.org/10.4315/0362-028X-60.11.1318.

Baron, F., Nau, F., Guérin-Dubiard, C., Bonnassie, S., Gautier, M., Andrews, S.C., Jan, S., 2016. Egg white versus *Salmonella* Enteritidis! A harsh medium meets a resilient pathogen. Food Microbiol. 53, 82–93. https://doi.org/10.1016/j.fm.2015.09.009.

Baron, F., Nau, F., Guérin-Dubiard, C., Gonnet, F., Dubois, J.-J., Gautier, M., 2003. Effect of dry heating on the microbiological quality, functional properties, and natural bacteriostatic ability of egg white after reconstitution. J. Food Protect. 66, 825–832. https://doi.org/10.4315/0362-028x-66.5.825.

Bister, B., Bischoff, D., Nicholson, G.J., Valdebenito, M., Schneider, K., Winkelmann, G., Hantke, K., Süssmuth, R.D., 2004. The structure of salmochelins: C-glucosylated enterobactins of *Salmonella enterica*. Biometals 17, 471–481. https://doi.org/10.1023/b:biom.0000029432.69418.6a.

Cebrián, G., Mañas, P., Condón, S., 2015. Relationship between membrane permeabilization and sensitization of *S. aureus* to sodium chloride upon exposure to Pulsed Electric Fields. Innovat. Food Sci. Emerg. Technol. 32, 91–100. https://doi.org/10.1016/j.ifset.2015.09.017.

Cerasi, M., Liu, J.Z., Ammendola, S., Poe, A.J., Petrarca, P., Pesciaroli, M., Pasquali, P., Raffatellu, M., Battistoni, A., 2014. The ZupT transporter plays an important role in zinc homeostasis and contributes to *Salmonella enterica* virulence. Metallomics 6, 845–853. https://doi.org/10.1039/c3mt00352c.

Chart, H., Rowe, B., 1993. Iron restriction and the growth of *Salmonella enteritidis*. Epidemiol. Infect. 110, 41–47. https://doi.org/10.1017/S0950268800050664.

Clavijo, R.I., Loui, C., Andersen, G.L., Riley, L.W., Lu, S., 2006. Identification of genes associated with survival of *Salmonella enterica* serovar Enteritidis in chicken egg albumen. Appl. Environ. Microbiol. 72, 1055–1064. https://doi.org/10.1128/AEM.72.2.1055-1064.2006.

Conesa, R., Periago, P.M., Esnoz, A., López, A., Palop, A., 2003. Prediction of *Bacillus subtilis* spore survival after a combined non-isothermal-isothermal heat treatment. Eur. Food Res. Technol. 217, 319–324. https://doi.org/10.1007/s00217-003-0749-5.

Correnti, C., Clifton, M.C., Abergel, R.J., Allred, B., Hoette, T.M., Ruiz, M., Cancedda, R., Raymond, K.N., Descalzi, F., Strong, R.K., 2011. Galline Ex-FABP is an antibacterial siderocalin and a lysophosphatidic acid sensor functioning through dual ligand specificities. Structure 19, 1796–1806. https://doi.org/10.1016/j.str.2011.09.019.

De Reu, K., Grijspeerdt, K., Messens, W., Heyndrickx, M., Uyttendaele, M., Debevere, J., Herman, L., 2006. Eggshell factors influencing eggshell penetration and whole egg contamination by different bacteria, including *Salmonella enteritidis*. Int. J. Food Microbiol. 112, 253–260. https://doi.org/10.1016/j.ijfoodmicro.2006.04.011.

Dostal, A., Gagnon, M., Chassard, C., Zimmermann, M.B., O'Mahony, L., Lacroix, C., 2014. *Salmonella* adhesion, invasion and cellular immune responses are differentially affected by iron concentrations in a combined *in vitro* gut fermentation-cell model. PLoS One 9, e93549. https://doi.org/10.1371/journal.pone.0093549.

EFSA, (European Food Safety Authority), 2021. The European Union one health 2019 zoonoses report. EFSA J. 19, e06406 https://doi.org/10.2903/j.efsa.2021.6406.

Ellison, R.T., Giehl, T.J., LaForce, F.M., 1988. Damage of the outer membrane of enteric gram-negative bacteria by lactoferrin and transferrin. Infect. Immun. 56, 2774–2781. https://doi.org/10.1128/iai.56.11.2774-2781.1988.

Gantois, I., Ducatelle, R., Pasmans, F., Haesebrouck, F., Gast, R., Humphrey, T.J., Van Immerseel, F., 2009. Mechanisms of egg contamination by *Salmonella* Enteritidis. FEMS Microbiol. Rev. 33, 718–738. https://doi.org/10.1111/j.1574-6976.2008.00161.x.

Garibaldi, J.A., 1970. Role of microbial iron transport compounds in bacterial spoilage of eggs. Appl. Microbiol. 20, 558–560. https://doi.org/10.1128/am.20.4.558-560.1970.

Guérin-Dubiard, C., Pasco, M., Mollé, D., Désert, C., Croguennec, T., Nau, F., 2006. Proteomic analysis of hen egg white. J. Agric. Food Chem. 54, 3901–3910. https://doi.org/10.1021/jf0529969.

Guillén, S., Marcén, M., Álvarez, I., Mañas, P., Cebrián, G., 2021. Influence of the initial cell number on the growth fitness of *Salmonella* Enteritidis in raw and pasteurized liquid whole egg, egg white, and egg yolk. Foods 10, 1621. https://doi.org/10.3390/foods10071621.

Guillén, S., Marcén, M., Álvarez, I., Mañas, P., Cebrián, G., 2020a. Influence of the initial cell number on the growth fitness of *Salmonella* Enteritidis in raw and pasteurized liquid whole egg. Proceedings 70, 21. https://doi.org/10.3390/foods_2020-07661.

Guillén, S., Marcén, M., Mañas, P., Cebrián, G., 2020b. Differences in resistance to different environmental stresses and non-thermal food preservation technologies among *Salmonella enterica* subsp. *enterica* strains. Food Res. Int. 132, 109042. https://doi.org/10.1016/j.foodres.2020.109042.

Gurtler, J.B., Conner, D.E., 2009. Survival and growth of *Salmonella* Enteritidis in liquid egg products varying by temperature, product composition, and carbon dioxide concentration. Foodb. Pathog. Dis. 6, 561–567. https://doi.org/10.1089/fpd.2008.0202.

Guyot, N., Réhault-Godbert, S., Slugocki, C., Harichaux, G., Labas, V., Helloin, E., Nys, Y., 2016. Characterization of egg white antibacterial properties during the first half of incubation: a comparative study between embryonated and unfertilized eggs. Poult. Sci. 95, 2956–2970. https://doi.org/10.3382/ps/pew271.

Hantke, K., Nicholson, G., Rabsch, W., Winkelmann, G., 2003. Salmochelins, siderophores of *Salmonella enterica* and uropathogenic *Escherichia coli* strains, are recognized by the outer membrane receptor IroN. Proc. Natl. Acad. Sci. U.S.A. 100, 3677–3682. https://doi.org/10.1073/pnas.0737682100.

Huang, X., Zhou, X., Jia, B., Li, N., Jia, J., He, M., He, Y., Qin, X., Cui, Y., Shi, C., Liu, Y., Shi, X., 2019. Transcriptional sequencing uncovers survival mechanisms of *Salmonella enterica* serovar Enteritidis in antibacterial egg white. mSphere 4. https://doi.org/10.1128/mSphere.00700-18.

Jakočiūnė, D., Bisgaard, M., Hervé, G., Protais, J., Olsen, J.E., Chemaly, M., 2014. Effects of environmental conditions on growth and survival of *Salmonella* in pasteurized whole egg. Int. J. Food Microbiol. 184, 27–30. https://doi.org/10.1016/j.ijfoodmicro.2014.03.015.

Janakiraman, A., Slauch, J.M., 2000. The putative iron transport system SitABCD encoded on SPI1 is required for full virulence of *Salmonella typhimurium*. Mol. Microbiol. 35, 1146–1155. https://doi.org/10.1046/j.1365-2958.2000.01783.x.

Julien, L.A., Fau, C., Baron, F., Bonnassie, S., Guérin-Dubiard, C., Nau, F., Gautier, M., Karatzas, K.A., Jan, S., Andrews, S.C., 2020. The three lipocalins of egg-white: only Ex-FABP inhibits siderophore-dependent iron sequestration by *Salmonella* Enteritidis. Front. Microbiol. 11 https://doi.org/10.3389/fmicb.2020.00913.

Kammler, M., Schön, C., Hantke, K., 1993. Characterization of the ferrous iron uptake system of *Escherichia coli*. J. Bacteriol. 175, 6212–6219. https://doi.org/10.1128/jb.175.19.6212-6219.1993.

Kang, M.S., Park, J.H., Kim, H.J., 2021. Predictive modeling for the growth of *Salmonella* spp. in liquid egg white and application of scenario-based risk estimation. Microorganisms 9, 486. https://doi.org/10.3390/microorganisms9030486.

Keller, L.H., Benson, C.E., Krotec, K., Eckroade, R.J., 1995. *Salmonella enteritidis* colonization of the reproductive tract and forming and freshly laid eggs of chickens. Infect. Immun. 63, 2443–2449. https://doi.org/10.1128/IAI.63.7.2443-2449.1995.

Kim, Y.-J., Moon, H.-J., Lee, S.-K., Song, B.-R., Lim, J.-S., Heo, E.-J., Park, H.-J., Wee, S.-H., Moon, J.-S., 2018. Development and validation of predictive model for *Salmonella* growth in unpasteurized liquid eggs. Korean J. Food Sci. Anim. Resour. 38, 442–450. https://doi.org/10.5851/kosfa.2018.38.3.442.

Lau, C.K.Y., Krewulak, K.D., Vogel, H.J., 2016. Bacterial ferrous iron transport: the Feo system. FEMS Microbiol. Rev. 40, 273–298. https://doi.org/10.1093/femsre/fuv049.

Lechevalier, V., Guérin-Dubiard, C., Anton, M., Beaumal, V., David Briand, E., Gillard, A., Le Gouar, Y., Musikaphun, N., Tanguy, G., Pasco, M., Dupont, D., Nau, F., 2017. Pasteurisation of liquid whole egg: optimal heat treatments in relation to its functional, nutritional and allergenic properties. J. Food Eng. 195, 137–149. https://doi.org/10.1016/j.jfoodeng.2016.10.007.

Legros, J., Jan, S., Bonnassie, S., Gautier, M., Croguennec, T., Pezennec, S., Cochet, M.-F., Nau, F., Andrews, S.C., Baron, F., 2021. The role of ovotransferrin in egg-white antimicrobial activity: a review. Foods 10, 823. https://doi.org/10.3390/foods10040823.

Li-Chan, E.C.Y., Powrie, W.D., Nakai, S., 1995. The chemistry of eggs and egg products. In: Stadelman, W.J., Cotterill, O.J. (Eds.), Egg Science and Technology. The Haworth Press Inc., 10 Alice St., Binghamton, NY 13904-1580, pp. 105–175.

Lin, L.N., Mason, A.B., Woodworth, R.C., Brandts, J.F., 1994. Calorimetric studies of serum transferrin and ovotransferrin. Estimates of domain interactions, and study of the kinetic complexities of ferric ion binding. Biochemistry 33, 1881–1888. https://doi.org/10.1021/bi00173a035.

Mademidis, A., Köster, W., 1998. Transport activity of FhuA, FhuC, FhuD, and FhuB derivatives in a system free of polar effects, and stoichiometry of components involved in ferrichrome uptake. Mol. Gen. Genet. 258, 156–165. https://doi.org/10.1007/s004380050718.

Messens, W., Duboccage, L., Grijspeerdt, K., Heyndrickx, M., Herman, L., 2004. Growth of *Salmonella* serovars in hens' egg albumen as affected by storage prior to inoculation. Food Microbiol. 21, 25–32. https://doi.org/10.1016/S0740-0020(03)00045-5.

Messens, W., Grijspeerdt, K., Herman, L., 2005. Eggshell penetration by *Salmonella*: a review. Worlds Poultry Sci. J. 61, 71–86. https://doi.org/10.1079/WPS200443.

Moon, H.J., Lim, J.G., Yoon, K.S., 2016. Comparative study of change in *Salmonella* Enteritidis and *Salmonella* Typhimurium populations in egg white and yolk. J. Food Hyg. Saf. 31, 342–348. https://doi.org/10.13103/JFHS.2016.31.5.342.

Müller, S.I., Valdebenito, M., Hantke, K., 2009. Salmochelin, the long-overlooked catecholate siderophore of *Salmonella*. Biometals 22, 691–695. https://doi.org/10.1007/s10534-009-9217-4.

Ng, H., Garibaldi, J.A., Ijichi, K., Mihara, K.L., 1979. Pasteurization of salted whole egg inoculated with *Arizona* or *Salmonella*. Appl. Environ. Microbiol. 37, 1091–1095. https://doi.org/10.1128/aem.37.6.1091-1095.1979.

Pucci, F., Kwasigroch, J.M., Rooman, M., 2017. SCooP: an accurate and fast predictor of protein stability curves as a function of temperature. Bioinformatics 33, 3415–3422. https://doi.org/10.1093/bioinformatics/btx417.

Ratledge, C., Dover, L.G., 2000. Iron metabolism in pathogenic bacteria. Annu. Rev. Microbiol. 54, 881–941. https://doi.org/10.1146/annurev.micro.54.1.881.

Raymond, K.N., Dertz, E.A., Kim, S.S., 2003. Enterobactin: an archetype for microbial iron transport. Proc. Natl. Acad. Sci. U.S.A. 100, 3584–3588. https://doi.org/10.1073/pnas.0630018100.

Rusnak, F., Sakaitani, M., Drueckhammer, D., Reichert, J., Walsh, C.T., 1991. Biosynthesis of the *Escherichia coli* siderophore enterobactin: sequence of the *entF* gene, expression and purification of EntF, and analysis of covalent phosphopantetheine. Biochemistry 30, 2916–2927. https://doi.org/10.1021/bi00225a027.

Schade, A.L., Caroline, L., 1944. Raw hen egg white and the role of iron in growth inhibition of *Shigella dysenteriae, Staphylococcus aureus, Escherichia coli* and *Saccharomyces cerevisiae*. Science 100, 14–15. https://doi.org/10.1126/science.100.2584.14.

Scholz, R.L., Greenberg, E.P., 2015. Sociality in *Escherichia Coli*: enterochelin is a private good at low cell density and can be shared at high cell density. J. Bacteriol. 197, 2122–2128. https://doi.org/10.1128/JB.02596-14.

Timoney, J.F., Shivaprasad, H.L., Baker, R.C., Rowe, B., 1989. Egg transmission after infection of hens with *Salmonella enteritidis* phage type 4. Vet. Rec. 125, 600–601.

Wellawa, D.H., Allan, B., White, A.P., Köster, W., 2020. Iron-uptake systems of chicken-associated *Salmonella* serovars and their role in colonizing the avian host. Microorganisms 8, 1203. https://doi.org/10.3390/microorganisms8081203.

Zaharik, M.L., Cullen, V.L., Fung, A.M., Libby, S.J., Kujat Choy, S.L., Coburn, B., Kehres, D.G., Maguire, M.E., Fang, F.C., Finlay, B.B., 2004. The *Salmonella enterica* serovar Typhimurium divalent cation transport systems MntH and SitABCD are essential for virulence in an *Nramp1G169* murine typhoid model. Infect. Immun. 72, 5522–5525. https://doi.org/10.1128/IAI.72.9.5522-5525.2004.