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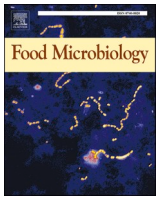

Antimicrobial defenses of table eggs: Importance of antibacterial proteins in egg white as a function of hen age in an extended production cycle

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ABSTRACT

The importance of egg natural defences to prevent bacterial contamination and their relation with hen age in extended production cycles were evaluated. Egg-white from eggs of different hen age groups (up 100-weeks-old) and lines (Hy-Line white and brown) were inoculated with Gram-positive *Staphylococcus aureus* or Gram-negative *Salmonella Typhimurium*, ranging from 10^3-10^6 CFU/mL. Our results show that concentrations of egg-white lysozyme and, particularly, ovotransferrin are important to modulate bacterial survival in a dose-dependent matter. Depending on protein concentration, their effect ranges from bactericidal to bacteriostatic, with a threshold for bacterial contamination that depends also on hen age and line. The concentrations of lysozyme and ovotransferrin increased with hen age (up to 2 and 22 w/w% of total protein, respectively), and eggs laid by older hens exhibited the greatest potential to prevent the growth of the highest *Salmonella* inoculum (10^6 CFU/mL). *Salmonella*-penetration experiments demonstrated that non-contaminated eggs display significantly higher concentrations of antimicrobial proteins. However, eggs from older hens needed a higher concentration of these proteins (>20% ovotransferrin) to prevent bacterial contamination, showing that antimicrobial protein concentrations in egg-whites was not the only factor influencing bacterial contamination. Finally, this study demonstrated that egg-white of eggs produced by old hens are less prone to contamination by *Salmonella*.

1. Introduction

The chicken egg is one of the most complete and inexpensive sources of protein, lipids and vitamins in the human diet (Réhault-Godbert et al., 2019). However, eggs are also a common vehicle for food-borne diseases (i.e., salmonellosis) (EFSA, 2018; Gantois et al., 2009). In the European Union (EU), nearly 100,000 human cases of salmonellosis are reported each year, and over 1 million in the United States, although these numbers are declining due to a variety of regulatory and sanitation protocols (CDC, 2021; EFSA, 2018). The potential for transfer of *Salmonella* spp. from the contaminated eggshell surface to the egg interior, with heightened risk to the consumer, has been underlined (Luber, 2009).

The egg contents are protected against physical impact, dehydration, and microbial contamination by the eggshell, a thin mineral layer (about 0.35 mm thick in chickens), which is pierced by respiratory pores that allow gaseous exchange (Hincke et al., 2012). The eggshell surface is covered by the cuticle, a very thin organic coating, rich in antimicrobial proteins, that seals the respiratory pores and acts as an effective physical and chemical barrier against bacterial penetration through the eggshell (Bain et al., 2013; De Reu et al., 2006; Kulshreshtha et al., 2018). There are two main mechanisms of egg contamination: vertical transmission (transovarian route) during eggshell formation in the oviduct, in contrast to horizontal transmission (*trans*-shell penetration) that occurs following oviposition (Gantois et al., 2009). Bacteria that pass through the eggshell and associated membranes reach the egg white (albumen), which is an unfavourable medium for bacterial growth and mobility due to its high viscosity, high pH and potent antimicrobial proteins (Guyot

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et al., 2020; Réhault-Godbert et al., 2011; 2013; Sellier et al., 2007; Whenham et al., 2015). The antimicrobial activities of protective proteins can act either through direct interaction with the bacterial cell wall (lysozyme, avian beta-defensins) or indirectly by decreasing the bioavailability of growth factors such as iron (ovotransferrin) or biotin (avidin). For example, lysozyme can limit bacterial growth as it hydrolyses peptidoglycan bonds and weakens the bacterial cell wall. On the other hand, ovotransferrin limits bacterial growth by iron deprivation due to its high capacity to chelate iron (Legros et al., 2021). Additionally, ovotransferrin can also limit bacterial growth as it increases membrane permeability (Aguilera et al., 2003).

The bacteria that are found on the egg surface are mostly Gram-positive, but bacteria that contaminate the egg contents are normally Gram-negative (i.e., *Salmonella*) (Legros et al., 2021; Sellier et al., 2007). *Salmonella* is especially problematic, as it is able to resist to a large extent the egg white antimicrobial defenses; once bacterial cells reach the nutrient-rich yolk, they divide very rapidly and contaminate the egg contents. Thus, eggshell integrity and egg white antimicrobial properties are of fundamental importance for the food safety of table eggs and egg products. However, relevant eggshell properties (thickness, breaking strength, cuticle deposition) are highly variable and depend on many factors including hen age, genetics, nutrition and housing (Bain et al., 2016; Dunn et al., 2009; Kulshreshtha et al., 2021; Nys, 2017). For instance, there is a decrease of eggshell quality with hen age, with an increase of up to 30% in the number of eggs with cracked and damaged shell by the end of the laying cycle (normally about 70 weeks of age), which increases the risk of egg contamination (Bain et al., 2016; De Reu et al., 2006). Moreover, egg internal quality (i.e., viscosity/Haugh units) and egg white antimicrobial properties also depend on hen age, genetics and storage conditions (Sellier et al., 2007; Travel et al., 2011).

A current industrial goal is to improve lay persistency until hens reach 100 weeks of age (from the current 70 weeks), in order to increase the sustainability of egg production (Bain et al., 2016). Nevertheless, extended production cycles will challenge hen health, and exacerbation of egg quality problems are anticipated (Alfonso-Carrillo et al., 2021; Nys, 2017). Information is lacking on the changes occurring in egg white composition and resistance to bacterial contamination in eggs in long production cycles (up to 100 weeks). To address this deficiency, we have evaluated the protein profile and antimicrobial properties of egg white in eggs laid by hens from different age groups (up to 100 weeks old) and lines (brown and white), using model Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Salmonella Typhimurium*) bacteria. More importantly, here we report for the first time, the maximum initial bacterial load which is fully inhibited by egg white as a function of antimicrobial protein concentration.

On the other hand, egg contamination by horizontal transmission in freshly laid eggs is mainly determined by eggshell properties (Bain et al., 2013; Muñoz et al., 2015). The susceptibility of egg contamination during longer exposure times (i.e., during storage) will be determined by the antimicrobial properties of egg white, coupled with the bacterial capacity to grow in this harsh environment. To address this aspect, we present egg white protein data concerning the resistance of eggs to *Salmonella* contamination during 21 days of storage.

This study presents comprehensive *in vitro* and *in ovo* analyses of the importance and effectiveness of egg white antimicrobial properties against bacterial contamination. It also provides new information about changes with hen age in egg white chemical composition (protein profile) using an advanced analytical method (UPLC-Mass spectroscopy). The results of this study improve our understanding of the food safety of eggs laid by older hens at the end of long production cycles (up to 100 weeks).

2. Materials and methods

2.1. Samples

Freshly laid eggs from Hy-Line brown and white hens were obtained from a local farm (Avícola Garrido-García S.L, Spain). Thirty eggs laid by hens of each age group (Y, 35 weeks; O, 100 weeks) and line (W, white; B, brown) were randomly selected for the study on albumen protein profile and antimicrobial properties.

Another set of eggs from different age groups (25, 35 and 52 weeks old) from the brown line (Hyline) were exposed to a suspension of *Salmonella* cells, stored for 21 days at room temperature, and then evaluated for *Salmonella* contamination following the methodology by Muñoz et al. (2015). A selection of 10 egg whites from each age group (30 eggs in total) of which 5 had been found to be contaminated with *Salmonella* and 5 had remained sterile were analyzed for protein concentration using the method described below. These egg white samples were kept frozen (−20 °C) until being analyzed.

Egg white from selected eggs was evaluated for protein profile analysis. Individual egg whites were collected and homogenized prior to sampling. Each egg white sample (1 mL) was diluted 11-fold with 0.8% NaCl (Awadé and Efstathiou, 1999), gently stirred overnight at 4 °C, and filtered through a 0.45 μm cellulose-acetate filter.

The total protein concentration of egg white from individual eggs was determined by the Bradford method, with bovine serum albumen as a reference standard, as per the manufacturer's protocol (Bio-Rad Laboratories, Spain). The concentrations of lysozyme and ovotransferrin were determined in individual eggs (N = 10 eggs per group), after separation by ultra-performance liquid chromatography (UPLC) (Waters ACQUITY UPLC binary system) on a BEH300 C4 1.7 μm column (2.1 × 50mm) interfaced with a time of flight (TOF) Mass spectrometer (Waters SYNAPT G2 instrument). Reversed-phase chromatography was performed using 0.1% formic acid in water as mobile phase A, and 0.1% formic acid in acetonitrile as mobile phase B. The flow rate and column temperature during chromatographic analyses were maintained at 0.2 mL/min and 50 °C, respectively. The diluted and filtered egg white solution (as described above) was loaded onto the column and eluted with an increasing acetonitrile concentration as indicated in Table S2. Eluted samples were analyzed after electrospray ionization in positive mode (ESI+) with the TOF Mass spectrometer. The desolvation gas and source temperatures were set to 400 °C and 100 °C, respectively, and the desolvation gas flow was maintained at 800 L/h. The capillary voltage was set at 2.5 kV. Protein concentrations were determined from the associated peak areas after calibration with standard solutions prepared from pure proteins (hen egg white lysozyme and ovotransferrin) from Sigma-Aldrich. Spectral data (Figure S1) were processed with Mass Lynx TM 4.1 software. The results are expressed in weight % (g protein × 100/100 g egg white protein). By using these units, instead of the traditionally used g protein/100 mL egg white, concentrations are not affected by variations in egg white density between samples collected from eggs of different hen age/line groups (Sloan et al., 2000).

Additionally, the protein profiles of selected egg white samples (2 eggs per age group and line) were assessed by SDS-PAGE, using both non-filtered and filtered egg white samples. No significant differences in the most intense bands and relative intensity among them were observed between filtered and non-filtered samples (Figure S2), indicating that no artefacts in measurement of these proteins were introduced by the filtration protocol.

2.2. In vitro microbiology

2.2.1. Susceptibility of Staphylococcus aureus and Salmonella Typhimurium to egg white

Staphylococcus aureus (*S. aureus)* and *Salmonella Typhimurium* strain LT2 (*S. Typhimurium*) (both strains from the Department of Microbiology collection at the University of Granada) were grown in trypticase soy

broth (TSB, VWR, Spain) at 37 °C with shaking at 220 rpm. For solid media experiments, the strains were cultured on trypticase soy agar (TSA, VWR, Spain). An aliquot of overnight cultures of *S. aureus* or *S. Typhimurium* were resuspended in 0.9% sterile NaCl and adjusted to 0.5 McFarland turbidity units (1.5×10^8 CFU/mL). This suspension was then diluted in 0.9% NaCl and used as inoculum for the following seven sets of experiments (one for each egg type and one as a control): 10 test tubes containing 1 mL of egg white from individual eggs in each group (WY = White egg, 35 week hen; WO = White egg, 100 week hen; BY = Brown egg, 35 week hen; BO = Brown egg, 100 week hen) and, as a control, 10 test tubes containing 1 mL of TSB. The final bacterial concentration in all tubes was 10^3 CFU/mL for *S. aureus* and 10^5 CFU/mL for *S. Typhimurium*. The higher inoculum concentration of the latter was determined to be suitable in preliminary experiments, in order to avoid the total inhibition of bacterial growth that occurred when 10^3 CFU/mL of *S. Typhimurium* was used as inoculum (as will be detailed below, i.e., Fig. 3), permitting correlations of bacterial reduction among groups to be established.

Additionally, 10 tubes of sterile TSB and 10 tubes from non-inoculated egg white from each group of eggs were used as negative control experiments. Aliquots were taken at t = 0 and 24 h, with incubation at 37 °C under agitation. Serial 10-fold dilutions in 0.9% sterile NaCl from all aliquots were plated on TSA in triplicate; colony counts were assessed after 24 h incubation at 37 °C.

2.2.2. Analysis of the viability of Salmonella Typhimurium in egg white at different inoculum concentrations

An overnight culture of *S. Typhimurium* was resuspended in 0.9% sterile NaCl, the turbidity adjusted to 0.5 McFarland units, and then diluted in NaCl to be used as inoculum. Samples of egg white (1 mL, gently mixed) were collected from 10 eggs from each experimental group (as above, BO, BY, WO and WY). For each egg, a non-diluted egg white sample was inoculated with *S. Typhimurium* to reach the final following concentrations: 10^3 CFU/mL, 10^4 CFU/mL, 10^5 CFU/mL and 10^6 CFU/mL. For all dilutions tested, a TSB solution inoculated at the same bacterial concentration was used as control. As stated earlier, TSB and non-inoculated egg white samples were used as negative controls. All tubes were incubated at 37 °C for 24 h under shaking. Finally, serial 10-fold dilutions in 0.9% sterile NaCl were plated on TSA in triplicate. After 24 h incubation at 37 °C, the colonies were counted.

2.3. Statistical analysis

Basic descriptive statistics were used to characterize all egg properties measured in this study (egg white protein characteristics, bacterial counts). Independent samples *t*-test or ANOVA tests were performed to compare egg properties. Pearson correlation analysis was performed to study relationships among the different properties measured. The level of significance chosen for all analyses was $p \leq 0.05$. The results are shown as average ± SD. The ANOVA test was done with post hoc comparisons by Bonferroni's test. Statistical differences between the treatments were considered significant when p-values were $p \leq 0.05$ (*), $p \leq 0.01$ (**) or $p \leq 0.001$ (***). All statistical analyses were performed using the software package SPSS 22.0 (IBM, New York, NY, USA) and GraphPad Prism version 8.4.2 for Windows, GraphPad Software (GraphPad Prism, San Diego, CA, USA).

3. Results

3.1. Total protein and antimicrobial protein concentrations in egg white

Total protein concentration of egg white from all eggs ranged from 85 mg/mL to 100 mg/mL, comparable to values reported in other studies (Sellier et al., 2007; Stadelman and Cotterill, 1995). However, egg white total protein concentrations showed notable differences between experimental groups, depending on hen age and line (Fig. 1). Egg white from brown eggs had a significantly higher total protein concentration compared to that of white eggs (100.4 ± 0.3 mg/mL in BY versus 89 ± 1 mg/mL in WY) (Fig. 1A). Moreover, there was a small but significant decrease in total protein concentration with increasing hen age (i.e., from 89 ± 1 mg/mL in WY, to 85 ± 2 mg/mL in WO). Interestingly, the concentrations of the major antimicrobial egg white proteins, especially ovotransferrin (Fig. 2C) increased significantly with hen age. However, there were no significant differences in concentrations of

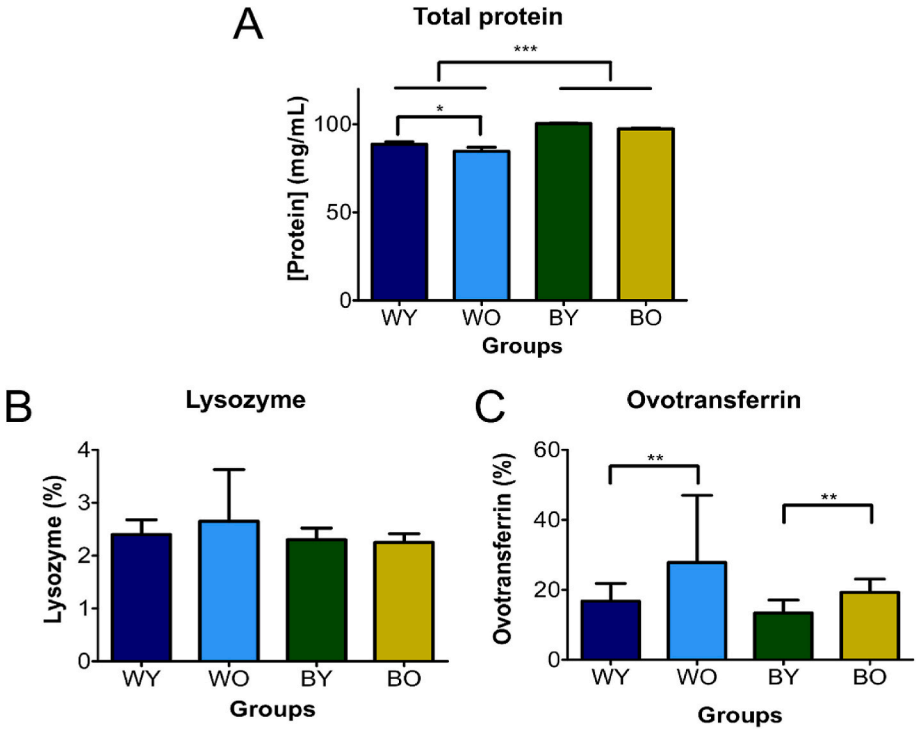

Fig. 1. Protein concentration in egg white from selected groups: White eggs, Young hens (WY); White eggs, Old hens (WO); Brown eggs, Young hens (BY); Brown eggs, Old hens (BO). A) Total protein measured by Bradford assay, and B) lysozyme, C) ovotransferrin concentrations determined by UPLC. % protein = g protein × 100/100 g egg white protein. Results are mean ± SD of 10 eggs per group, where $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

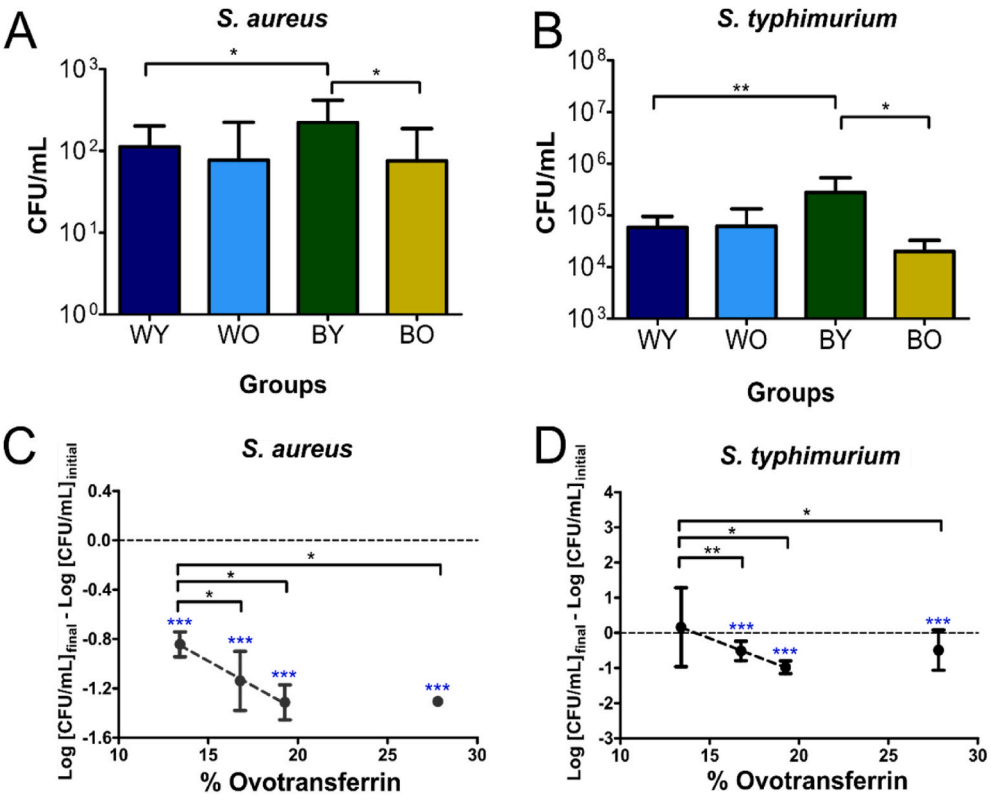

Fig. 2. A) and B) Bacterial growth in egg white samples from selected groups: WY, WO, BY and BO after 24 h of incubation with (A) *S. aureus* starting at 103 CFU/mL (initial inoculum) and (B) S. Typhimurium starting at 105 CFU/mL (initial inoculum). Bacterial counts are expressed as mean ± SD, of 10 eggs per group where $p \leq 0.05$ (*) and $p \leq 0.01$ (**). C) and D) Differences in bacterial growth after 24 h of incubation of (C) *S. aureus* and (D) S. Typhimurium compared to the inocula, as a function of the ovotransferrin concentration. Negative values indicate a reduction in the bacterial count (bactericidal effect of egg whites), 0 indicates an invariant bacterial count (bacteriostatic effect of egg whites) and positive values indicate an increase in the bacterial counts. Results are mean ± SD. Significant differences between data at the different ovotransferrin % (black *) and inoculum (blue *) are marked, $p \leq 0.05$ (*) and $p \leq 0.01$ (**). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

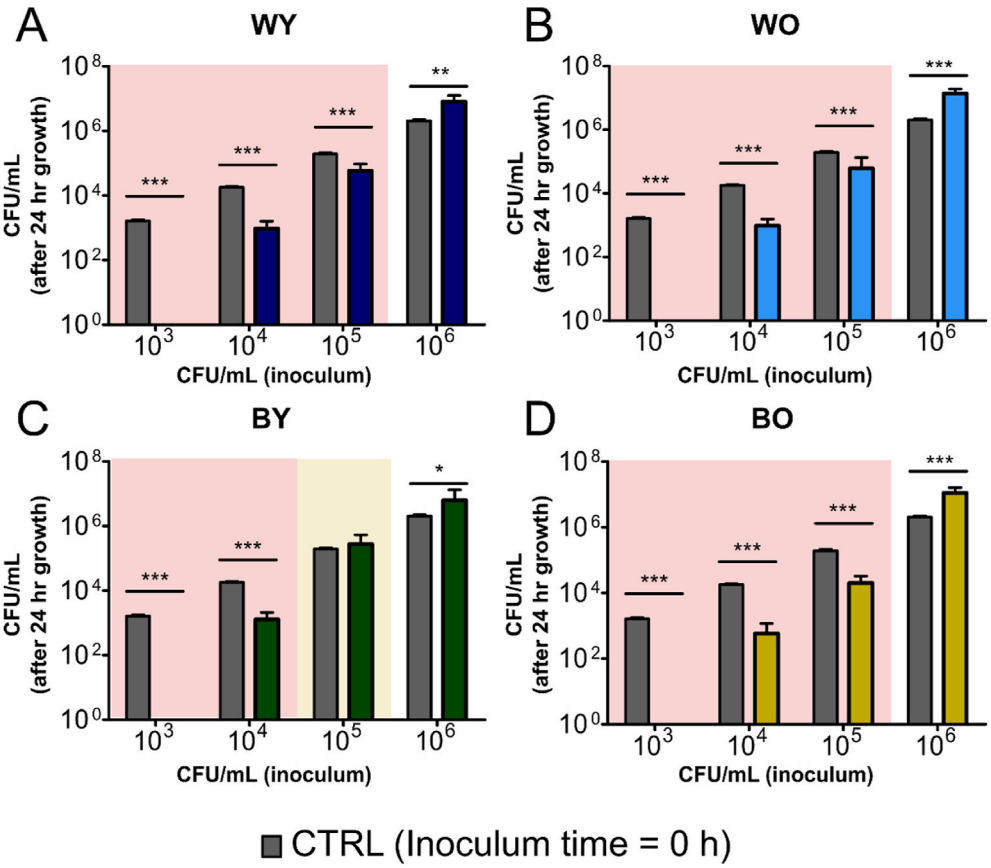

Fig. 3. Bacterial growth in egg white inoculated with different initial concentrations of S. Typhimurium in eggs from: A) White eggs, young hens; B) White eggs, old hens; C) Brown eggs, young hens; D) Brown eggs, old hens. Bacterial growth in egg white samples was measured after 24 h. Red background indicates bactericidal effect (reduction in bacterial counts) and yellow background indicate bacteriostatic effect (no change in bacterial counts with respect to the inoculum). In all panels, the grey bars indicate the initial inoculum. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

these proteins between bird lines. The total protein content of egg white in the white line decreased significantly with hen age (WY vs WO). While there was no difference in lysozyme content between lines or with hen age, the relative concentration of ovotransferrin increased with hen age in both brown and white lines. This suggests that oviduct physiology is altered in older hens, resulting in selective enrichment of antimicrobial ovotransferrin in the egg white.

3.2. Egg white antimicrobial properties as a function of hen age and line

To evaluate egg white antimicrobial properties, egg white samples (10 eggs per group) were inoculated with 10^3 CFU/mL of *S. aureus* or 10^5 CFU/mL of *S. Typhimurium*. *S. aureus:* After 24 h, a significant reduction in the bacterial counts by $\sim10^1$ CFU/mL was observed, especially in eggs from older hens indicating a potent bactericidal effect (Fig. 2A). *S. Typhimurium:* After 24 h, there was a differential behavior in terms of bacteria counts in egg whites, which depended on hen line and age. All egg whites showed a bacterial count (CFU/mL) significantly lower than that of the inoculum (10^5 CFU/mL), indicating a bactericidal effect, with the exception of BY egg whites, which showed a bacteria count of $(2.3 \pm 2.6) \times 10^5$ CFU/mL, not significantly different from that of the inoculum, indicating a bacteriostatic effect, as no bacterial growth was detected within these 24 h (Fig. 2B). This dissimilar behavior of egg whites as a function of hen line and age in terms of resistance against *Salmonella* prompted a more detailed study as described below (Fig. 3). In both cases, *S. aureus* and *S. Typhimurium* incubated for 24 h in TSB yielded CFU/mL of $\sim10^8$-10^9, indicating the viability and rapid growth of the inoculum.

It is interesting to note that there is a significant trend (Table S1) in the ability of the egg white to reduce bacterial growth (after 24 h incubation) as the concentration of ovotransferrin increases, at least up to a relative concentration of 19% (g ovotransferrin × 100/100 g egg white protein) (Fig. 2C and D, Table S1). Higher concentrations of ovotransferrin did not induce a greater reduction in the bacterial count.

3.3. Egg white antimicrobial properties as a function of salmonella concentration

To test the influence of the initial bacterial load on the antimicrobial properties of egg white, egg white samples were inoculated with increasing concentrations of *Salmonella Typhimurium,* from 10^3 CFU/mL to 10^6 CFU/mL, in 10-fold increments. At the lowest initial concentration (10^3 CFU/mL), no bacterial growth was detected (0 CFU/mL) after 24 h (Fig. 3). As the initial bacterial load increased, the ability of egg white to inhibit *Salmonella* growth decreased, although there were notable differences depending on hen age and line (see Fig. 3). At an initial concentration of up to 10^5 CFU/mL, there was a reduction in bacterial counts in egg white from all sources (bactericidal effect) with the exception of those from BY hens (Fig. 3C). At inoculum concentration 10^5 CFU/mL, a bacteriostatic effect (no reduction or increase) was observed in egg whites from BY hens. Curiously, it is precisely this group (BY) that demonstrated the lowest relative concentration of ovotransferrin in their egg whites (Fig. 1D), which further confirms the observation in Fig. 2D regarding the ovotransferrin dependence of the ability of egg white to prevent bacterial growth. For the highest initial bacterial concentration tested (10^6 CFU/mL), the antimicrobial capacity of egg white was overcome in all cases. As stated earlier, *S. Typhimurium* inocula incubated for 24 h in TSB yielded CFU/mL ranging from $\sim10^8$ to $\sim10^9$, respectively, for the lowest (10^3 CFU/mL) to the highest (10^6 CFU/mL) inoculum, indicating the viability of the inoculum.

Overall, these results indicate that egg white has potent antimicrobial properties with the capacity to inhibit *Salmonella* growth, if the initial bacterial load is below a certain threshold. This threshold is mainly dependent on hen age, being higher in eggs for older hens, but also depends on the hen line (egg white from white eggs inhibited to a greater extent than brown eggs, for younger hens). The higher capacity to inhibit bacterial growth in eggs from older hens was particularly correlated with the higher content of antimicrobial ovotransferrin in the egg white.

3.4. Salmonella contamination in whole eggs

We determined the resistance to *Salmonella* contamination in eggs from different hen age groups following exposure to a *Salmonella* suspension after 21 days of storage. The protein concentration in eggs that has viable bacteria (contaminated) or not (non-contaminated) were compared for each age group. A selection of egg contents were analyzed for the presence of viable bacteria. In the present study, protein concentration data from a selection of the stored egg whites from different hen age groups are reported to compare potential differences in the egg white protein profile between contaminated and non-contaminated eggs. Our results show that, firstly, the concentrations of the main egg white antimicrobial proteins (ovotransferrin and lysozyme) increased with hen age (Fig. 4), also in agreement with results shown in Fig. 1C.

Interestingly, the content of ovotransferrin was significantly higher in non-contaminated eggs. This result indicates that bacterial growth is suppressed (*Salmonella* negative, bactericidal effect) if the concentration of egg white ovotransferrin exceeded a certain threshold. However, the minimum concentration of ovotransferrin needed to suppress bacterial growth was always higher in older hens.

4. Discussion

The present work aimed to study the resistance of eggs to contamination by bacteria as a function of hen age in an extended production cycle (100 weeks). Microbes can infect eggs at different stages, including production, processing, preparation and consumption. For example, horizontal transmission occurs through penetration of the eggshell, while vertical transmission is tran-ovarian during egg formation (Gantois et al., 2009). Hen age is a major factor in food safety of eggs, since those laid by older hens have a poorer eggshell quality and are more susceptible to bacterial contamination (Bain et al., 2016; Hamilton et al., 1979; Rodriguez-Navarro et al., 2002). A good quality eggshell is an effective physical barrier and represents the first line of defence against bacterial contamination by horizontal transmission (Bain et al., 2013; De Reu et al., 2006; Dunn et al., 2019; Muñoz et al., 2015; Sparks and Board, 1984). Moreover, eggs with poor cuticle coverage and/or immature cuticle, are more susceptible to bacterial contamination. Nevertheless, in this study, bacterial contamination was independent of eggshell properties since the sample selection and experimental setup minimized differences in eggshell quality, as only eggs with good eggshell quality were selected.

On the other hand, egg internal quality (egg white viscosity, Haugh units) also decreases with hen age (Bain et al., 2016; Nys, 2017). Additionally, changes in egg white protein composition with hen age is a major factor to influence susceptibility to bacterial contamination of the egg content (Sellier et al., 2007; Vlčková et al., 2019; Guyot et al., 2020). In particular, egg white content of antimicrobial proteins (i.e., lysozyme, and especially ovotransferrin), control bacterial growth. For instance, this study shows that an egg white concentration of ovotransferrin of ~20% was enough to induce a bacteriostatic effect. In our samples, the relative concentration of lysozyme ranged from 0.5 to 2 w/w % (~0.5–~1.8 mg/mL), while that of ovotransferrin ranged from ~10 to ~22% w/w % (~10–~19.8 mg/mL). Our results are similar to those previously reported for lysozyme (2.2–4.5 mg/mL; ~3.4% of egg white protein) and for ovotransferrin (~12% of egg white protein) (Vidal et al., 2005; Stadelman and Cotterill, 1995).

During egg storage for up to 21 days, the proliferation of viable *Salmonella* cells in the egg interior was mainly determined by the capacity of egg white to suppress bacterial growth, and not by eggshell properties. In this context, our results demonstrate that eggs that remained uncontaminated after 21 days had a significantly higher

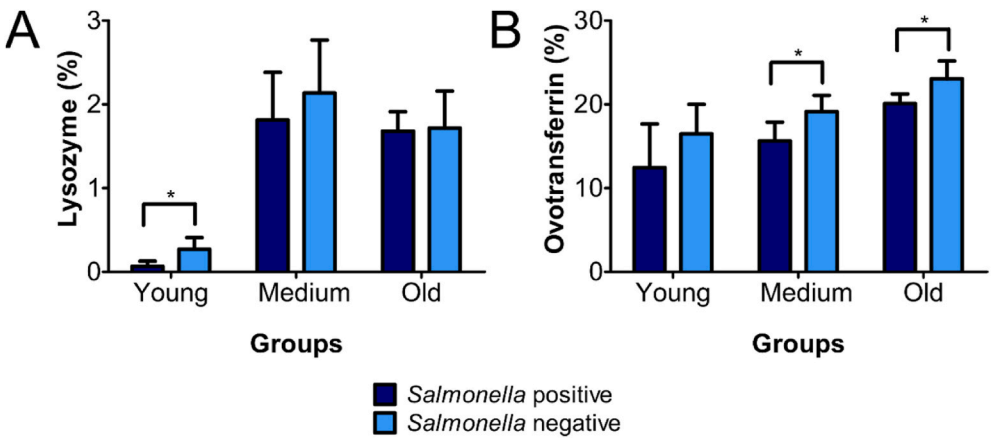

Fig. 4. Concentrations of antimicrobial egg white proteins in *Salmonella* positive and negative brown eggs for different hen age groups (Young, 25 weeks; Medium, 35 weeks; Old, 52 weeks). Bacterial survival in egg interior was assessed 21 days after exposure of intact egg to a Salmonella solution. A) Lysozyme (%); B) Ovotransferrin (%). Statistically significant differences ($P < 0.05$) are indicated with *. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

amount of both lysozyme and ovotransferrin in their egg white, indicating a strong correlation between the egg white antibacterial properties and the viability of *Salmonella*. This correlation is demonstrated in Fig. 2C and D for the *in vitro* experiments, in which the inocula concentrations could be more precisely normalized. These results are in agreement with other studies, which showed that higher concentrations of lysozyme and ovotransferrin in egg white were correlated with higher resistance against *Salmonella enteritis* (Sellier et al., 2007). Interestingly, we have demonstrated that this inhibition of bacterial growth extends to both Gram-positive *S. aureus* and Gram-negative *S. Typhimurium* bacterial species. The antimicrobial mechanisms of lysozyme and ovotransferrin have been previously described, and their cooperative action in egg white which suppresses the growth of Gram-negative bacteria such as *Salmonella* has been demonstrated (Baron et al., 1997; Legros et al., 2021; Lock and Board, 1992; Facon and Skura, 1996). *S. Typhimurium* is a generally useful model for nontyphoidal *Salmonella* (NTS) serovars, and has been characterized extensively; however, we acknowledge that this laboratory strain may not accurately represent NTS virulence due to absence of additional virulence factors, adaptations, and other fitness determinants (Cheng et al., 2019).

Moreover, our results demonstrate that the antimicrobial effect of lysozyme and ovotransferrin is dose-dependent (Figs. 2 and 3). On one hand, Fig. 2C and D shows that as the content of ovotransferrin increases (up to ~20%), there was a reduction in inoculum bacterial counts. On the other hand, Fig. 3 shows that the potential of these proteins to inhibit bacterial growth depends on the initial bacterial load. At low inoculum concentration (low bacteria/protein ratio), a strong bactericidal effect was observed in all egg whites, regardless of the hen line and/or age. As the initial bacteria load increased (at higher bacterial inoculum concentration; high bacteria/protein ratio), the bactericidal effect was weakened and only a bacteriostatic effect was observed. Our *in vitro* results are further supported by our *Salmonella*-penetration experiments, in which eggs that remained *Salmonella*-free during storage were shown to possess a significantly higher content of lysozyme and ovotransferrin (about 20%) (and therefore, a lower bacteria/protein ratio).

In the context of the effect of hen age on bacterial resistance, our results (Figs. 1 and 4) align with those of (Sellier et al., 2007) and, most significantly, reveal that the concentrations of egg white antimicrobial proteins (ovotransferrin, lysozyme) increase with hen age. While another study reported a decrease in antimicrobial egg white proteins with hen age, there are differences in hen line and rearing conditions from our study, which may be relevant (Vidal et al., 2005). A higher concentration of antimicrobial proteins could compensate for, on one hand, the poorer eggshell quality in eggs laid by older hens and, on the other, the lower viscosity in egg white, which, in turn, facilitates bacterial access to nutrients (mainly in the yolk). However, our results show that there is a minimum concentration of these proteins (i.e., ovotransferrin) necessary to inhibit bacterial growth, which increases with hen age (Fig. 4). Our hypothesis is that this increase may not be related to loss of protein activity, but rather to other factors contributing to egg white antimicrobial properties (i.e., viscosity, pH). As egg white physical properties deteriorate with hen age, a compensatory increase in concentration of antimicrobial egg white proteins would be necessary to reduce bacterial survival. Thus, other parameters such as viscosity and pH of egg white, or even opposing changes in the content of other (non-measured in our study) antimicrobial proteins might fully explain the resistance to bacterial contamination of eggs from hens from different lines and/or ages.

5. Conclusions

Our results show that concentrations of egg white proteins, particularly ovotransferrin, are a critical factor to prevent bacterial survival, by either *S. aureus* or *Salmonella,* in egg white. This egg white inhibitory activity is proportional to the ovotransferrin content in egg white (up to ~ 20%). This result was confirmed in experiments not only using egg white samples, but also in whole eggs (*Salmonella* penetration tests), where non-contaminated eggs displayed a higher content of antimicrobial proteins, especially ovotransferrin (about 20%). Additionally, this study showed that the ability of egg white to inhibit bacterial growth also depends on the initial bacterial load, varying from bactericidal at low inoculum (or low bacteria/protein ratio) to bacteriostatic as the inoculum concentration increases (or lower bacteria/protein ratio). Therefore, our results also demonstrate that although the egg white has potent antimicrobial properties, the capacity of egg white to prevent bacterial growth is limited by the concentration of antimicrobial proteins for a given initial bacterial load, and that these characteristics strongly depends on hen age.

All of all, the results from this study indicate that eggs produced by old hens (up to 100 weeks old) may remain safely free of viable *Salmonella* cells, provided that the eggshell remains intact. Most significantly, our results highlight the importance of egg white ovotransferrin to prevent bacterial growth (in particular, *Salmonella*). Therefore, breeding programs should aim to select hen lines that lay eggs in which the egg white is further enriched in ovotransferrin, particularly in older hens, for improved food safety.

Declaration of competing interest

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The manuscript, or its contents in some other form, has not been published previously by any of the authors and/or is not under consideration for publication in another journal at the time of submission. All authors have seen and approved the submission of the manuscript.

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2022.104068.

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