Characterization of egg white antibacterial properties during the first half of incubation: A comparative study between embryonated and unfertilized eggs

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Characterization of egg white antibacterial properties during the first half of incubation: A comparative study between embryonated and unfertilized eggs

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ABSTRACT Egg white is an important contributor to the protection of eggs against bacterial contaminations during the first half of incubation (day zero to 12), prior to the egg white transfer into the amniotic fluid to be orally absorbed by the embryo. This protective system relies on an arsenal of antimicrobial proteins and on intrinsic physicochemical properties that are generally unfavorable for bacterial multiplication and dissemination. Some changes in these parameters can be observed in egg white during egg storage and incubation. The aim of this work was to characterize changes in the antibacterial potential of egg white in embryonated eggs (FE) during the first half of incubation using unfertilized eggs (UF) as controls. Egg white samples were collected at day zero, 4, 8, and 12 and analyzed for pH, protein concentration, and protein profile. Antibacterial properties of egg white proteins were evaluated against *Listeria monocytogenes*, *Streptococcus uberis*, *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella* Enteritidis. During incubation, differential variations of egg white pH and protein concentrations were observed between UF and FE. At equal protein concentrations, similar activities against *L. monocytogenes* and *S. uberis* were observed for FE and UF egg white proteins. A progressive decline in these activities, however, was observed over incubation time, regardless of the egg group (UF or FE). SDS-PAGE analysis of egg white proteins during incubation revealed discrete changes in the profile of major proteins, whereas the stability of some less abundant antimicrobial proteins seemed more affected. To conclude, the antibacterial activity of egg white proteins progressively decreased during the first half of egg incubation, possibly resulting from the alteration of specific antimicrobial proteins. This apparent decline may be partly counterbalanced in embryonated eggs by the increase in egg white protein concentration. The antibacterial potential of egg white is very effective during early stages of embryonic development but its alteration during incubation suggests that extra-embryonic structures could then progressively ensure protective functions.

Key words: egg white, antibacterial property, incubation, embryonated egg, unfertilized egg

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INTRODUCTION

The bird egg is a complex structure ensuring the autonomous development of an embryo outside the mother's body. It contains nutrients and bioactive molecules but also a sophisticated system to protect the developing embryo against microbial assaults. The egg yolk, rich in various nutrients and highly susceptible to bacterial contaminations, is surrounded by the vitelline membranes, the egg white, and eggshell, which play key roles in egg protection. At oviposition, the egg content is germ-free and its protective system allows the unfertilized egg to be stored at room temperature for weeks without spoiling. The efficiency of egg protection is further established by the fact that only few bacteria can invade and multiply in the egg, *Salmonella enterica* Enteritidis still being the main threat for eggs and a major concern for human health. However, egg bacterial contaminations infrequently occur in the ovary or in the oviduct (vertical contamination) or following the crossing of altered eggshells (horizontal contamination).

Egg white complements the physical protection insured by the eggshell. It is a viscous biological fluid with antibacterial properties, mainly consisting of a salty solution of proteins (9.7–10.6%) (Li-Chan et al., 1995). Antibacterial properties of egg white are due to its physicochemical characteristics and to the presence of numerous antimicrobial proteins. Temperature is crucial in controlling bacterial growth in eggs, but egg white pH and viscosity also have a significant role in regulating the diffusion, growth, and survival of bacteria (e.g., *Salmonella*), either by acting directly on microorganisms (e.g., inhibition of *Salmonella* at alkaline pH or inhibition of cell motility by viscosity) or by modulating the activity of antimicrobial proteins. These

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physical characteristics change during egg storage. The egg white pH is close to neutrality at oviposition (7.6) and becomes alkaline after a few days of storage at room temperature, reaching pH values as high as 9.5. Alkalinity can directly inhibit bacterial growth and flagella synthesis (Baron, 1998; Maurer et al., 2005). The viscosity of the thick egg white, which tends to diminish during storage, inhibits cell motility and restrains the migration of bacteria towards the yolk. These physicochemical changes can therefore affect the ability of bacteria to grow in the egg white. Antimicrobial activity of egg white will be either bacteriostatic or bactericidal depending on the types of bacteria and on the conditions of egg storage temperature and duration. However, the mechanism by which egg white exerts its antimicrobial activity is complex, and the information on the relationship between egg white physical properties and activities of egg white antimicrobial proteins remains limited.

Egg white contains a large number of potential antibacterial proteins (Réhault-Godbert et al., 2011), including the well-known lysozyme and ovotransferrin. These proteins are major constituents of the egg white (respectively, 3.4 and 12% of total egg white proteins) and play a significant role in the egg white antibacterial activities (Baron and Jan, 2011). Lysozyme is a cationic protein (isoelectric point of 10.7) possessing a muramidase activity involved in the degradation of peptidoglycan, a constituent of the bacterial cell wall exposed at the surface of Gram-positive bacteria. Lytic activity of lysozyme is observed over a broad range of pH but a significant inhibition of this activity can be observed at alkaline pH (>9) (Smolelis and Hartsell, 1952; Ibrahim et al., 1994; Ibrahim et al., 2001a). Interestingly, the antimicrobial properties of lysozyme also rely on alternative mechanisms independent of muramidase activity (Ibrahim et al., 2001a; Masschalck et al., 2002). Ovotransferrin is an iron-binding molecule depriving the egg white environment of free iron, a metal essential for the growth of many bacteria. Iron deficiency due to ovotransferrin is considered the main factor preventing the growth of Gram-negative bacteria (Garibaldi, 1970; Tranter and Board, 1984; Baron et al., 1997). The chelating activity of ovotransferrin is reinforced at alkaline pH (Halbrooks et al., 2005). In addition to its bacteriostatic effect, ovotransferrin also exhibits bactericidal activities dependent on its ability to bind bacterial membranes (Superti et al., 2007). A recent study demonstrated that the bactericidal activity of ovotransferrin against *Bacillus cereus* occurs via a membrane disturbance mechanism (Baron et al., 2014). Besides these 2 major compounds, numerous putative antibacterial molecules have been identified in the egg during the last decade thanks to proteomics and predictive bioinformatic approaches. Ovalbumin-related protein X (**OVAX**), two defensins (gallin and avian β-defensin 11), and ovoinhibitor recently have been isolated and identified as antimicrobials (Herve-Grepinet et al., 2010; Bourin et al., 2011; Rehault-Godbert et al., 2013; Herve et al., 2014; Guyot et al., 2016). Among these, OVAX and avian β-defensin 11 (AvBD11) were purified using their property to bind heparin, as this negatively charged glycosaminoglycan is currently used to enrich fractions with antimicrobial proteins and peptides in plants or animals (Andersson et al., 2004; Malmstrom et al., 2009; Kumar et al., 2014; Lande et al., 2015). In hen egg white, OVAX is the major protein of the heparin-bound fraction of egg white and possesses antibacterial activities against *Listeria monocytogenes* (Rehault-Godbert et al., 2013). AvBD11 also has been isolated from egg white and vitelline membrane by heparin-affinity and exhibits a potent antibacterial activity against several Gram-positive (*Listeria monocytogenes*, *Staphylococcus aureus*) and Gram-negative (*Salmonella enterica*, *Escherichia coli*) bacteria (Herve-Grepinet et al., 2010). The influence of physicochemical properties of the milieu on the stability and antibacterial activity of these proteins present in egg white at low concentration and their contribution to any change in global antimicrobial activity of egg white remains to be investigated.

The presence of antibacterial protection is crucial throughout embryonic development. However, the inner part of eggshell is progressively dissolved to release the calcium required by the embryo during the second half of incubation and the egg white is progressively transferred to the amniotic fluid from d 11 onwards to be further orally absorbed by the embryo. In addition, the egg white of fertilized eggs undergoes many changes, such as variations of pH (Cunningham, 1974), alteration of lysozyme and MMP-2 activities (Cunningham, 1974; Rehault-Godbert et al., 2008), and degradation and complexation of several egg white proteins (Qiu et al., 2012a; Wang and Wu, 2014).

The aim of this study is to evaluate the antibacterial potential of egg white proteins during the first period of egg incubation to explore whether changes in egg white characteristics impact its antibacterial activity and embryonic protection. A comparative approach between embryonated and unfertilized eggs has been used to identify changes in the egg white physicochemical characteristics and antibacterial activities associated with the presence of a developing embryo throughout the first period of incubation. The antibacterial activity also has been assessed in the heparin-binding fraction of egg white enriched in antibacterial proteins (Guyot et al., 2016) to explore the role of some of these recently identified antibacterial molecules in the change of egg white protective activity.

MATERIALS AND METHODS

Ethical Statement

All experiments were carried out in accordance with the European Community council directives concerning the practice for the care and use of animals for scientific purposes and with the French Ministry on animal experimentation (French decree # 2013–118 of

February 1, 2013). The experimental unit UE-PEAT 1295, where birds were kept, has permission to rear birds (decree # B37-175-1 of August 28th 2012 delivered by the "Prefecture of Indre-et-Loire" following the inspection of the Direction of Veterinary Services).

Storage and Incubation of Eggs

Freshly laid eggs were collected from 2 groups of animals: non-inseminated hens (**NIH**, unfertilized control) and inseminated hens (**IH**) (SA51 hen line, SASSO, Sabres, France). Twenty-one eggs (from the NIH group) collected just after laying were immediately processed and the others (eggs from IH or NIH groups) were first stored 3 d at 16 to 17°C (80 to 85% relative humidity) then transferred to an egg incubator (37.7°C, 45% humidity) for various durations ranging from zero to 12 days. Egg storage and incubation were carried out in the INRA hatchery (PEAT, INRA, Nouzilly, France). Twenty-one NIH eggs and 21 IH eggs were processed after the 3 d storage period, at the start of incubation (d 0). At designated time points of incubation (d 4, 8, and 12), IH eggs were candled to identify fertilized embryonated eggs (**IH-FE**), "clear" eggs (**IH-UF**, defined as mainly unfertilized eggs; however, a small proportion of very early embryonic mortality may not be excluded), and those fertilized but with late embryonic mortality (**IH-FM**). Twenty-one eggs of each group (NIH-UF, IH-UF, IH-FE, and IH-FM) were removed from the incubator and immediately sampled for further analyses. Sufficient numbers of IH-UF and IH-FM eggs, classically present at low levels within IH eggs, were obtained from a total of 6,200 IH eggs. Experimental design is summarized in Figure 1.

Egg White Sampling and Determination of pH and Protein Concentration

Eggs were flamed with absolute ethanol to sterilize egg surfaces and were maintained under sterile conditions. Egg whites were collected, pooled (3 eggs per pool) and homogenized on ice using an ultra-turrax device (T 18 basic ULTRA-TURRAX®, IKA-Werke, Staufen, Germany). A total of 7 pools (corresponding to 7 × 3 eggs) were prepared for each condition. The pH was measured in the egg white homogenate and egg white pools were stored at −20°C until use. Protein concentration in each egg white pool was determined using DC Protein Assay Kit (Bio-Rad, Marnes-la-Coquette, France) according to the manufacturer's instructions, with diluted samples (1/150 to 1/500 in ultrapure water depending on samples) and using Bovine Serum Albumin (concentration range: 0 to 2 mg/mL in ultrapure water) to perform the standard curve.

Heparin-affinity Chromatography to Study Ovalbumin-related Protein X and Other Less Abundant Antimicrobial Egg White Proteins

Egg white proteins (1 g diluted in 8.5 mL of ultrapure water) were mixed with 50 mM Tris-HCl, 150 mM NaCl, pH 7.4 (22.5 mL) and incubated overnight with 0.6 g of heparin-Sepharose beads (CL-6B, GE Healthcare, Velizy-Villacoublay, France) at 4°C under gentle agitation. Beads were extensively washed with 50 mM Tris-HCl, 150 mM NaCl, pH 7.4, packed in a 1 mL polypropylene column (QIAGEN, Courtaboeuf, France) and washed again with 50 mM Tris-HCl, 150 mM NaCl (pH 7.4). Heparin-bound proteins were eluted using 50 mM Tris-HCl, 1 M NaCl (pH 7.4). Proteins were concentrated and desalted using 3 kDa cut-off Amicon Ultra centrifugal filters (Merck Millipore, Molsheim, France) according to the manufacturer's recommendations.

SDS-PAGE Analysis

Proteins were diluted in non-reducing Laemmli SDS-sample buffer and loaded on a 4 to 20% gradient or 15% polyacrylamide gel. Electrophoresis was conducted at 100 V in a Mini-PROTEAN®II electrophoresis cell (Bio-Rad, Marnes-la-Coquette, France). Proteins were stained with Coomassie Blue.

Mass Spectrometry Analysis

Protein bands from SDS-PAGE were excised and washed in water, acetonitrile (1:1) for 5 min followed by a second wash in 100% acetonitrile for 10 minutes. Cysteine reduction and alkylation were performed by

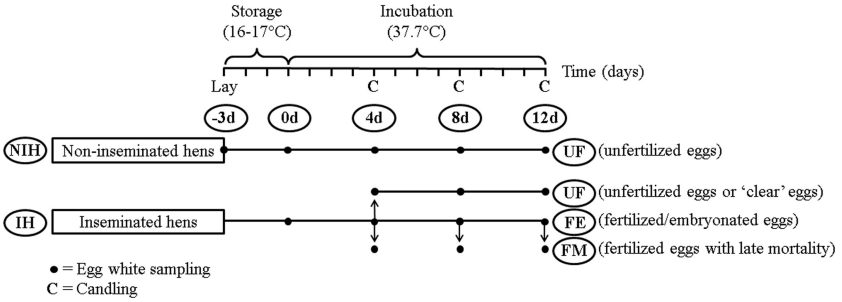

Figure 1. Experimental design.

successive incubations in solutions of 10 mM dithiothreitol, 50 mM NH_4HCO_3 for 30 min at 56°C and 55 mM iodoacetamide, 50 mM NH_4HCO_3 for 20 min at room temperature in the dark, respectively. Gel slices were washed by incubation in 0.1% formic acid, acetonitrile (1:1) for 10 min followed by incubation in acetonitrile for 15 minutes. Proteins were digested overnight in 25 mM NH_4HCO_3 with 12.5 ng/μL trypsin (Sequencing Grade, Roche, Paris). The resulting peptides were extracted from the gel using incubation in 0.1% formic acid, acetonitrile (1:1) for 10 min followed by incubation in acetonitrile. The 2 collected extractions were pooled with the initial digestion supernatant, dried in a Speed-Vac, reconstituted with 20 μL of 0.1% formic acid, 2% acetonitrile, and sonicated for 10 min. Digested peptides were analyzed by on-line nanoflow liquid chromatography tandem mass spectrometry (nanoLC-MS/MS). All experiments were performed on an Ultimate® 3000 RSLC Ultra High Pressure Liquid Chromatographer (Dionex, Amsterdam, the Netherlands) coupled to a dual linear ion trap LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Samples were desalted and concentrated using a precolumn Acclaim PepMap 100 C_{18} trap (3 μm particles, 100 μm inner diameter x 2 cm long) and separated using a reverse-phase analytic column Acclaim PepMap C18 (3 μm particles, 75 μm inner diameter x 50 cm long) with a flow rate of 300 nL/min. The column was equilibrated with 96% of solvent A (0.1% formic acid, 2% acetonitrile) and 4% solvent B (84% acetonitrile, 15.9% H_2O, 0.1% formic acid, v/v) and the gradient was from 4 to 55% of solvent B for 60 min followed by 99% of B for 20 minutes. The eluate was nanoelectrosprayed through a Thermo Finnigan Nanospray Ion Source 1 with a SilicaTip emitter of 15 μm inner diameter (New Objective, Woburn, MA). Data acquisitions were automatically performed in positive mode in data-dependent mode using high resolution full scan MS spectra (R=60,000) and low-resolution CID-MS/MS. In the scan range of m/z 300 to 1,800, the 20 most intense peptide ions with charge states $\geq$2 were sequentially isolated (isolation width, 2 m/z; 1 microscan) and fragmented (Qz 0.25, activation time 10 ms, collision energy 35).

Polydimethylcyclosiloxane (m/z, 445.1200025) ions were used for internal calibration. Raw data files were converted to MGF with Proteome Discoverer software (version 1.4; Thermo Fisher Scientific, San Jose, CA). Precursor mass range of 350 to 5,000 Da and signal to noise ratio of 1.5 were the criteria used for generation of peak lists. MS/MS ion searches were performed using Mascot search engine v 2.3 (Matrix Science, London, UK) against the chordata, sections of a locally maintained copy of nr NCBI (downloaded July 2015). The parameters used for database searches included trypsin as a protease with 2 missed cleavages allowed, and carbamidomethylcysteine, oxidation of methionine, and N-terminal protein acetylation as variable modifications. The tolerance of the ions was set to 5 ppm for parent and 0.8 Da for fragment ion matches. Mascot results were subjected to Scaffold software (v 3.6.4, Proteome Software, Portland, OR). Peptide and protein identification was done by the Peptide and Protein Prophet algorithms with a probability of 95.0% (Keller et al., 2002; Nesvizhskii et al., 2003). From the mass spectrometry results, only proteins identified with at least 2 exclusive peptides were considered. A false discovery rate was calculated as <1% at the peptide or protein level. The abundance of identified proteins was estimated by calculating the exponentially modified protein abundance index (emPAI) using Scaffold software (Ishihama et al., 2005).

Bacterial Growth Analysis

All bacterial strains used in this study (*Staphylococcus aureus* CIRMBP-476, *Listeria monocytogenes* CIRMBP-711, *Streptococcus uberis* CIRMBP-637, *Escherichia coli* CIRMBP-945, and *Salmonella enterica* sv. Enteritidis CIRMBP-733) were from the International Center of Microbial Resources dedicated to Pathogenic Bacteria (CIRM-BP, ISP, INRA, Nouzilly, France, http://www6.inra.fr/cirm). Bacterial growth analysis was carried out according to the protocol described elsewhere (Bedrani et al., 2013a,b) with little modifications. Briefly, bacteria were cultivated in Tryptic Soy Broth for *L. monocytogenes*, in Mueller-Hinton + 5% horse serum for *S. uberis* and in Mueller-Hinton for *S. aureus*, *E. coli*, and *S.* Enteritidis. One-hundred-fifty μL of proteins at 50, 25, 12.5, and 6.25 mg/mL (egg white proteins) or at 1.4 mg/mL (heparin-bound egg white proteins) in ultrapure water were added in a 100-well sterile microtiter plate and inoculated with 150 μL of bacterial cultures in mid-exponential growth phase to a final concentration of about 10^5 cfu/mL. The sterility of the culture medium and egg white was checked in each test. The microplate was incubated at 37°C in an automatic growth curves analyzer (Bioscreen C ®, Thermo Fisher Scientific, Saint-Herblain, France). The optical density (**OD**) at 600 nm was measured every 45 min for 22.5 h. Experiments with each sample were repeated at least twice. Growth rate, lag time, and maximal optical density (**max OD**) of the growth curve of each sample were calculated from the data generated by the automatic growth curves analyzer using the program GrowthRates 1.9 (Hall et al., 2014).

Statistics were carried out using Minitab 17 (Coventry, UK). One-way Anova was carried out to compare the effects of pre-incubation periods (-3d or 0d) or protein concentrations relative to control (no egg white protein) at the start of incubation (0d). Two- or three-way variance analyses (GLM procedure) were performed when analyzing the effect of levels of protein (3.125, 6.25, or 12.5 mg/mL) and that of pre-incubation periods (-3d and 0d) or that of duration of incubation (0d to 12d). The protein concentrations largely impacted the bacterial growth ($P < 0.001$); therefore, in

Table 2, two-way analyses were performed (GLM procedure) at each protein concentration to analyze the effect of duration of incubation and egg status and to compare means modified by duration of incubation or egg status (Tukey's test) at each concentration.

RESULTS

All eggs used in the present study were obtained from inseminated or non-inseminated hens and called IH and NIH eggs, respectively. All NIH eggs are unfertilized (NIH-UF). Freshly laid eggs (abbreviated as -3d eggs) were initially stored for 3 d at 16 to 17°C then incubated at 37.7°C for zero, 4, 8, and 12 d (abbreviated as 0d, 4d, 8d, and 12d, respectively). The candling of a large number of incubated IH eggs (6,200 IH eggs in total) at d 4, 8, and 12 allowed to differentiate and identify fertilized embryonated eggs (IH-FE), fertilized eggs exhibiting late mortality (IH-FM), and "clear" eggs (IH-UF, unfertilized or early mortality). Four types of egg whites (NIH-UF, IH-UF, IH-FE, and IH-FM) were therefore constituted at these designated time points and used for biochemical and microbiological analyses, while only 2 groups (NIH-UF and IH-FE) were considered at the initial time of incubation (0d). The experimental design is summarized in Figure 1.

Physicochemical Modifications of the Egg White During the Egg Incubation

The pH and the protein concentration were determined in all egg white samples (7 pools tested for each condition, 3 individual egg whites per pool) to follow their evolution during incubation. First, the effect of the 3 d pre-incubation period at 16°C with 85% relative humidity on egg white pH and protein concentration in NIH-UF eggs was verified, knowing that in practice, eggs are stored from 3 to 10 d before incubation. As demonstrated in Figure 2, the initial pH at lay was lower than that found at the start of

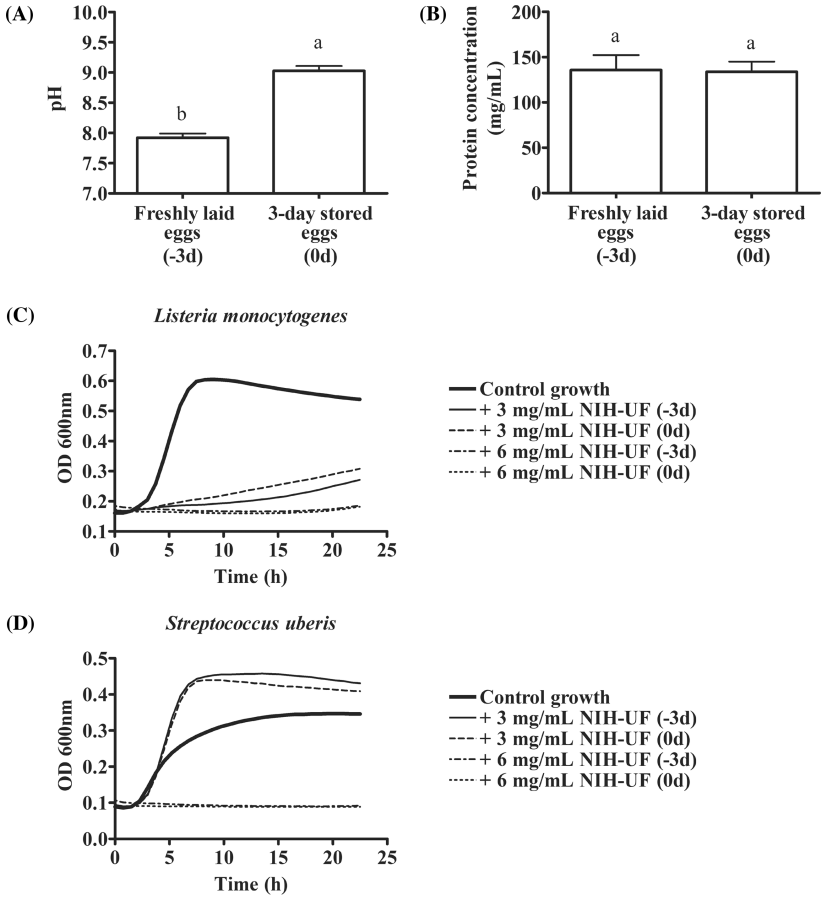

Figure 2. Effect of the pre-incubation storage period on egg white pH and protein concentration and on the antibacterial potential of egg white proteins. (A, B) Comparison of egg white pH (A) and protein concentration (B) values between freshly laid NIH eggs (-3d) and NIH eggs stored for 3 d at 16 to 17°C (0d). Values are means ± standard deviation of 7 pools of egg whites (3 eggs per pool). Bars with no common letter differ significantly according to the Tukey's test ($P < 0.05$). (C, D) Growth of *Listeria monocytogenes* (C) and *Streptococcus uberis* (D) in presence of egg white proteins of freshly laid eggs (-3d) and eggs stored for 3 d at 16 to 17°C (0d). The growth of bacteria was determined in presence of egg white proteins at 3.125 and 6.25 mg/mL. TSB medium and Mueller-Hinton medium (+5% horse serum), containing NIH-UF egg white proteins, were, respectively, inoculated with 10^5 cfu/mL of *L. monocytogenes* and *S. uberis*. Bacterial growth was monitored at 37°C by measuring the turbidity at 600 nm. Growth control was carried out in the same conditions without adding egg white proteins. Each curve represents the average growth curve obtained with n = 7 pools of egg whites (3 eggs per pool). The experiment was repeated 3 times. NIH, non-inseminated hens group; UF, unfertilized eggs.

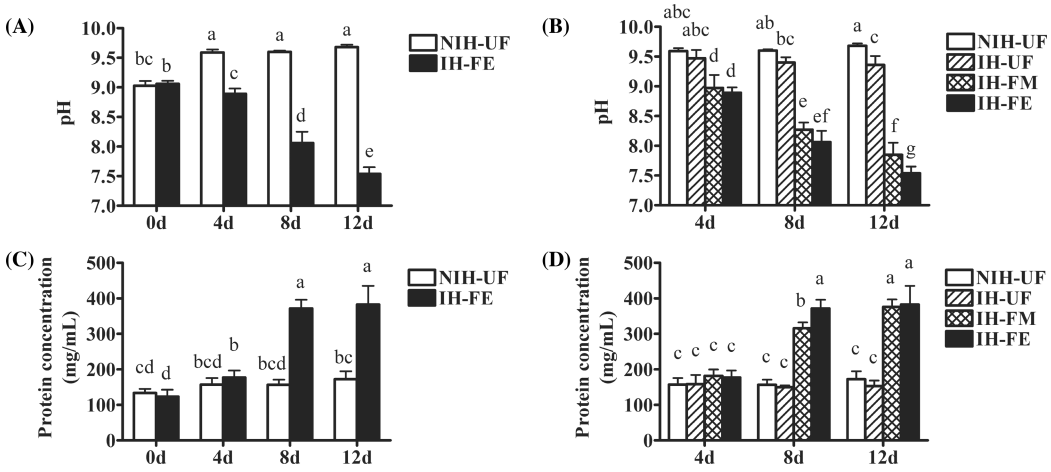

Figure 3. Changes in egg white pH and protein concentration during the first half of incubation. The pH (A, B) and protein concentrations (C, D) were measured in egg whites of 3-day-old eggs incubated for zero, 4, 8, and 12 d at 37.7°C (respectively, identified as 0d, 4d, 8d, and 12d). (A, C) Evolution of pH (A) and protein concentration (C) in NIH-UF (non-inseminated hens, unfertilized) and IH-FE (inseminated hens, fertilized embryonated) eggs during incubation. (B, D) Comparison of NIH-UF (non-inseminated hens, unfertilized), IH-UF (inseminated hens, unfertilized), IH-FM (inseminated hens, fertilized with late embryonic mortality), and IH-FE (inseminated hens, fertilized embryonated) eggs for pH (B) and protein concentration (D) values at different stages of incubation (4d, 8d, and 12d). Values are means ± standard deviation of 7 pools of egg whites (3 eggs per pool). Bars with no common letter differ significantly according to the Tukey's test ($P < 0.05$).

incubation after 3 d of storage (7.9 vs. 9, respectively in -3d and 0d eggs, $P < 0.001$, Figure 2A), while no significant variation in protein concentrations was observed ($P = 0.795$, Figure 2B). Results in Figure 3A and 3C clearly show a diverging evolution of these 2 parameters between NIH-UF and IH-FE eggs during incubation. As expected, egg whites of IH-FE and NIH-UF eggs were at pH 9 at the start of incubation (0d) due to the 3 d egg storage, but this value increased up to 9.5 in the NIH-UF group whereas it progressively decreased to 7.5 in the IH-FE group during the 12 d of incubation (12d) (Figure 3A). The effects of the egg status (unfertilized, embryonated) and the duration of incubation on egg white pH were significant ($P < 0.001$). A dramatic increase in the concentration of egg white proteins (from 120 to 380 mg/mL) also was observed in IH-FE eggs during incubation by comparison to NIH-UF eggs (130 to 170 mg/mL) (Figure 3C). This rise mainly occurred between d 4 (4d) and d 8 (8d). Egg white protein concentration was significantly affected by the egg status (unfertilized, embryonated) and the duration of incubation ($P < 0.001$). Additionally, we studied in parallel the physicochemical properties of "clear" eggs (IH-UF) and eggs with late mortality (IH-FM). Values of NIH-UF and IH-FE, shown in Figures 3A and C, were further compared with IH-UF and IH-FM (Figures 3B and D) at d 4, 8, and 12 (IH-UF and IH-FM not being detected at 0d). As expected, "clear" eggs (IH-UF) underwent similar features as unfertilized eggs (NIH-UF), whereas eggs with late mortality (IH-FM) resembled embryonated eggs (IH-FE) at d 4 (Figures 3B and D). Later on (at d 8 and 12), slight but significant differences were observed between NIH-UF and IH-UF eggs, and between IH-FM and IH-FE eggs. Egg white pH and protein concentration were significantly affected by the egg status (NIH-UF, IH-UF, IH-FM, and IH-FE) and the duration of incubation ($P < 0.001$).

Effects of Egg Incubation on the Antibacterial Properties of Egg White Proteins

The antibacterial properties of egg white proteins were analyzed in NIH-UF and IH-FE eggs during incubation. Given the differences observed in terms of protein concentrations, identical quantities of proteins were tested and compared. The antibacterial property of egg white proteins was evaluated by determining their inhibitory effect on the growth of selected bacteria in well-defined conditions (see Materials and Methods section). Five bacterial strains belonging to the species *Listeria monocytogenes*, *Streptococcus uberis*, *Staphyloccocus aureus*, *Escherichia coli* and *Salmonella enterica* Enteritidis were used in this study. No dramatic inhibition of the growth of *S. aureus*, *E. coli*, and *S.* Enteritidis was detected in our conditions with our samples at a concentration as high as 25 mg/mL (data not shown). In contrast, *L. monocytogenes* and *S. uberis* were significantly inhibited at this concentration (result not shown) and at lower concentration (Figures 2C and D, Table 1). The dose-response effect ($P < 0.001$) from 12.5 to 3.125 mg/mL on the growth rate, lag time, and max OD of both bacteria is described in Table 1. Higher susceptibility to egg white proteins was observed for *L. monocytogenes* in our conditions. The 3 d storage of eggs had no effect on the growth rate and lag time of both bacteria (Table 1), although a trend towards an increase in the growth rate of *L. monocytogenes* was observed during this period ($P = 0.076$). In contrast, longer egg incubation time had a greater impact on egg

Table 1. Effects of egg white proteins on the growth of *Listeria monocytogenes* and *Streptococcus uberis* as influenced by the 3-d storage period.

		Listeria monocytogenes							*Streptococcus uberis*						
			Duration of egg storage			*P*-values				Duration of egg storage			*P*-values		
Growth parameter	EW protein concentration	Control growth[2]	-3d	0d	Pooled SD	EW concentration	Duration	EW concentration x Duration	Control growth[2]	-3d	0d	Pooled SD	EW concentration	Duration	EW concentration x Duration
Growth rate[1] (1000 ∗ LnOD.min^{-1})	0 mg/mL	27.3 ± 19.01							19.3 ± 1.8						
	3.125 mg/mL		4.1^{a,b}	5.1^{a}						16.7^{a}	17.1^{a}				
	6.25 mg/mL		3.0^{b,c}	3.8^{a,b}	1.2	$<$0.001	0.076	0.676		0.9^{b}	1.4^{b}	0.9	$<$0.001	0.305	0.679
	12.5 mg/mL		1.4^{c}	1.6^{c}						0.8^{b}	0.7^{b}				
Lag time[1] (min)	0 mg/mL	37 ± 55							49 ± 33						
	3.125 mg/mL		50^{b}	90^{b}						96	89	42	–	0.667	–
	6.25 mg/mL		1,239^{a}	1,199^{a}	102	$<$0.001	0.997	0.322		$>$1,350	$>$1,350				
	12.5 mg/mL		$>$1,350	$>$1,350						$>$1,350	$>$1,350				
Max OD[1]	0 mg/mL	0.440							0.266						
	3.125 mg/mL	± 0.049	0.118^{a}	0.155^{a}					± 0.028	0.386^{a}	0.361^{b}				
	6.25 mg/mL		0.036^{b}	0.029^{b}	0.032	$<$0.001	0.477	0.128		0.023^{c}	0.011^{c}	0.011	$<$0.001	0.001	0.029
	12.5 mg/mL		0.036^{b}	0.028^{b}						0.020^{c}	0.019^{c}				

EW: egg white; SD: standard deviation.

[a–c]For each bacterium and each growth parameter, means followed by distinct uppercase letters differ significantly by Tukey's test (5%).

[1]Each value represents the mean of 6 to 7 pools of 3 egg white samples. Two-way Anova (GLM procedure) and comparisons of means were carried out for each bacterium on the 2 periods and 3 concentrations only when proteins were present (3.125 to 12.5 mg/mL).

[2]Mean values of 10 control curves (±SD).

white protein antibacterial activities, which were progressively impaired. Growth parameters of *L. monocytogenes* in the presence of egg white proteins were improved (increased growth rate, reduced lag time, and superior max OD) as the duration of egg incubation increased ($P < 0.05$, Table 2, Figures 4A and B), suggesting a progressive loss of egg white antibacterial activities, as clearly observed at lower protein concentrations. The ability of egg white proteins to inhibit the growth rate of *S. uberis* was also significantly impaired during incubation ($P < 0.001$, Table 2, Figures 4C and D). Importantly, the egg status (unfertilized, embryonated) had no dramatic effect on the ability of egg white proteins to inhibit both bacteria (Table 2); only the lag time of *L. monocytogenes* was higher in embryonated egg at the lowest level of egg white protein ($P < 0.05$). Lag time and max OD also were affected by egg status at the highest concentration, whereas the bacterial growth rate was highly inhibited in all cases.

Effects of Egg Incubation on the Protein Profiles of Egg White Proteins

Protein profiles were analyzed by SDS-PAGE for each sample to verify the integrity of the most abundant egg white proteins during incubation. As demonstrated in Figures 5A and B, the stability of the major egg white constituents was not greatly affected during incubation. Only slight differences could be observed. In particular, a faint band at 30 to 35 kDa (identified in Figures 5A and B) probably resulting from ovotransferrin and ovalbumin degradation (Rehault-Godbert et al., 2010) appeared from d 4 of incubation and its intensity progressively increased up to 12 days. Moreover, the intensity of the protein band at 14 kDa (commonly known as lysozyme) seemed to vary during incubation, being the more intense on d 4 (Figures 5A and B). Overall, unfertilized (NIH-UF) and embryonated (IH-FE) egg white samples shared similar protein profiles, no significant differences being visually observed between the 2 groups (Figures 5A and B).

Effects of Egg Incubation on the Protein Profiles and Antibacterial Activity of the Heparin-bound Egg White Proteins

The integrity of less abundant egg white antimicrobial proteins was examined after fractionation of egg white samples by heparin-affinity chromatography. This fraction has recently been employed to enrich and isolate antibacterial proteins such as OVAX and AvBD11, which both exhibit antimicrobial activity against *L. monocytogenes* (Herve-Grepinet et al., 2010; Rehault-Godbert et al., 2013; Guyot et al., 2016). The SDS-PAGE analysis of the heparin-bound fraction of egg white proteins is represented in Figures 5C and D. Modifications of the protein profile of these minor egg white components were observed during incubation. In particular, a band at ∼15 to 20 kDa present in freshly laid eggs (-3d) and in 3 d stored eggs (0d) dissipated after 4 d of incubation in unfertilized (NIH-UF) and embryonated (IH-FE) eggs (Figures 5C and D). Another protein band, at ∼25 to 30 kDa, became visible in unfertilized eggs after 4 d of incubation (Figure 5C). The intensity of this band, absent in embryonated eggs, progressively increased up to 12 days. It is also noteworthy that the level of the major band of the heparin-bound fraction (at 50 kDa), known as OVAX, seemed to slightly decrease during incubation in unfertilized eggs (Figure 5C), whereas no change was observed in embryonated eggs (Figure 5D). Bands of interest were excised and analyzed by mass spectrometry (Tables 3 and 4). The 15 to 20 kDa band in the unfertilized sample at oviposition (-3d) was mainly composed of beta-microseminoprotein-like (gi|513191195), lysozyme, TIMP-2, and gallinacin-11 (also called AvBD11) (Table 3). The 25 to 30 kDa band in the unfertilized sample at d 12 (12d) mainly corresponded to lysozyme, avidin, OVAX, Hep21 protein, ovalbumin, and ovotransferrin (Table 4). Heparin-bound fractions were tested for their ability to inhibit the growth of *Listeria monocytogenes*, *Streptococcus uberis*, and *Escherichia coli*. In our experimental conditions, no antibacterial activity was demonstrated against the 2 latter (*S. uberis* and *E. coli*) at a concentration as high as 0.7 mg/mL (data not shown), whereas the growth of *L. monocytogenes* was inhibited by the heparin-bound fraction (Figure 6). The duration of incubation seemed to have little effect on the activity of this egg white fraction against *L. monocytogenes* in both unfertilized and embryonated eggs (Figure 6).

DISCUSSION

The egg white has an important function regarding the nutrition of the embryo and its protection against bacterial contaminations. Its antibacterial properties rely on a myriad of active antimicrobial proteins and on specific conditions of pH and viscosity. The aim of the present study was to investigate the changes in these antibacterial defenses during incubation and to point out specific modifications induced by the embryonic development. Two groups of eggs (NIH, non-inseminated hens; IH, inseminated hens) were stored for 3 d in standard storage conditions (16°C, 85% humidity) then incubated at 37.7°C in an egg incubator for various durations up to 12 days. Egg whites were collected at zero, 4, 8, and 12 days of incubation to reveal any changes in pH, protein concentration, protein profile, or antibacterial potential of the egg white. Storing eggs at moderate temperature before incubation is a common practice in hatcheries to coordinate hatcheries' activities and anticipate demand; therefore, the effect of this initial period of storage also was investigated and discussed in our study. Storage of fertile eggs at this temperature during 3 to 7 d stops embryonic development prior to incubation. Optimal hatchability is achieved with these

Table 2. Effects of egg white proteins on the growth of *Listeria monocytogenes* and *Streptococcus uberis* as influenced by egg status (unfertilized *vs* embryonated) and the duration of incubation.

			Listeria monocytogenes								*Streptococcus uberis*							
			Duration of egg incubation					*P*-values			Duration of egg incubation					*P*-values		
Growth parameter	EW protein concentration	Egg status	0d	4d	8d	12d	Pooled SD	Egg status	Duration	Egg status x Duration	0d	4d	8d	12d	Pooled SD	Egg status	Duration	Egg status x Duration
Growth rate[1] ($1000 * \text{LnOD.min}^{-1}$)	3.125 mg/mL	Unfertilized	5.1^{d}	6.4^{b-d}	7.4^{a-d}	9.2^{a}	1.5	0.730	<0.001	0.260	17.1^{a}	$15.4^{a,b}$	$15.5^{a,b}$	$15.5^{a,b}$	1.2	0.817	<0.001	0.003
		Embryonated	6.4^{b-d}	$5.6^{c,d}$	7.9^{a-c}	$8.9^{a,b}$					$16.0^{a,b}$	14.2^{b}	16.4^{a}	17.2^{a}				
	6.25 mg/mL	Unfertilized	$3.8^{a,b}$	$3.7^{a,b}$	2.9^{b}	$4.8^{a,b}$	1.3	0.514	0.002	0.245	$1.4^{b,c}$	6.0^{a-c}	$9.2^{a,b}$	12.0^{a}	4.7	0.910	<0.001	0.427
		Embryonated	$4.1^{a,b}$	2.7^{b}	$3.9^{a,b}$	5.4^{a}					0.7^{c}	$3.0^{b,c}$	6.8^{a-c}	13.9^{a}				
	12.5 mg/mL	Unfertilized	$1.6^{a,b}$	$2.8^{a,b}$	$2.4^{a,b}$	$2.4^{a,b}$	1.3	0.206	0.031	0.084	$0.7^{b,c}$	0.1^{c}	$0.5^{b,c}$	$3.5^{b,c}$	2.3	0.003	<0.001	0.024
		Embryonated	1.1^{b}	1.0^{b}	$2.1^{a,b}$	3.3^{a}					$0.5^{b,c}$	0.3^{c}	$4.2^{a,b}$	7.4^{a}				
Lag time[1] (min)	3.125 mg/mL	Unfertilized	$90^{a,b}$	$92^{a,b}$	63^{b}	60^{b}	52	0.045	0.005	0.341	89^{a}	86^{a}	91^{a}	80^{a}	36	0.545	0.637	0.972
		Embryonated	159^{a}	$123^{a,b}$	$72^{a,b}$	64^{b}					100^{a}	91^{a}	99^{a}	79^{a}				
	6.25 mg/mL	Unfertilized	$1,199^{a}$	$1,076^{a}$	673^{b}	119^{c}	221	0.012	<0.001	0.089	>1,350	464^{a}	547^{a}	227^{a}	470	0.670	0.095	0.798
		Embryonated	$1,260^{a}$	$1,210^{a}$	686^{b}	535^{b}					>1,350	640^{a}	484^{a}	153^{a}				
	12.5 mg/mL	Unfertilized	>1,350	$1,122^{a}$	988^{a}	650^{b}	191	<0.001	<0.001	0.317	>1,350	>1,350	>1,350	422^{a}	397	0.270	0.148	–
		Embryonated	>1,350	$1,277^{a}$	$1,227^{a}$	$1,035^{a}$					>1,350	>1,350	501^{a}	181^{a}				
Max OD[1]	3.125 mg/mL	Unfertilized	0.155^{d}	0.286^{b-d}	$0.394^{a,b}$	0.430^{a}	0.081	0.328	<0.001	0.122	0.361^{a}	0.358^{a}	0.360^{a}	0.355^{a}	0.019	<0.001	0.087	0.308
		Embryonated	$0.254^{c,d}$	0.331^{a-c}	0.369^{a-c}	$0.397^{a,b}$					$0.351^{a,b}$	$0.342^{a,b}$	$0.330^{a,b}$	0.322^{b}				
	6.25 mg/mL	Unfertilized	$0.029^{b,c}$	$0.044^{b,c}$	0.085^{a-c}	0.194^{a}	0.067	0.682	<0.001	0.341	0.013^{b}	$0.113^{a,b}$	$0.184^{a,b}$	0.331^{a}	0.130	0.653	<0.001	0.906
		Embryonated	0.026^{c}	$0.044^{b,c}$	$0.146^{a,b}$	0.165^{a}					0.011^{b}	0.067^{b}	$0.200^{a,b}$	0.295^{a}				
	12.5 mg/mL	Unfertilized	$0.028^{b,c}$	$0.027^{b,c}$	0.055^{b}	0.093^{a}	0.017	<0.001	<0.001	0.001	0.019^{b}	0.021^{b}	0.024^{b}	0.075^{b}	0.049	<0.001	<0.001	0.002
		Embryonated	0.012^{c}	$0.039^{b,c}$	$0.027^{b,c}$	0.050^{b}					0.029^{b}	0.036^{b}	0.094^{b}	0.221^{a}				

EW: egg white; SD: standard deviation.

[a–d]For each bacterium, each growth parameter and for each egg white protein concentration considered, means followed by distinct uppercase letters differ significantly by Tukey's test (5%).

[1]Each value represents the mean of 6 to 7 pools of 3 egg white samples. Two-way Anova (GLM) and comparison of means between egg status and duration of incubation were carried out for each bacterium and for each egg white protein concentration.

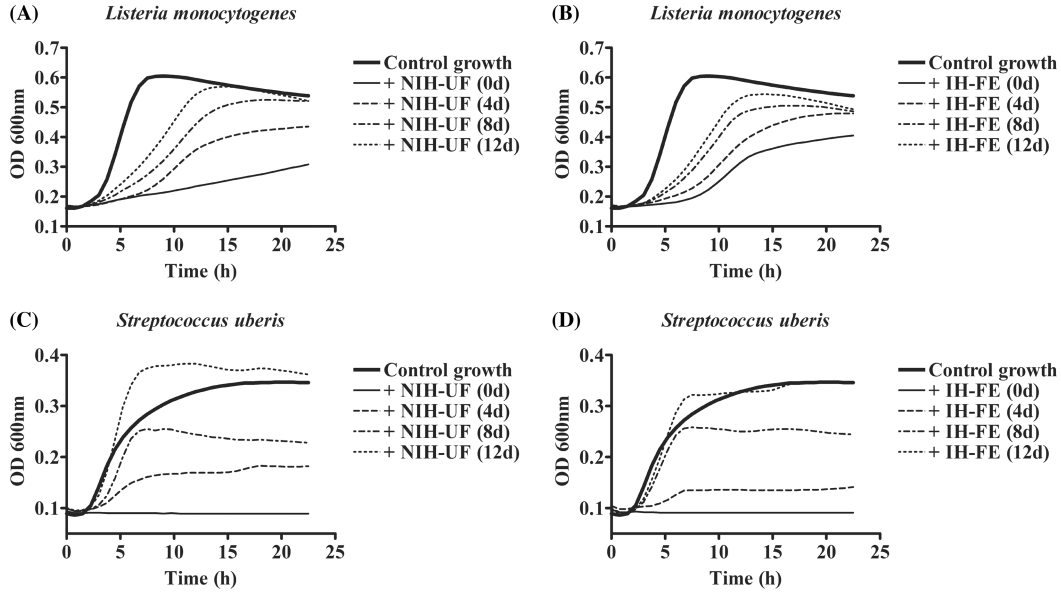

Figure 4. Effect of egg incubation on the ability of egg white proteins to inhibit the growth of *Listeria monocytogenes* and *Streptococcus uberis*. The growth of *Listeria monocytogenes* (with 3.125 mg/mL egg white proteins in TSB medium) and *Streptococcus uberis* (with 6.25 mg/mL egg white proteins in Mueller-Hinton medium +5% horse serum) was monitored at 37°C by measuring the turbidity at 600 nm. Control growth was carried out in the same conditions without egg white proteins. (A, C) Effects of unfertilized egg white proteins (NIH-UF) at different stages of incubation (0d, 4d, 8d, and 12d) on the growth of *L. monocytogenes* (A) and *S. uberis* (C). (B, D) Effects of embryonated egg white proteins (IH-FE) at different stages of incubation (0d, 4d, 8d, and 12d) on the growth of *L. monocytogenes* (B) and *S. uberis* (D). NIH-UF, unfertilized eggs produced by non-inseminated hens; IH-FE, fertilized embryonated eggs produced by inseminated hens. Each curve represents the average growth curve obtained with n = 7 pools of egg whites (3 eggs per pool). The experiment was repeated 3 times.

pre-incubatory storage conditions, while the incubation of fresh eggs or excessively stored eggs can deeply affect embryonic development and subsequent hatchability (Brake et al., 1997), thereby highlighting the importance of egg white quality (pH, viscosity) at the onset of incubation. It is noteworthy that this pre-incubatory period also occurs naturally in birds that lay a clutch of eggs before starting incubation.

The pH of the egg white can have an influence on its antibacterial properties, either by altering the growth potential of bacteria or via the regulation of the activity of antibacterial egg proteins. The pH also may have an impact on protein stability. A divergent evolution of egg white pH was observed between the NIH-UF and IH-FE eggs during incubation — a progressive neutralization of pH was noticed in embryonated eggs within 12 d of incubation, while the pH remained alkaline in unfertilized eggs during this period. These pH changes are consistent with literature data (Cunningham, 1974; Qiu et al., 2012a; Wang and Wu, 2014). The decline in pH seems to start after the second day of incubation (Fang et al., 2012). The pH of the albumen depends on the equilibrium between dissolved CO_2, HCO_3^-, CO_3^{2-} and protein (Li-Chan et al., 1995). HCO_3^- and CO_3^{2-} concentrations are governed by the partial pressure of CO_2 in the egg environment, which is the main factor responsible for egg white pH variations. The freshly laid egg white is enriched in carbon dioxide (under dissolved and combined form) that makes the egg white pH close to neutrality. During storage of unfertilized eggs, CO_2 progressively escapes through the pores of the eggshell leading to the alkalinization of egg white. This elevation of pH was confirmed in our study (pH 7.9 at lay, pH 9 after 3 d at 16°C; pH 9.5 after 4 additional d at 37.7°C). The alkaline environment of egg white is generally unfavorable for bacterial growth, which is usually optimal at pH 6.5 to 7.5. It also enhances the iron-chelating activity of ovotransferrin (Tranter and Board, 1984), but inhibits in counterpart the muramidase activity of lysozyme (Smolelis and Hartsell, 1952; Ibrahim et al., 1994; Ibrahim et al., 2001a). The drop in pH observed in embryonated eggs during incubation likely results from the secretion of hydrogen ions by the blastoderm and the diffusion of the CO_2 produced by developing tissues (Dawes, 1975), making the pH of the embryonic microenvironment optimal for embryonic development (Reijrink et al., 2008). It might favor the growth of some bacteria being closer to their optimal pH but in terms of antibacterial effect, this change should have a positive impact on activities of certain antimicrobial proteins such as lysozyme, the optimal pH of which is around 5 to 7 (Smolelis and Hartsell, 1952; Ibrahim et al., 1994; Ibrahim et al., 2001a).

The egg white is composed of water (~88%) and proteins (9.7–10.6%), carbohydrates (0.4 to 0.9%), ash (0.5 to 0.6%), and lipids (0.03%) (Li-Chan et al., 1995). A 3-fold increase in the concentration of egg white proteins was observed in embryonated eggs within 8 to 12 d of incubation, while only a slight increase occurred in unfertilized eggs. No further protein synthesis occurs in the egg white after oviposition and we visually observed a reduction in egg white volume after 8 d of

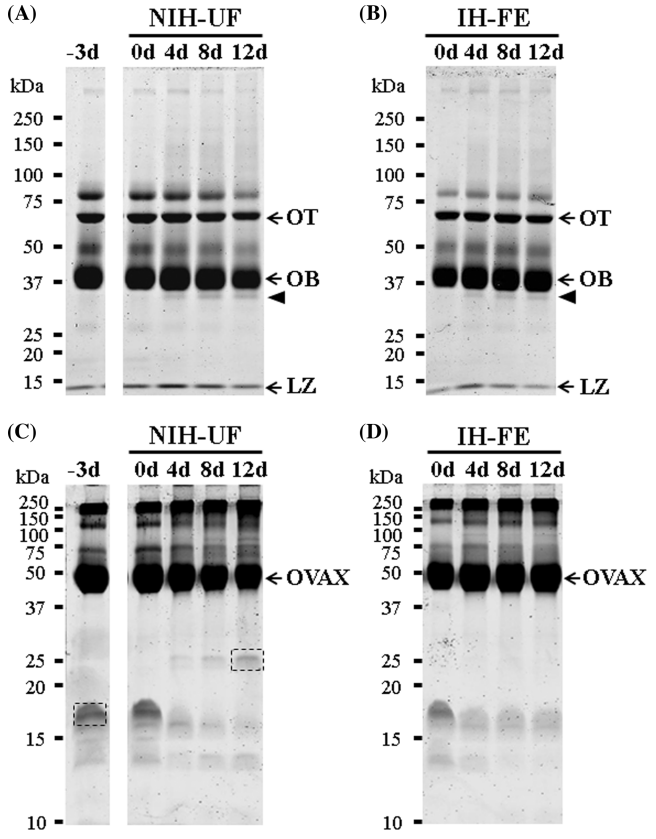

Figure 5. Protein profiles of egg white proteins during egg incubation. (A, B) Whole egg white proteins of unfertilized (A, NIH-UF) and fertilized embryonated (B, IH-FE) eggs at different stages of incubation (0d, 4d, 8d, and 12d) analyzed by SDS-PAGE (4 to 20% gradient polyacrylamide gel) under non-reducing conditions (8 μg of proteins per well). -3d represents control freshly laid eggs (before the pre-incubation period). (◀) indicates a proteolytic fragment at 30 to 35 kDa generated during incubation. OT, ovotransferrin; OB, ovalbumin; LZ, lysozyme. (C, D) Heparin-bound egg white proteins of unfertilized (C, NIH-UF) and fertilized embryonated (D, IH-FE) eggs at different stages of incubation (0d, 4d, 8d, and 12d) analyzed by SDS-PAGE analysis (15% polyacrylamide gel) under non-reducing conditions (13 μg of proteins per well). Egg whites of each condition were pooled and fractionated using heparin-affinity chromatography. -3d represents control freshly laid eggs (before the pre-incubation period). Protein bands identified in the dotted squares were further analyzed by mass spectrometry. OVAX, ovalbumin-related protein X.

incubation, which suggests that this change was due to a loss of water rather than an increase in protein amounts/secretion. This is in agreement with the decreased level of water in egg albumen shown during the first part of incubation (Romanoff and Romanoff, 1933). A large amount of water is used during the formation of embryonic membranes for the associated extraembryonic fluids (amniotic and chorioallantoic fluids) and for the growth of the embryo, while another part of the water dissipates by evaporation. Another study showed that the weight of egg white at 10 to 12 d of incubation is about 30% of that of non-incubated eggs (Carinci and Manzoli-Guidotti, 1968). The amplitude of this weight loss, which is due to a decrease in water content, is consistent with the increased protein concentrations in IH-FE egg whites, which may have potential effects on its protein activities.

Egg white contains large amounts of active antibacterial proteins, including lysozyme and ovotransferrin, which are among the most substantial (respectively, 3.5 and 12% of total egg white proteins), but also a number of other less abundant compounds. These antimicrobials together with the physicochemical characteristics of egg white are very efficient to prevent bacterial growth, since only *Salmonella enterica* Enteritidis is able to survive in egg white (Gantois et al., 2009). In the present study, global antibacterial activities of egg white proteins was evaluated by measuring their effects on the growth of several Gram-positive and Gram-negative bacteria in optimal culture conditions (nutrient-rich culture media at pH 7.4). We found that *Listeria monocytogenes* and *Streptococcus uberis* were inhibited in proportion to the degree of dilution of egg white, whereas the growth of *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella* Enteritidis was not or slightly impaired in our experimental conditions. The growth of the 2 latter is usually inhibited in raw egg white, due to alkaline pH and to the presence of ovotransferrin, which possesses iron-chelating activities and contributes in inhibiting the growth of iron-dependent bacteria, like *S.* Enteritidis, by decreasing bioavailability of free iron. The use of a buffered nutrient-rich medium and diluted egg whites clearly interfered with these 2 physicochemical antibacterial factors (alkaline pH, lack of free iron) and therefore did not allow the inhibition of *E. coli* and *S.* Enteritidis. In contrast, inhibitory activities against *Listeria monocytogenes* and *Streptococcus uberis* were revealed by this technique, in agreement with a previous work (Bedrani et al., 2013b). The advantage of this method is that it allows a direct evaluation of the contribution of egg white proteins in the antibacterial activity without any interference with other antimicrobial factors (viscosity and alkaline pH). Among egg white proteins, lysozyme is likely involved in the inhibition of the growth of *Listeria monocytogenes* as purified hen egg white lysozyme is active against this Gram-positive pathogen (Hughey and Johnson, 1987), but synergistic or interfering activities or both from other egg white proteins is not excluded. The observed decline in the anti-*Listeria* activity of NIH-UF and IH-FE egg white proteins during the incubation might therefore be the consequence of the decreased lysozyme activity. In this respect, a previous study indeed demonstrated that lysozyme activity in fertile egg white decreases rapidly during the first 12 d of incubation, reaching about 20% of the initial activity at d 17 of incubation (Cunningham, 1974). It was suggested that such a decline in lysozyme activity could result from the interaction of lysozyme with conalbumin (ovotransferrin), ovomucin or ovalbumins. Reduction of lysozyme activity also was observed in unfertilized eggs maintained at 35°C, despite the high stability of egg white proteins (Feeney et al., 1952). It is noteworthy that, in our study, the alteration of the antibacterial activity of egg white proteins during incubation occurred regardless of the presence of an embryo, no

Table 3. Proteins identified by mass spectrometry in the 15 to 20 kDa band of heparin-bound fraction of unfertilized egg whites (NIH-UF) at the start of incubation (0d).

Identified Proteins	Accession number	Gene ID	MW	Exclusive spectrum count	Quantitative value (emPAI)
PREDICTED: beta-microseminoprotein-like	gi|513191195	101750704	12 kDa	307	753.79
Lysozyme	gi|229157	396218	14 kDa	12	29.273
Metalloproteinase inhibitor 2	gi|514711419	374178	24 kDa	11	26.834
Gallinacin-11 precursor	gi|49169808	414876	12 kDa	6	14.637

Table 4. Proteins identified by mass spectrometry in the 25 to 30 kDa band of heparin-bound fraction of unfertilized egg whites (NIH-UF) at the twelfth day of incubation (12d).

Identified Proteins	Accession number	Gene ID	MW	Exclusive spectrum count	Quantitative value (emPAI)
Lysozyme	gi|229157	396218	14 kDa	703	503.16
	gi|229916		14 kDa	44	433.02
	gi|220899139		16 kDa	10	196.11
Avidin	gi|451889	396260	17 kDa	34	24.335
Ovalbumin-related protein X	gi|510032768	420898	45 kDa	30	21.472
Hep21 protein	gi|316995812	395192	12 kDa	10	7.1574
Ovalbumin	gi|223299	396058	43 kDa	9	6.4416
Ovotransferrin	gi|71274079	396241	78 kDa	7	5.0102
Ovoglobulin G2	gi|385145523	395882	47 kDa	4	2.8629
PREDICTED: alpha-2-macroglobulin-like 1 isoform X3	gi|513161014	418254	147 kDa	3	2.1472
PREDICTED: tumor necrosis factor receptor superfamily member 6B isoform X2	gi|118100703	395096	33 kDa	3	2.1472

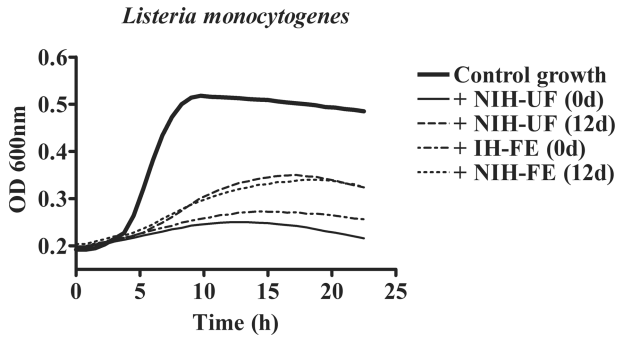

Figure 6. Effect of egg incubation on the ability of egg white heparin-bound proteins to inhibit the growth of *Listeria monocytogenes*. TSB medium containing heparin-binding proteins at 0.7 mg/mL was inoculated with 10^5 cfu/mL of *L. monocytogenes* and incubated at 37°C. Bacterial growth was monitored by measuring the turbidity at 600 nm. Control growth was carried out in the same conditions without adding heparin-binding proteins. NIH-UF (unfertilized eggs from non-inseminated hens) and IH-FE (fertilized embryonated eggs from inseminated hens) samples were analyzed at the start (0d) and at d 12 (12d) of incubation. Each curve represents a single preparation of heparin-bound proteins obtained with a pool of all samples for each condition. The experiment was repeated 2 times.

difference being observed between the 2 groups (NIH-UF and IH-FE) using the same protein amounts. It is likely that this apparent weakening of the egg protection is counterbalanced in embryonated egg white, at least in part, by the loss of water and the subsequent increase in protein concentration. A rise in protein concentration indeed should theoretically be accompanied by a reinforcement of antimicrobial activities and a higher viscosity. In this regard, IH-FE samples at d 8 and 12 of incubation were found to retain strong inhibitory activities against *L. monocytogenes* and *S. uberis*, when protein amounts were doubled (see Table 2). This hypothesis is also supported by the fact that the total amount of egg white proteins remains rather stable during the first half of incubation. Previous studies suggest that egg white proteins are not significantly absorbed by the embryo nor transferred to fetal fluids before d 12 of incubation, but mostly during the second half of the incubation (Romanoff and Romanoff, 1933; Carinci and Manzoli-Guidotti, 1968).

Any alterations of protein integrity may have an impact on protein activities. For this reason, it was hypothesized that protein degradation might occur in eggs during incubation, explaining part of the decline in the antibacterial activities of egg white proteins. Nevertheless, it should be noted that, in some cases, protein fragments also can exhibit antibacterial properties, as demonstrated for peptides generated in vitro by the hydrolysis of the egg white proteins lysozyme, ovotransferrin, and ovalbumin (Ibrahim et al., 2000; Ibrahim et al., 2001b; Mine et al., 2004; Pellegrini et al., 2004). The stability of egg proteins is highly challenged by both the temperature and the duration of incubation, but also by the variations of pH occurring in eggs. Our SDS-PAGE analysis, however, revealed a relative stability of the major proteins of egg white during the first 12 d of incubation, although peptides resulting from protein degradation cannot be detected by this technique. The apparent stability of egg white proteins observed in our experimental conditions also was observed in previous studies (Feeney et al., 1952; Rehault-Godbert et al., 2010; Omana et al., 2011; Qiu et al., 2012a), whereas other authors observed major changes by 12 d of incubation (Cunningham, 1974) or 15 d of storage at 37°C (Qiu et al., 2012b). Recent proteomic analyses by 2D

gel electrophoresis of fertile eggs revealed changes in several egg white protein spots during incubation (Qiu et al., 2012a; Liu et al., 2013; Wang and Wu, 2014; Liu et al., 2015). According to our results, the integrity of the 14 kDa band (identified as lysozyme in the literature) does not seem to be compromised but, curiously, the intensity of this band was higher at d 4 of incubation while antibacterial activities were decreased compared to that at the start of incubation (Figure 4, Table 2). The decrease in the 14 kDa band intensity at d 12 cannot solely explain the decreased antibacterial activity of egg white observed at d 12 compared to d 4. The reason for this increase at d 4 remains unclear but similar variations were observed in a recent proteomic study with several lysozyme spots that were increased during the first 5 d of incubation and then decreased from the fifth to ninth day of incubation (Wang and Wu, 2014). The only protein fragment visualized by SDS-PAGE in our study was a faint band at 30 to 35 kDa progressively appearing during incubation in both embryonated (IH-FE) and unfertilized (NIH-UF) eggs (Figure 5). Similar fragments were previously observed in unfertilized eggs stored at 37°C and identified by mass spectrometry as fragments of ovalbumin and ovotransferrin (Rehault-Godbert et al., 2010). Fragments of clusterin (protein with molecular chaperone function) and ovalbumin were detected in the range of 30 to 35 kDa according to a proteomic study on the egg white of fertile and unfertilized eggs incubated for 7 d (Qiu et al., 2012a). Given the divergent pH observed in the egg white during incubation between embryonated and unfertilized eggs, it can be concluded that the generation of this protein fragment observed in both groups is not pH-dependent but rather related to the temperature of incubation, which might activate latent proteases such as aminopeptidase Ey, transmembrane protease serine 9, carboxypeptidase D, and aminopeptidase A (Mann and Mann, 2011). In this regard no protein fragments were detected by SDS-PAGE in the egg white from eggs stored for up to 30 d at 20°C or 4°C (Rehault-Godbert et al., 2010). In a recent proteomic study (2D gel electrophoresis combined with MALDI-TOF MS/MS), it also was suggested that the alterations observed for most of the identified egg white protein spots during incubation, which were similar between fertilized and unfertilized eggs, likely result from incubation temperature rather than the presence of the embryo (Qiu et al., 2012a). Some differences between the 2 groups also were revealed for a few protein spots identified as clusterin, ovoinhibitor, lysozyme, and OVAY, which were suggested to be pH-dependent. Further investigations are needed to explain the intensity changes of protein bands observed during incubation and to evaluate the effects of such modifications on egg physiology.

While the integrity of major egg white proteins was not or little affected by incubation conditions, some less abundant proteins seem to be more susceptible to change. In particular, a protein band of the heparin-bound fraction at 15 to 20 kDa, mainly composed of beta-microseminoprotein-like (gi|513191195) (Table 3), rapidly disappeared during the first 4 d of incubation (Figure 5). Our group has recently revealed that beta-microseminoprotein-like (gi|513191195) possesses antibacterial activities (Guyot et al., 2016). The 15 to 20 kDa band also contains other known antibacterial proteins such as lysozyme and AvBD11 (gallinacin-11). It is unclear whether these proteins are degraded, complexed, or transferred in another egg compartment during egg incubation but it is likely that the loss of the native form of these proteins has repercussion on egg bioactivities, given the various biological functions mediated by heparin-binding proteins and carbohydrate-protein interactions. Our results support that, in comparison to major egg white proteins, the stability of low abundant egg white proteins can be greatly altered during incubation. The inhibitory activity of the heparin-bound egg white proteins against *Listeria monocytogenes* is probably not mainly attributed to these proteins but rather to the major protein of this fraction, ovalbumin-related protein X (OVAX), recently identified as an anti-*Listeria* agent (Rehault-Godbert et al., 2013), although we believe that all these molecules act synergistically. Furthermore, it is interesting to note that a 25 to 30 kDa protein band, mainly composed of lysozyme and avidin, was appearing during incubation in the heparin-bound egg white fraction of incubated unfertilized eggs (Figure 5C). The absence of this band in the egg white of embryonated eggs (Figure 5D) suggests that its formation may be influenced by pH or proteolytic degradation. The molecular weights of lysozyme and avidin monomer are, respectively, 14 kDa and 16 kDa, which suggests that they might interact with each other or that they exist as multimers. Multimerization of lysozyme is a known process that depends on particular conditions such as lysozyme concentration, ionic strength, and pH. Dimerization of lysozyme is likely to occur at alkaline pH (Sophianopoulos and Van Holde, 1961; Thomas et al., 1996), which is consistent with the alkaline pH measured in unfertilized eggs during incubation. Dimerization of lysozyme was found also to occur in egg vitelline membrane during egg storage and a reduced activity of this dimer was observed against *Bacillus megaterium* (Back, 1984). It was then suggested that dimerization during storage could contribute, at least in part, to the loss of lysozyme activity (Feeney et al., 1952). Avidin also was identified in the 25 to 30 kDa band of our study, suggesting that it may correspond to avidin-avidin or avidin-lysozyme complexes. Avidin is a homotetrameric protein with the ability to bind biotin. Avidin-lysozyme interactions have been reported previously in connection with the presence of contaminating lysozyme in several avidin preparations isolated from egg white (Durance, 1987). Further investigations suggest that avidin-lysozyme interactions can result from electrostatic and hydrophobic interactions (Wijewickreme, 1990).

In conclusion, the present study demonstrated that some antibacterial properties of egg white proteins (against *Listeria monocytogenes* and *Streptococcus uberis*) are progressively impaired during incubation in both embryonated and unfertilized eggs, possibly resulting from the degradation of some egg white proteins. Although these protein alterations seem mostly independent of the presence of an embryo, some important differences related to the egg white physicochemical properties are observed between embryonated and unfertilized eggs: Unfertilized egg white is mainly characterized by alkaline conditions maintained during incubation, while embryonated egg white progressively undergoes a pH neutralization combined with increasing protein concentration. We hypothesize that the apparent alteration of antibacterial defenses in embryonated eggs is likely counterbalanced by the increase in protein concentration, which might enhance or stabilize the activity of antibacterial proteins. Whereas egg white proteins mainly contribute to egg protection during the first half of incubation, it seems that they are rather used as a nutrient during the second half of incubation. During this latter period, egg white proteins are indeed known to be rapidly transferred to amniotic fluid and then absorbed orally by the embryo. It is not known whether egg white antimicrobials are impaired during the transfer to the other compartment but it is hypothesized that the loss of the egg white-associated protection is counterbalanced by other defense systems (possibly the chorioallantoic membrane or the chorioallantoic fluid or both) that appear during embryonic development, to ensure the continuous protection of the embryo.

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