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Importance of eggshell cuticle composition and maturity for avoiding trans-shell *Salmonella* contamination in chicken eggs

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ABSTRACT

The cuticle coating the eggshell surface is the first line of defense of the egg against bacterial ingress. However, the cuticle properties (i.e., thickness, degree of coverage, chemical composition) have a very large natural variability and this work analyzed how this variability influence the risk of eggs being contaminated by Salmonella. Microbial growth on the eggshell surface as well as the incidence of Salmonella penetration in eggs increases significantly with hen age for the groups (25, 35, and 52 weeks) considered in this study. It shows also that the cuticle is most effective against bacterial penetration between 6 and 72 h after eggs have being laid when this coating is fully mature and has not dried excessively. In contrast, freshly laid eggs can be easily contaminated as they have an immature cuticle which is not able to resist bacterial penetration. This study show also that the chemical composition of the mature cuticle determines the risk of trans-shell contamination by Salmonella. In particular, it shows that eggs with a cuticle rich in proteins have a decreased shell permeability and greater resistance against *Salmonella* penetration. The novel analytical technique used here to quantify the cuticle quality (based on infrared spectroscopy; ATR-FTIR) could be used in assisted selection programs aimed to improve the quality and safety of eggs.

1. Introduction

Hen eggs are a popular and inexpensive source of proteins which are extensively consumed around the world (Nys, Bain, & Van Immerseel, 2011). However, they are also commonly associated to food-poisoning outbreaks due to their contamination with pathogenic bacteria (i.e., *Salmonella* enteritis). Only in the EU, there is an estimated incidence of over 100.000 cases of salmonellosis per year, that being a serious health problem with an important economic impact (EFSA., 2009; Gastois et al., 2009; Messens, Grijspeerdt, & Herman, 2005; Solomon et al., 1997). Thus, it is important to take active actions to reduce the risk of such contamination. Contamination of the egg can occur either prior to the oviposition, due to infection of the reproductive organs, in contaminated flocks, and/or during or after the oviposition, by trans-shell penetration, which is considered the prevalent route for bacterial contamination (Board & Tranter, 1995; Gantois et al., 2009; Messens et al., 2005). Cracked eggs or eggs with a poor eggshell quality (which can account for over 6% of total production) can be more easily contaminated with bacteria and pose an important risk to consumers as they need to be downgraded causing important economic losses to producers (Dunn et al., 2010; Hamilton, Hollands, Voisey, & Grunder, 1979; Washburn, 1982).

The egg is protected against bacterial contamination by the eggshell and the shell membranes, which if intact, act together as an effective physical barrier against bacterial penetration (Board & Tranter, 1995; De Reu et al., 2006; Jonchere et al., 2010). Even if the eggshell integrity is good, the mineral shell is still perforated by many pores that allow gas and water exchange necessary for the developing chick embryo but also possibilities microbial ingress and contamination of the egg content (Hincke, Nys, Gautron, Mann, & Rodríguez-Navarro, 2012). The cuticle, a very thin (up to 12 um) organic layer, coats the eggshell surface and plugs the eggshell pore opening, thus limiting the movement of particles, water and

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bacteria through the shell (Board & Halls, 1973; Board & Tranter, 1995; De Reu et al., 2006). It is composed mainly of proteins (glycoproteins) (90%) and, in a lesser proportion, polysaccharides and lipids (Baker & Balch, 1962; Hasiak, Vadehra, & Baker, 1970; Rodríguez-Navarro, Domínguez-Gasca, Muñoz, Ortega-Huertas, 2013; Rose-Martel, Du, & Hincke, 2012; Wedral, Vadehra, & Baker, 1974). Some of the cuticle proteins (lysozyme C, ovotransferrin, ovocalyxin and ovocleidin) and lipid components have been shown to have a potent antimicrobial activity and could significantly contribute to egg safety especially considering that it is the first barrier that microorganisms encounters (Jonchere et al., 2010; Rose-Martel et al., 2012; Wellman-Labadie, Picman, & Hincke, 2008, 2010). In fact, eggs with an absent or partially removed cuticle are more susceptible to bacterial contamination (Bain et al., 2013; Board & Halls, 1973; De Reu et al., 2006; Messens et al., 2005; Sparks & Board, 1984). Therefore, there is a great interest in studying the different factors that contribute to cuticle and eggshell quality to improve the safety and quality of eggs (Bain et al., 2013; Dunn et al., 2009; Jonchere et al., 2010; Nys et el., 2011; Solomon, 1997).

Eggshell quality and, particularly, cuticle properties are highly variable as they are influenced by a wide array of factors including hen age, genetics, and diet as well as hen housing (Bain et al., 2013; Board & Halls, 1973; Board & Tranter, 1995; Dunn et al., 2009; Leleu, Messens, De Reu, De Preter, & Herman, 2011; Nys et al., 2011; Solomon, 1997; Rodriguez-Navarro et al., 2013). Additionally, it has been shown that the cuticle composition is not constant and it changes with hen age and as it matures (Hasiak et al., 1970; Rodriguez-Navarro et al., 2013).

To evaluate how the natural variability of cuticle properties (i.e., thickness, degree of coverage, composition) can modify the risk of trans-shell contamination by bacteria, we have conducted a detailed study in which we determined the incidence of *Salmonella* penetrated eggs in eggs for hens of different ages and in which the cuticle properties are expected to vary substantially. The influence of cuticle maturity was also tested by evaluating bacterial penetration in freshly laid eggs exposed to *Salmonella* at different times after oviposition. Relevant cuticle properties (e.g., thickness, degree of coverage, morphology and chemical composition) were analyzed using complementary analytical techniques such as scanning electron microscopy (SEM) and attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR) and their influence on Salmonella penetration was evaluated. ATR-FTIR spectroscopy is especially suited for the analysis of the eggshell cuticle quality as it provide detailed information about the chemical composition and thickness of this coating (Rodriguez-Navarro et al., 2013). Furthermore, it probes the cuticle chemical composition in its natural state without the need of extraction. Also, ATR signal penetration is limited to about 2 microns which is typically the thickness of the cuticle. However, being a local measurement, it does not provide information of the extent of the cuticle to evaluate the risk of eggs being contaminated coverage on the egg. Thus, for a more complete characterization of the cuticle quality, SEM observation was used to determine the cuticle coverage and the degree of exposure of shell pores. To the authors' knowledge, this is the first study evaluating how the natural variability in cuticle properties influences bacterial penetration. The study provides a new and comprehensive approach by *Salmonella* or other pathogens.

2. Material and methods

2.1. Samples

Hyline plus brown hen eggs were collected, immediately after being laid, directly from a local farm with no history of *Salmonella* contamination (Avícola Garrido-García S.L, Albolote, Spain). To study the effect of hen age, eggs laid by hens of different age groups (25, 35 or 52 weeks old) were selected. For each hen age group studied, a total of 180 freshly laid eggs were collected. To evaluate the effect of cuticle maturity, subsets of 60 eggs, from each hen age group, were exposed to Salmonella suspension after 3, 6 or 72 h from oviposition, representing eggs with an immature, mature or dry cuticle, respectively. Eggs were kept at 20 °C throughout the experiments.

2.2. Bacterial strain and culture conditions

Experiments were conducted using *Salmonella arizonae* from the collection of the Department of Microbiology of the University of Granada. The strain was maintained at 4 °C on Tryptone Soya Agar, TSA (Oxoid, Hampshire, UK). Overnight cultures of *Salmonella* spp. at 30 °C in TSB (Oxoid) were used for inoculation of the ringer solutions (Sigma–Aldrich, St. Louis, MO, USA) used for all microbial experiments. After inoculation of the ringer solution, bacteria were allowed a 20 min adaptation period before egg contamination.

2.3. Egg inoculation

Eggs were immersed in a with a 6 log cfu/mL *Salmonella* suspension in ringer solution. To evaluate the effect of cuticle maturity, subsets of 60 eggs, from each hen age group, were inoculated after 3, 6 or 72 h from oviposition, representing eggs with an immature, mature or dry cuticle, respectively. Eggs were placed in pairs in sterile bags containing 20 mL of the microbial suspension. The eggs were gently massaged for 3 min, avoiding to wet the sharp end (used for later analyses), allowing for bacterial transfer from the suspension to the eggshell. This method guaranteed a microbial contamination of *S. arizonae* of 3–5 log cfu per egg. Next, eggs were placed into a plastic box to ensure a constant humidity and kept at 20° C during the entire duration of the experiment (21 days). Between 36 and 48 h following contamination, 30 eggs from each set were examined to determine surface total microbial load, *S. arizonae* penetration and cuticle composition and eggshell properties. At day 21 after contamination, the remaining 30 eggs were examined to determine the same parameters. Control experiments using the same number of eggs but without exposing them to *Salmonella* suspension were run in parallel to determine the total microbial surface load of non-contaminated eggs and to verify the absence of *Salmonella* in the egg content in the control.

2.4. Surface microbial analyses

To measure the microbial surface load on the eggshell surface, eggs were placed individually in sterile bags containing 10 mL of ringer solution and gently massaged for 1 min (without wetting the non-contaminated sharp end) to transfer the microorganisms present in the eggshell to the saline solution. After that, 100 μL of the appropriate dilutions were plated by triplicate onto TSA and incubated at 30 °C for 48 h prior to enumeration. Total microbial counts were referred as log cfu/shell after applying the correcting factor (×10) corresponding to the total volume (10 mL) where the bacterial suspension for each egg was obtained.

2.5. Penetration of Salmonella

After determining the surface microbial counts, each egg was pulverized with 70% ethanol and flamed (approx. 5 s) twice before breaking it aseptically (modified from De Reu et al., 2006). Dilutions 1:10 of homogenised egg contents were prepared in sterile buffered peptone water (BPW) (Oxoid) and incubated at 30 °C for 24 h. After