Intrinsic and extrinsic factors influencing eggshell penetration by *Salmonella* Enteritidis

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**Summary**

To prevent penetration of *Salmonella* into eggs, a clear understanding of various factors influencing bacterial penetration could be a valuable tool in planning intervention strategies. Penetration was assessed by replacing the egg contents with a selective medium that allowed visualising *Salmonella* growth on the inside of the shell by candling. Only eggshells without visual abnormalities were used.

Eggs from one ISA-Brown Warren laying flock were used to determine the effect of hen age and storage conditions. A faster appearance of penetration spots was observed with increasing storage temperatures possibly caused by increased growth rates of SE on the agar. Although shell characteristics differed with hen age, hen age did not influence SE penetration. Eggshell penetration varied greatly between eggs bought at a warehouse. Only 6% of the eggs from “free range” hens and 16% of the “white” eggs were contaminated. The remaining eggs (bio, columbus and brown) were contaminated at a frequency of ca 33%.

Eggs from both ISA-Brown Warren and Bovan Goldline hens kept in 2 types of housing system (furnished cage versus aviary) and fed 2 types of feed (standard feed versus feed containing corn cob mix) were tested. The breed of the hen neither the housing system did affect the eggshell penetration by SE. Eggs from hens fed CCM were more frequently penetrated. Hence, the feed can play an important role to reduce eggshell penetration.

No correlations were observed between any of the shell characteristics studied and the ability of SE to penetrate the shell, although the egg(shell) characteristics of the various eggs differed greatly. One factor that is of major significance is the growth of SE on the shell because shell contamination at the end of storage and SE penetration were highly correlated. Thus, SE growth on the shell should be prevented.

**Introduction**

In 2003 in Belgium, *Salmonella enterica* serovar Enteritidis (SE) was the most frequently isolated *Salmonella* serovar (72%) (Imberechts and Dierick, 2004). Foods of animal origin, especially poultry, poultry products, eggs and egg products, are often implicated in sporadic cases and outbreaks of human salmonellosis (Bryan and Doyle, 1995). Foods associated with SE outbreaks include eggs and egg products in 68.2% of the cases (WHO, 2001).

Two possible routes of *Salmonella* contamination of intact eggs have been considered: direct contamination of yolk or albumen originating from *Salmonella* infection of the reproductive organs before the eggs are covered by shell, and *Salmonella* penetration through the eggshell after the eggs are covered by the shell (Miyamoto et al., 1998). The egg possesses three physical barriers to the latter transmission. These are a hydrophobic proteinaceous layer (the cuticle) which overlies the shell and the pore mouths, the crystalline shell itself and the membranes which separate the shell and the albumen (Haigh and Betts, 1991). While the numerous pores of the eggshell represent portals of entry, their function as primary routes of transfer is of secondary importance to structural defects that offer a much easier route (Nascimento et al., 1992; Solomon, 1997). The mature cuticle interferes with bacterial invasion by closing the pores (Berrang et al., 1999). Conflicting results on the effect of shell thickness on bacterial penetration have been found (Messens et al., 2005).

The objective of our study was to determine the effect of the environmental conditions the eggs are exposed to on eggshell penetration by *Salmonella*. Also the influence of various hen related factors on eggshell quality and eggshell penetration by *Salmonella* was studied. First, the influence of hen age was evaluated. Second, it was checked to what extent bacterial penetration differs for eggs bought in...
a warehouse. Third, the influence of breed of the hen, housing system and feed was studied. For each study, the eggshell characteristics were determined and it was tried to evaluate how they could be linked to bacterial penetration.

**Materials and methods**

**EGGS**

Eggshell crack detection was based on acoustic resonance frequency analysis. Eggs were also candled and stored overnight at 20°C until use. Only intact eggs were used.

**AGAR MOULDING TECHNIQUE**

An agar moulding technique developed by Board and Board (1967) was used to visualise SE penetration. The egg contents were replaced by sterile molten (50°C) plate count agar containing 25 ppm streptomycin and 0.1% 2,3,5-triphenyl tetrazolium chloride. The agar-filled eggs were inoculated, placed in the climate chamber and candled, daily at first and three times a week later, to record the number of red colonies visible on the molten agar. Where bacterial penetration had occurred, the streptomycin-resistant SE bacteria grew and reduced the tetrazolium compound to formazan, which is red in colour.

**INOCULATION AND STORAGE**

SE MB1409 was used, an own isolate from an egg content, that was made resistant to streptomycin. Whole and agar-filled eggs were exposed to the streptomycin resistant SE MB1409 by dipping for 1 min in the immersion solution (saline (0.85% NaCl) containing on average $5.3 \times 10^6$ colony-forming units SE (cfu)/ml). Both the eggs and the immersion solution were at 20°C. This resulted in ca 2.6 log cells per eggshell. All eggs remained at ambient conditions until dry and then placed in a climate chamber at 20°C and 60% relative humidity (RH), unless otherwise stated.

**DETERMINATION OF SHELL CONTAMINATION**

At the end of storage, the shell contamination of all agar-filled eggs was examined. Each egg was placed in a plastic bag containing 10 ml PBS and its shell was rubbed by hand for 1 min. Enumeration of *Salmonella* in the PBS solution was done by plating out 1 ml and 100 µl of serial dilutions on XLD agar and incubation for 24 h at 37°C. In case of no counts, the PBS solution was enriched with BPW and incubated overnight at 37°C before plating.

In case of no colonies in 1 ml PBS solution, but a positive plate after enrichment of this PBS solution, counts were given 5 cfu/eggshell. In case of no colonies after enrichment, counts were given of 1 cfu/eggshell to allow a log transformation.

**CUTICLE STAINING, POROSITY AND SHELL THICKNESS**

When the penetration experiment was completed, the cuticle was stained by immersion in an aqueous solution containing per litre 7.2 g Tartrazine and 2.8 g Green S for a period of 1 min. The shell was then rinsed in distilled water to remove excess dye prior to drying (Board and Halls, 1973).

The shell porosity was determined using a method described by Tyler (1953). Pieces of shell taken from the entire egg were immersed for 25 sec in 65% nitric acid. The pieces were rinsed with distilled water, placed under a light microscope (eye-piece $\times 10$; objective $\times 10$) and the amount of pores visible on each area focussed on (ca 3 mm$^2$) was counted.

The shell thickness was determined with a micrometer on three places. The mean value was used for calculations.

The dynamic stiffness and the damping ratio of each egg were determined using the lab scale acoustic test apparatus.
EXPERIMENT 1
Eggs from ISA-Brown Warren hens were collected at the beginning (26 weeks), middle (42 weeks), end (65 weeks) and late end (69 weeks) of lay (Department of Animal Nutrition and Husbandry, section Small Stock Husbandry, Agricultural Research Centre-Ghent, Belgium). 100 eggs were used for each condition. At the middle of lay, eggs were stored at temperatures between 15 and 25°C and RH between 45 and 75%.

EXPERIMENT 2
Various types of eggs were bought at a warehouse (Table 1) to obtain a wide range of egg(shell) characteristics. The LSL hens were moulted 4 months before collection of the eggs.

Table 1 Types of eggs used in experiment 2.

<table>
<thead>
<tr>
<th>Type of egg</th>
<th>Colour</th>
<th>Size</th>
<th>Breed of the hen</th>
<th>Hen age</th>
<th>Number of eggs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bio</td>
<td>Brown</td>
<td>#</td>
<td>Shaver</td>
<td>32 weeks</td>
<td>50</td>
</tr>
<tr>
<td>Free range</td>
<td>Brown</td>
<td>L</td>
<td>Bovans</td>
<td>39 weeks</td>
<td>50</td>
</tr>
<tr>
<td>Columbus</td>
<td>Brown</td>
<td>L</td>
<td>ISA-Brown Warren</td>
<td>21 weeks</td>
<td>50</td>
</tr>
<tr>
<td>White</td>
<td>White</td>
<td>L</td>
<td>LSL (moulted)</td>
<td>88 weeks</td>
<td>50</td>
</tr>
<tr>
<td>Brown</td>
<td>Brown</td>
<td>L</td>
<td>ISA-Brown Warren</td>
<td>63 weeks</td>
<td>50</td>
</tr>
</tbody>
</table>

EXPERIMENT 3
Eggs from 2 breeds (ISA-Brown Warren versus Bovans Goldline) of hens at 25 weeks of age, kept in 2 types of housing system (furnished cage versus aviary) and fed 2 types of feed (standard feed versus feed containing corn cob mix) (Table 2) were collected at the Provincial Centre for Applied Poultry Research (Provincial Services for Agriculture and Horticulture of the Province of Antwerp, Geel, Belgium).

Table 2 Types of eggs used in experiment 3.

<table>
<thead>
<tr>
<th>Code</th>
<th>Breed of the hen</th>
<th>Housing system</th>
<th>Feed</th>
<th>Number of eggs</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>ISA-Brown Warren</td>
<td>Furnished cage</td>
<td>Standard</td>
<td>50</td>
</tr>
<tr>
<td>B</td>
<td>Bovans Goldline</td>
<td>Furnished cage</td>
<td>Standard</td>
<td>50</td>
</tr>
<tr>
<td>C</td>
<td>Bovans Goldline</td>
<td>Aviary</td>
<td>Corn cob mix (CCM) added</td>
<td>50</td>
</tr>
<tr>
<td>D</td>
<td>Bovans Goldline</td>
<td>Aviary</td>
<td>Standard</td>
<td>50</td>
</tr>
</tbody>
</table>

Results

EXPERIMENT 1
The effect of the hen age on the percentage of penetrated eggshells as a function of storage time is depicted in Figure 1a. At 20°C, most eggshells were penetrated on day 3. The laying period did not significantly influence the fraction of penetrated eggshells although a trend was visible towards a lower fraction of penetrated shells as the flock aged. On average 39.1% of the eggshells were penetrated at 20 days of storage.

The eggshell penetration of agar-filled eggs that were stored at various environmental conditions is shown in Figure 1b. Eggs were from hens at the middle of lay. Eggshell penetration by SE MB1409 varied depending on the temperature of storage and particularly the initial rate of penetration was affected. At higher temperatures a faster appearance of red spots on the agar within the eggs was visible: at 25°C, eggshell penetration was observed most frequently at day 2; at 20°C and 15°C, most eggshells were penetrated at day 3 and day 6, respectively.

Although Salmonella penetration was not influenced by the hen age, hen age affected some eggshell characteristics (Table 3). At the middle of lay (week 42 of hen age), the mean shell thickness, the mean cuticle deposition and the mean amount of pores were highest.
Table 3  Egg(shell) characteristics of eggs from ISA-Brown Warren hens at various ages\(^*\).

<table>
<thead>
<tr>
<th>Laying period</th>
<th>(N)</th>
<th>Egg weight (g)</th>
<th>Shell thickness (mm)</th>
<th>Cuticle score</th>
<th>Number of pores per shell</th>
<th>Salmonella shell contamination (log cfu/shell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beginning of lay</td>
<td>100</td>
<td>57.3 ± 3.9(^a)</td>
<td>0.417 ± 0.033(^b)</td>
<td>162 ± 24(^b)</td>
<td>2200 ± 1200(^b)</td>
<td>1.4 ± 1.8(^b)</td>
</tr>
<tr>
<td>Middle of lay</td>
<td>95</td>
<td>63.4 ± 4.0(^b)</td>
<td>0.455 ± 0.030(^d)</td>
<td>184 ± 27(^a)</td>
<td>3100 ± 1400(^c)</td>
<td>0.8 ± 1.5(^a)</td>
</tr>
<tr>
<td>End of lay</td>
<td>96</td>
<td>65.1 ± 4.7(^c)</td>
<td>0.395 ± 0.028(^b)</td>
<td>167 ± 20(^b)</td>
<td>2000 ± 1100(^b)</td>
<td>1.3 ± 1.6(^b)</td>
</tr>
<tr>
<td>Late end of lay</td>
<td>98</td>
<td>64 ± 5.7(^b)</td>
<td>0.386 ± 0.030(^a)</td>
<td>154 ± 26(^c)</td>
<td>1200 ± 860(^a)</td>
<td>0.7 ± 1.6(^a)</td>
</tr>
</tbody>
</table>

\(P\) *** *** *** *** **

Values are means ± SD, and means in the same column without common superscripts are significantly different (Duncan test).

\(N\) = number of eggs sampled; ND = not determined; **\(P<0.01\); ***\(P<0.001\).

EXPERIMENT 2

Eggshell penetration of a wide range of eggs was assessed (Figure 1c). At the end of storage, only 6% of the eggs from “free range” hens and 16% of the “white” eggs were contaminated. The remaining eggs (“bio”, “Columbus”, “brown”) were contaminated at a frequency of ca 33%.

Egg(shell) characteristics of these various eggs differed greatly (Table 4). The shells of eggs from “free range” hens and these of the “white” eggs were thinnest. The cuticle score of “white” and “brown” eggs were worst. Also, parameters of acoustic resonance frequency analysis differed.

Table 4  Egg(shell) characteristics of various eggs from a warehouse\(^*\).

<table>
<thead>
<tr>
<th>Type of egg</th>
<th>(N)</th>
<th>Egg weight (g)</th>
<th>Shell thickness (mm)</th>
<th>Cuticle score</th>
<th>Number of pores per shell</th>
<th>(k_{\text{dy}n})×100 (N/m)</th>
<th>Damping (%)</th>
<th>Salmonella shell contamination (log cfu/shell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bio</td>
<td>20</td>
<td>60 ± 4(^a)</td>
<td>0.40 ± 0(^b)</td>
<td>179 ± 32(^a)</td>
<td>1100 ± 500</td>
<td>14 900 ± 800(^b)</td>
<td>3.6 ± 1.2</td>
<td>0.8 ± 1.4(^b)</td>
</tr>
<tr>
<td>Free range</td>
<td>20</td>
<td>67 ± 3(^c)</td>
<td>0.38 ± 0.02(^a)</td>
<td>186 ± 22(^b)</td>
<td>1500 ± 800</td>
<td>15 000 ± 1000(^b)</td>
<td>2.9 ± 0.7</td>
<td>0.1 ± 0.5(^a)</td>
</tr>
<tr>
<td>Columbus</td>
<td>20</td>
<td>63 ± 3(^c)</td>
<td>0.41 ± 0.03(^b)</td>
<td>174 ± 29(^a)</td>
<td>1500 ± 800</td>
<td>14 100 ± 7000(^a)</td>
<td>3.8 ± 0.4</td>
<td>0.8 ± 1.6(^b)</td>
</tr>
<tr>
<td>White</td>
<td>20</td>
<td>70 ± 4(^d)</td>
<td>0.37 ± 0.03(^a)</td>
<td>132 ± 49(^b)</td>
<td>16 500 ± 1100</td>
<td>16 000 ± 8000(^c)</td>
<td>2.4 ± 0.6</td>
<td>0.7 ± 1.7(^b)</td>
</tr>
<tr>
<td>Brown</td>
<td>20</td>
<td>67 ± 2(^c)</td>
<td>0.41 ± 0.03(^b)</td>
<td>141 ± 1000</td>
<td>16 000 ± 8000(^c)</td>
<td>2.9 ± 0.6</td>
<td>0.9 ± 1.5(^b)</td>
<td></td>
</tr>
</tbody>
</table>

\(\*) *** *** *** NS *** *** *

Values are means ± SD, and means in the same column without common superscripts are significantly different (Duncan test).

\(N\) = number of eggs sampled; NS = not significant; *\(P<0.05\); ***\(P<0.001\).

EXPERIMENT 3

Eggshell penetration of eggs from 2 breeds, kept in 2 types of housing systems and fed 2 types of feed was assessed (Figure 1d). In this experiment, the breed of the hen did not affect the eggshell penetration by SE (\(A\) versus \(B\); \(P=0.84\)). Also, the housing system (\(B\) versus \(D\); \(P=0.55\)) did not give different penetration probabilities. The feed on the other hand influenced the fraction of penetrated eggshells: eggs from hens fed CCM were more frequently penetrated (although not significant: \(P=0.16\)) (\(C\) versus \(D\)).

Only the number of pores and the damping parameter were slightly affected by the type of egg in this experiment.
Figure 1  Effect of storage time on the fraction of penetrated eggshells upon storage of agar-filled eggs at 20°C-60% RH unless otherwise stated. Various types of eggs were used; see for (a) and (b) experiment 1, (c) experiment 2, (d) experiment 3.
**Correlations between shell characteristics and SE penetration**

All penetration results (920 eggs in total) obtained so far were pooled to analyse whether SE penetration is correlated with one of the egg(shell) characteristic studied. The probability of SE penetration through the eggshell was not correlated with the egg weight, shell thickness, number of pores, cuticle deposition on the shell, dynamic shell stiffness or damping. The shell contamination at the end of storage ($P<0.0001$) correlated with SE penetration (Figure 2).

![Figure 2: Correlation between eggshell penetration and *Salmonella* contamination on the shell at the end of storage.](image)

**Discussion**

Temperature enhanced the rate of appearance of red spots on the agar within the eggs, possibly due to the faster growth of SE on the agar at higher temperatures and not solely due to the faster speed of penetration. At the end of storage, the same fraction of eggs finally got contaminated, indicating that eggshells are prone to get penetrated at the same frequency at all conditions studied. Although the shell characteristics studied changed throughout the hen age, hen age and ability to penetrate were not correlated. This is not surprising as none of the shell characteristics studied influenced SE penetration. First, the number of pores of the eggshell did not affect the ability of SE to penetrate the shell. This is in accordance to what has been found by Nascimento *et al.* (1992) but is conflicting to earlier findings (Kraft *et al.*, 1958). Second, no relationship was found between shell thickness and the likelihood of SE to penetrate the eggshell, in agreement with other studies (Kraft *et al.*, 1958; Williams *et al.*, 1968). Sauter and Petersen (1974) however observed that eggs with low specific gravity, and hence thinner shells, were more likely to be penetrated by *Salmonella*. Third, the ability of SE to penetrate the eggshell did not depend on the cuticle deposition. The first-line defence of the cuticular layer has already been questioned by Nascimento *et al.* (1992). It should be noted that only intact eggs were selected for our study. As flock age increased towards the late end of lay, more eggshells showed abnormalities. These abnormalities will give rise to very frequent eggshell penetration (13 eggshells were penetrated on a total of 14 eggshells with cracks, i.e. 93%). Even when taking into account these abnormalities, eggs from older hens were not penetrated more frequently.

Eggshell penetration probabilities of a wide range of eggs bought at a warehouse differed greatly. Eggs from free range hens and white eggs were least frequently penetrated. The limited influence of hen age and shell thickness can be deduced from this experiment as well, as these eggs had thinner shells and the white eggs were from old moulted hens. The breed of the hen could have influenced the
penetration probabilities, as the results were grouped: the eggs with more frequent eggshell penetration were from ISA-Brown Warren or Sheaver hens. The eggs with less frequent penetration were from LSL and Bovan hens. Because this experiment was not standardised (hen age differed, some moulting occurred, feed differed), conclusions can not be drawn. Also, the addition of oyster shell supplement to the "free range" hens could have led to the lower eggshell penetration frequency. The different housing systems could also have affected eggshell penetration. A standardised experiment including eggs from both ISA-Brown Warren and Bovan Goldline hens revealed that the breed of the hen did not affect the eggshell penetration by SE. Also, the housing system (furnished cage versus aviary) did not give different penetration probabilities. The feed on the other hand influenced the fraction of penetrated eggshells: eggs from hens fed corn cob mix were more frequently penetrated. Hence, the feed administered to the hens could be very important for preventing of bacterial penetration. More research should be done on this topic.

The outgrowth of SE on the shell during storage significantly influenced the penetration ability. Eggshells that were penetrated had a significantly higher contamination on the shell at the end of storage. Why some eggs allow SE growth on the shell and others do not need to be investigated.

Acknowledgements
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References