Penetration of *Salmonella* Enteritidis through the vitelline membrane of hen's eggs as affected by its strength during the laying period

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Abbreviated title: Vitelline membrane : penetration and strength

Summary

Eggs have been implicated as leading source of human salmonellosis caused by *Salmonella enterica* serovar Enteritidis (SE). Although SE is not often deposited directly inside the yolks of naturally contaminated eggs, penetration through the vitelline membrane to reach the yolk contents could result in rapid bacterial multiplication. This study was conducted to determine whether the penetration of SE through the vitelline membrane is affected by the vitelline membrane strength (VMS). The VMS and penetration were determined for successive eggs. At the beginning, middle and end of lay, five series of successive eggs were collected of 24 hens from one laying flock. For the penetration study, an *in vitro* egg contamination model was used enabling sampling of the yolk contents as a function of egg storage (up to three weeks). The VMS decreased from the beginning to the middle of lay, but subsequently stayed

constant until the end of lay. The proportion of vitelline membrane samples that were penetrated, was hardly affected by the laying period. The time of penetration varied strongly within the group of hens and within one hen. Combining all results, only a slight but significant correlation (R=0.1692 ; p=0.011) was found between the VMS and the moment of penetration.

Keywords: *Salmonella* Enteritidis, hen's egg, vitelline membrane, penetration, vitelline membrane strength

Introduction

Th Eggs have been implicated as leading source of human salmonellosis caused by Salmonella enterica serovar Enteritidis (SE) (EFSA, 2007). Contamination of eggs can occur via two possible routes : horizontal or vertical. The term horizontal contamination is used when SE penetrates the eggshell and the shell membranes after contamination of the shell surface. Vertical contamination can occur when SE is introduced from infected reproductive tissues to eggs. So freshly laid eggs can be contaminated with SE at various sites – the yolk, the vitelline membrane, the albumen, the shell membranes or the eggshell originating from the infection of the ovary, the infundibulum, the magnum, the isthmus or the shell gland respectively. SE contamination is observed most frequently on the vitelline membrane or the albumen (Gast et al., 2002; Gast and Beard, 1992). SE can survive in the albumen and at sufficiently high temperatures even grow and migrate to the yolk (Grijspeerdt et al., 2005). The yolk is an excellent medium for growth at supportive temperatures. As a consequence, SE penetration through the vitelline membrane can allow rapid and extensive multiplication. Thus it is of interest to investigate the correlation between the SE penetration through the vitelline membrane and its characteristics.

Materials and methods

Eggs

Eggs from 24 Lohman hens, maintained in conventional cages, were obtained from a test flock at the Animal Sciences Unit (Institute for Agricultural and Fisheries Research, ILVO, Belgium). At the beginning (27-29 weeks of hen age), middle (42-44 weeks of hen age) and end (57-60 weeks of hen age) of lay, five series of successive eggs were

collected. Of each two successive eggs, the first one was used to measure vitelline membrane penetration and the second one to determine the vitelline membrane strength.

Bacterial strain and cultures

Salmonella enterica serovar Enteritidis (SE) MB1409 was used, a strain that was isolated at our laboratory from egg contents. To allow inoculating the yolk membrane with a replicable number of cells, a stock solution of this strain was stored in buffered peptone water (BPW, Oxoid, Basingstoke, Hampshire, UK) with 15% glycerol and kept at -80°C. Before use, the strain was resuscitated at 20°C for 70 minutes, after a tenfold dilution in BPW. This resuscitated solution was then diluted to obtain an inoculum solution with on average 546 ± 212 (standard error of 67.12) cells of SE per 10 µl.

Vitelline membrane penetration

The eggshells were first decontaminated by dipping the eggs in hydrogen peroxide (30%) for 10 seconds, followed by sprinkling the eggs with 75% ethanol and burning off the alcohol during circa 5 seconds, as described in De Reu et al. (2007). The eggs were cracked and the yolk separated from the albumen, using a sterilised egg divider. The yolk was gently rolled down a sterile and moist paper towel that soaked up the remaining albumen. The intact yolk was placed in a modified Falcon tube having a septum at the bottom enabling to withdraw samples from the yolk contents over time (Fleischman et al., 2003). The yolk was first inoculated with 10 µl of the inoculum solution. Then, above the inoculated yolk, pasteurised liquid albumen (Coco vite, Lodewijckx nv, Veerle-Laakdal, Belgium) was introduced and the tubes were stored in a climate chamber (Termaks KBP 6395 F, Solheimsviken, Norway) at 20°C and 60% relative humidity for up to 21 days.

At regular times – 3, 4, 5, 7, 10, 14 and 21days after inoculation – a needle (1.1 x 40 mm) was put through the decontaminated septum to enter the yolk and withdraw a sample of the yolk at 0.5-1.0 cm under the top surface of the membrane. The sample was plated on xylose lysine desoxycholate agar plates (XLD agar, Oxoid), using a spiral plater and the plates were incubated at 37°C for 24 h.

Vitelline membrane strength (VMS)

The measurements of the VMS were carried out using a texture analyser (Lloyd Instruments Limited, Fareham, Hampshire, UK) with a 20 N load cell and a cylindrical probe with a diameter of 2 mm. The egg was cracked and the egg contents were placed on a plastic plate. Then the probe was placed at the center of the top of the yolk and the compression test with a speed of 42 mm/min was started and the VMS measured.

Statistical analysis

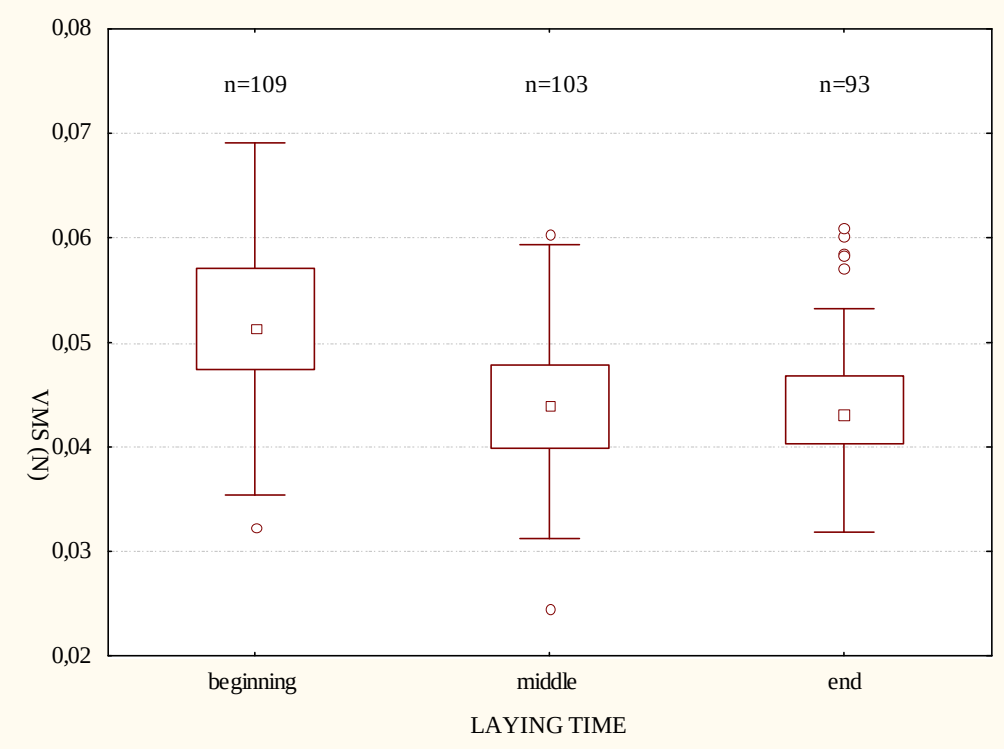
A one-way analysis of variance (ANOVA) was used to assess whether the mean VMS is influenced by the laying period. The Tukey's Honest Significant Difference test was used to identify pairs with significant differences. The standard Pearson correlation coefficient (r) and the associated significance level was calculated using VMS-VMS and VMS-moment of penetration. The significance level α was set at 0.05. All analysis were done in Statistica 8.0 (Statsoft Inc., Tulsa, Okla.).

Results

The proportion of vitelline membrane samples that were penetrated during storage, was hardly affected by the laying period. After one week of storage, 43, 57 and 55% of the vitelline membranes of eggs collected at the beginning, middle and end of lay respectively, were penetrated. At the end of storage, respectively 9, 12 and 14% of the vitelline membranes were not penetrated. The moment of penetration varied strongly both for eggs from various hens and from one hen.

Figure 1 shows the VMS of eggs collected at the beginning, middle and end of lay. The mean VMS of eggs collected at the beginning of lay (0.0520 ± 0.0076 N; n=109) versus the middle of lay (0.0437 ± 0.0061 N; n=103) differed significantly. However, there was no significant difference between the mean VMS of eggs collected at the middle and the end of lay (0.0440 ± 0.0061 N; n=93).

Figure 1 : Vitelline membrane strength of eggs collected at the beginning, middle and end of lay. The number of vitelline membranes tested (n) is shown. The small square within the box marks the median. The boundaries of the box represent the 25th and 75th percentiles. Whiskers above and below the box indicate the 10th and 90th percentiles. The outliers are shown.

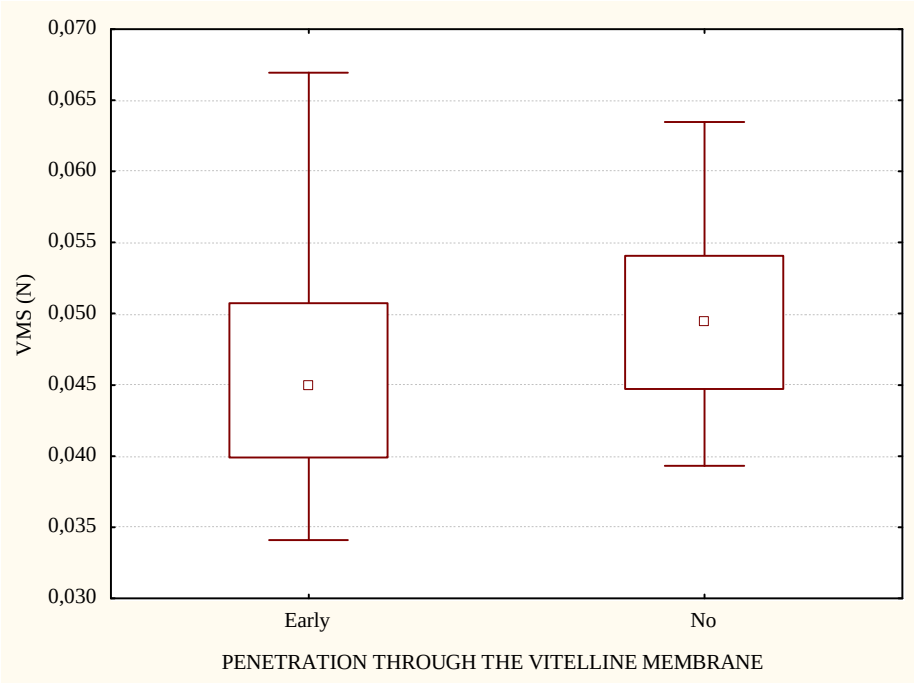


To be certain that successive eggs can be used to investigate the correlation between the VMS and the moment of penetration, it needed to be determined that successive eggs of the same hen have comparable VMS values. Combining results at the beginning, middle and end of lay, the correlation in VMS of eggs from the same hen was studied. A significant ($p < 0.001$) and strong correlation (Pearson correlation coefficient $r = 0.6151$) was found between the VMS of eggs from one hen being laid with two days difference. As the time between lay increased, the Pearson correlation coefficient decreased (to 0.5117 with 7 days difference in laying time and 0.4005 with 19 days difference).

When combining the results of the vitelline membrane penetration and the VMS of eggs collected at the beginning, middle and end of lay, a slight ($r = 0.1692$) but significant ($p = 0.011$) correlation was found between the time of penetration through the vitelline membrane and the VMS of two successive eggs of the same hen.

Taking into account only the extremes of penetration, meaning penetration at day three, four or five versus no penetration, there's a significant difference in the mean VMS values. The VMS was significantly ($p = 0.014$) lower for the first compared to the latter.

Figure 2. Vitelline membrane strength of the eggs succeeding eggs with early (at day three, four or five) or no penetration through the vitelline membrane. The small square within the box marks the median. The boundaries of the box represent the 25th and 75th percentiles. Whiskers above and below the box indicate the 10th and 90th percentiles.



Conclusion

Due to the significant and strong correlation between VMS of eggs from one hen being laid with two days difference, we can suggest that successive eggs have a comparable VMS. Consequently, successive eggs can be used to test the hypothesis that the occurrence of SE penetration through the vitelline membrane is correlated with the VMS. This study displays a slight but significant correlation between both. It can thus be assumed that a higher VMS leads to a longer resistance of the vitelline membrane against penetration by SE.

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