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Eggshell penetration of hen's eggs by *Salmonella enterica* serovar Enteritidis upon various storage conditions

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Abstract 1. The survival and penetration of *Salmonella enterica* serovar Enteritidis (SE) inoculated on the eggshell was examined upon storage for up to 20 d at real-life conditions (15 to 25°C and 45 to 75% relative humidity (RH)).

2. Penetration was assessed by emptying the egg contents and filling the eggs with a selective medium that allowed visualising *Salmonella* growth on the inside of the shell and membrane complex.

3. The study of survival on the eggshells was based on viable counts and showed that numbers of surviving organisms decreased over time. Survival was inversely related to storage temperature and RH. Although the average counts decreased over time, a limited proportion of shells carried high numbers of SE at all storage conditions.

4. Penetration spots were observed earlier using an increased storage temperature due to increased growth rates of SE on the agar. After 20 d of storage a similar percentage (*c*. 44·7%) of eggshells became penetrated, irrespective of the storage conditions tested in this study.

5. The higher the *Salmonella* shell contamination at the end of storage, the higher the probability that the eggshell was penetrated. *Salmonella* shell counts exceeding 4 log cfu yielded more than a 90% probability of eggshell penetration occurring.

INTRODUCTION

Eggshells can be penetrated by various bacteria, among which is *Salmonella enterica* serovar Enteritidis (SE). This serotype is linked with human salmonellosis cases caused by the consumption of shell eggs and egg products. In Belgium, in 2004, 9543 cases were reported, of which 63·6% were caused by serovar Enteritidis (Collard and Bertrand, 2005). Various studies have been conducted to examine the probability of *Salmonella* penetration through the eggshell. The major extrinsic factors identified as being important to the transmission of *Salmonella* through the shell are: bacterial strain, temperature differential, moisture, number of organisms present on the eggshell and storage conditions. The most important intrinsic factor affecting SE penetration is the presence of shell defects, such as cracks (Messens *et al.*, 2005*b*).

The process responsible for eggshell contamination by infected birds is not yet clear. Shell contamination most likely depends on either intestinal and oviduct infection. The egg surface can be contaminated with faeces containing *Salmonella* during expulsion of the egg from the hen, that is, intestinal infection (Gast and Beard, 1990). The egg surface can also be contaminated within the hen reproductive system after formation of the shell, that is, oviduct contamination (Humphrey *et al.*, 1991). Following oviposition, the shell acquires contamination from all surfaces with which it makes contact and the extent of contamination is directly related to the cleanliness of these surfaces (Board and Tranter, 1995). Contamination in egg-packing plants may also be

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a significant contributory factor to external contamination of shell eggs by *Salmonella* (Davies and Breslin, 2003*a*) and other bacteria (De Reu *et al.*, 2005).

Salmonella organisms on eggshells can die rapidly during storage but survival is enhanced by low temperatures (Rizk *et al.*, 1966; Baker, 1990; Braun *et al.*, 1999; Radkowski, 2002), especially when relative humidities (RHs) are low (Simmons *et al.*, 1970). Salmonellae probably survive for a longer time under lower temperature due to the slower metabolism induced by the disadvantageous conditions on the dry eggshell surface (Radkowski, 2002). The presence of *Salmonella* on the surface of the shell can give rise to cross-contamination in the kitchen and to eggshell penetration.

This study was designed to examine the effect of storage conditions on survival/growth of SE on the eggshell and on the ability of SE to penetrate the shell. The storage conditions were specified in such a way to resemble the environment the eggs may be exposed to in Western Europe: in the egg-laying farm in summer (25°C–75% RH) or winter (15°C–75% RH), in the egg-packing plant (15°C–45% RH to 20°C–60% RH) and in the store in the summer (25°C–45% RH) or winter (15°C–75% RH) (personal communication with François Porin, INRA-LORIA, Ivry sur Seine, France).

MATERIALS AND METHODS

Eggs

Eggs from ISA Brown Warren hens maintained in conventional cages were obtained from a test flock at the ILVO Animal Unit, Merelboke, Belgium). Eggs were collected in the period January to June 2003 at the middle (34 to 51 weeks of hen age) of lay. No egg out of 30 eggs sampled tested positively for antibiotics (Premi® Test-Egg kit) or *Salmonella* antibodies (Desmidt *et al.*, 1996). Only intact eggs assessed by candling without visual faecal or egg contents contamination of the shell were taken. The eggs were stored overnight at 20°C until use and were only 24 h post lay at the start of the study.

Bacterial strain and cultures

S. Enteritidis MB1409 was used, a strain that was isolated at our laboratory from egg contents and made resistant to streptomycin. Bacteria were resuscitated, then plated and incubated at 37°C as previously described (Messens *et al.*, 2005*a*). Next, one colony was grown overnight at 37°C in 9 ml BPW containing 25 ppm streptomycin and diluted (to 10^{-2}) with phosphate buffered saline (PBS; Oxoid, Basingstoke, UK). The count of viable SE cells in this immersion solution was $5{\cdot}3 \times 10^6$ colony-forming units (cfu)/ml. Enumeration was done by serial decimal dilution as described (Messens *et al.*, 2005*a*).

Agar moulding technique

An agar moulding technique was used (Messens *et al.*, 2005*a*), in which the egg contents were replaced by sterile molten (50°C) PCA (Oxoid) containing 25 ppm streptomycin and 1 g/l 2, 3, 5-triphenyl tetrazolium chloride (TTC; Sigma-Aldrich, Bornem, Belgium). Adding streptomycin to the agar ensured that only resistant bacteria could grow, thus inhibiting competitors of the resistant SE. TTC is reduced by growing bacteria, resulting in dark red colours. At the end of the experiment, all agar-moulded eggs were aseptically opened. On some eggs the inside of the inner membrane was swabbed at the red spots with a sterile cotton-tipped applicator. The applicator was used to transfer the bacteria to a plate of xylose lysine desoxycholate (XLD; Oxoid) agar and incubated for 24 h at 37°C. Growth of *Salmonella* was obvious on the plates.

The agar-filled eggs were inoculated and placed in the climate chamber. Penetration was recorded as the number of red colonies visible on the agar when the agar-filled eggs were candled carefully, preventing cross-contamination, daily during the first week and three times a week later. A TSA (Oxoid) plate was inoculated with the SE strain and simultaneously incubated in the climate chamber to control for the time gap for growth. This is necessary because penetration can only be recorded by observing growth of *Salmonella* on the agar.

Inoculation and storage

For each experiment, *c.* 340 whole eggs and 100 agar-filled eggs were exposed to streptomycin resistant SE MB1409 by dipping for one minute in the immersion solution. Both the eggs and the immersion solution were at 20°C. All eggs remained at ambient conditions until dry and were then placed in a climate chamber (Termaks KBP 6395 F, Solheimsviken, Norway) for up to 20 d.

Determination of shell contamination

On the day of inoculation the *Salmonella* shell contamination of 20 whole eggs was analysed to determine the inoculation dose. Also, 3, 8, 15 and 20 d later, *c.* 80 whole eggs were taken at random from the climate chamber and their

Table 1. *Percentage of eggshells positive for Salmonella by enrichment and percentage of eggshells having counts > 2·57 log cfu/shell (that is, mean inoculation count; in italics) as a function of storage time at various environmental conditions*[1]

	Temperature and relative humidity of egg storage				
Storage time (d)	15°C–45% RH	15°C–75% RH	20°C–60% RH	25°C–45% RH	25°C–75% RH
3	91·3 (82·8–96·4)^{c}	42·5 (31·5–54·1)^{b}	93·7 (85·9–97·9)^{c}	91·3 (82·8–96·4)^{c}	14·2 (7·4–24·1)^{a}
	3·8 (0·8–10·6)	*10·0 (4·4–18·8)*	*1·3 (0–6·9)*	*6·3 (2·1–14·0)*	*10·4 (4·6–19·5)*
8	88·8 (79·7–94·7)^{d}	27·9 (18·4–39·1)^{b}	76·3 (65·2–85·3)^{cd}	66·7 (55·1–76·9)^{c}	15·4 (8·2–25·3)^{a}
	6·3 (2·1–14·0)	*11·4 (5·3–20·5)*	*6·6 (2·1–14·7)*	*6·4 (2·1–14·3)*	*11·5 (5·4–20·8)*
15	87·5 (78·2–93·8)^{d}	20·0 (11·9–30·4)^{ab}	46·7 (35·1–58·6)^{c}	29·5 (19·7–40·9)^{bc}	10·1 (4·5–19·0)^{a}
	3·8 (0·8–10·6)^{ab}	*15·0 (8·0–24·7)^{b}*	*0 (0–3·9)^{a}*	*3·9 (0·8–10·8)^{ab}*	*8·9 (3·6–17·4)^{ab}*
20	71·3 (60·1–80·8)^{c}	25·3 (16·2–36·4)^{ab}	32·4 (22·0–44·3)^{b}	20·0 (11·9–30·4)^{ab}	8·8 (3·6–17·2)^{a}
	6·3 (2·1–14·0)	*15·2 (8·1–25·0)*	*2·7 (0·3–9·4)*	*3·8 (0·8–10·6)*	*7·5 (8·8–15·6)*

[1]Seventy-four to 80 eggs sampled. Means and 95% confidence interval (between parentheses) are given. Values in the same row without common superscripts are significantly different.

shell contamination was examined. At the end of storage, the *Salmonella* shell contamination of all agar-filled eggs was also determined.

Each egg was gently washed in 10 ml PBS (Oxoid) in a sterile plastic bag by rubbing the surface of the eggs, through the plastic bag, for one minute. Enumeration of *Salmonella* in the PBS solution was done by plating out 1 ml on XLD agar (Oxoid) and incubation for 24 h at 37°C. In case of high counts, serial decimal dilutions of the PBS solution (stored refrigerated overnight) in 1/4-strength Ringer solution (Oxoid) were made and plated out on XLD agar (Oxoid). In case of no counts, the PBS solution was enriched with BPW (Oxoid) at a ratio of 1:3 (v:v), incubated overnight at 37°C and a loop full of this solution was streaked on to an XLD (Oxoid) plate and incubated for 24 h at 37°C. When there were no colonies in 1 ml PBS solution, but there was a positive plate after enrichment of this PBS solution, a count of 5 cfu/eggshell (or 10 ml PBS solution) was given. When there were no colonies after enrichment, a count of 1 cfu/eggshell was given to allow log transformation.

Statistical analysis

One-way ANOVA was used to study the effect of the storage conditions on *Salmonella* shell contamination using Statistica 6.1 (Statsoft Inc., Tulsa, USA). Non-central interval estimation in the power analysis module was used to calculate the confidence intervals (CI) at 95% confidence of the percentage of penetrated eggshells at the various storage conditions.

RESULTS

Survival of *Salmonella* on the eggshell

The survival of SE MB1409, inoculated on the shell of whole eggs, was assessed during storage at various environmental conditions. The mean initial contamination dose was $2{\cdot}57 \pm 0{\cdot}80$ log cells per egg.

The percentage of shells positive for *Salmonella* by enrichment is depicted in Table 1. With increasing storage time, the percentage of contaminated shells decreased and was most pronounced at 75% RH. While more than 90% of shells remained contaminated after 3 d of storage at 60% and 45% RH, at 75% RH fewer than 43% remained contaminated. Particularly when combined with the higher temperature of 25°C, contaminated shells became scarce. At 45% RH, survival at 15°C was higher than at 25°C. When the eggs were stored at 20°C–60% RH, survival was comparable to storage at 25°C–45% RH.

The survival experiments showed considerable variability. Average *Salmonella* counts on the shell declined over time (Figure 1). However, following inoculation, bacteria either died off partly or completely (shells negative by enrichment, Table 1) or started to grow up to a maximum value of 7 log cfu/shell. Growth was observed in a similar proportion of eggs irrespective of the storage conditions tested (Table 1). Thus, although a large percentage (71·3%) of eggshells were positive after enrichment at 15°C–45% RH; growth was observed on only 6·3% of the eggshells, that is, on 8·8% of the eggshells that were positive after enrichment. In contrast, at 25°C–75% RH, only 8·8% of eggs were positive after enrichment, but on 86% of the positive shells growth was observed.

Influence of storage conditions on eggshell penetration

Eggshell penetration results of agar-filled eggs stored at various environmental conditions are shown in Figure 2. Storage temperature affected the initial rate of penetration by SE MB1409.

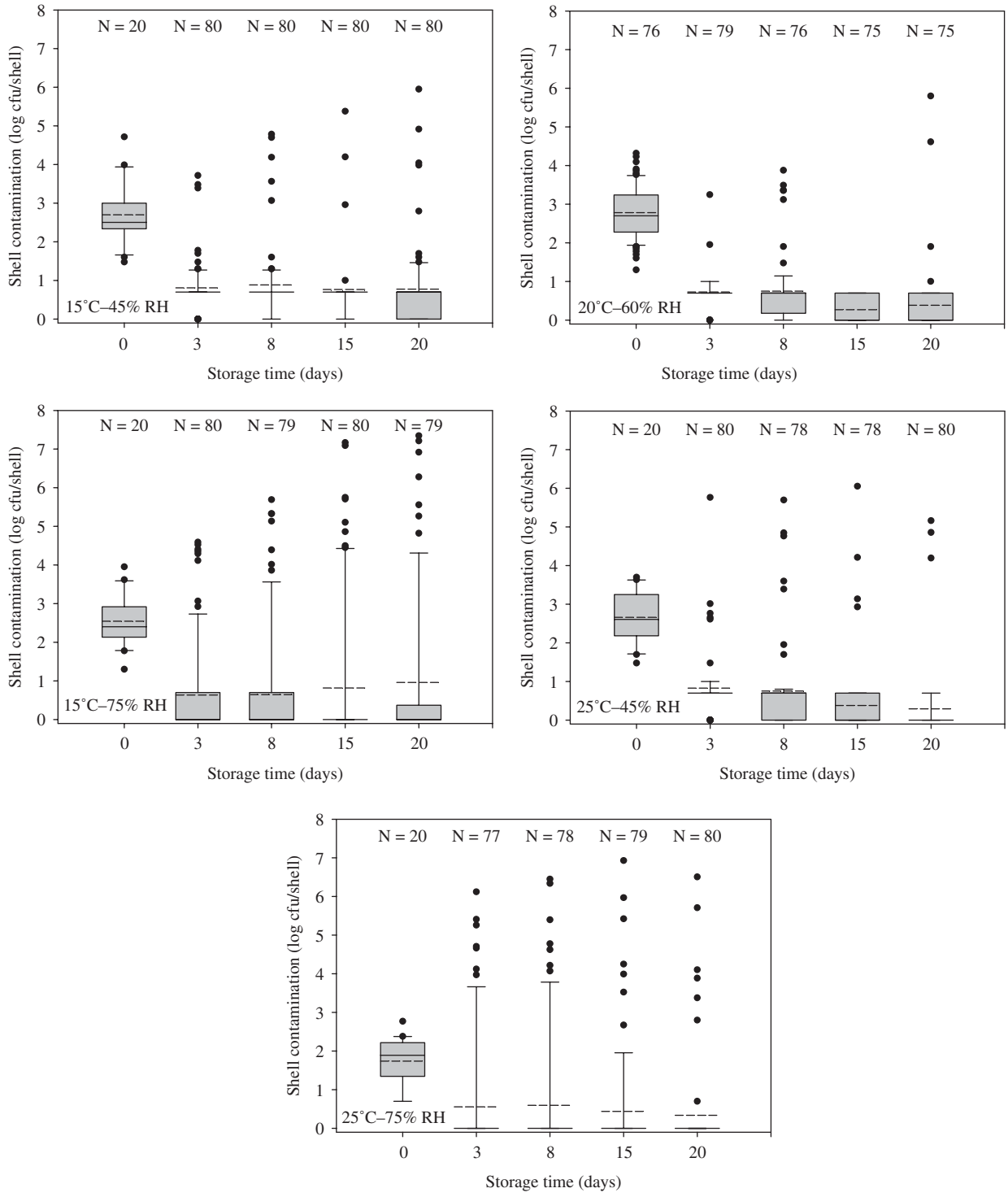

Figure 1. Salmonella *shell contamination on whole eggs as a function of storage time for various environmental conditions. The number of eggs sampled (N) is shown. The solid line within the box marks the median, while the dashed line marks the mean. The boundaries of the box represent the 25th and 75th percentiles. Whiskers above and below the box indicate the 10th and 90th percentiles. The outliers, all data points that lie outside the 10th and 90th percentiles, are shown as symbols.*

At higher temperatures red spots on the agar within the eggs appeared more quickly; at 25°C, shell penetration was observed most frequently at d 2; at 20 and 15°C, most shells were penetrated at d 3 and d 6, respectively. At d 7, 8 and 13, more than 95% of shell penetration was observed at 25, 20 and 15°C, respectively. Relative humidity of storage was not a significant factor. At the end of storage, on average 44·7% of eggs were contaminated. Although the percentage of penetrated eggs ranged from 35·4% (CI 25·9 to 45·8%) to 51·5% (CI 41·3 to 61·7%) for the various storage conditions, the differences were not significant.

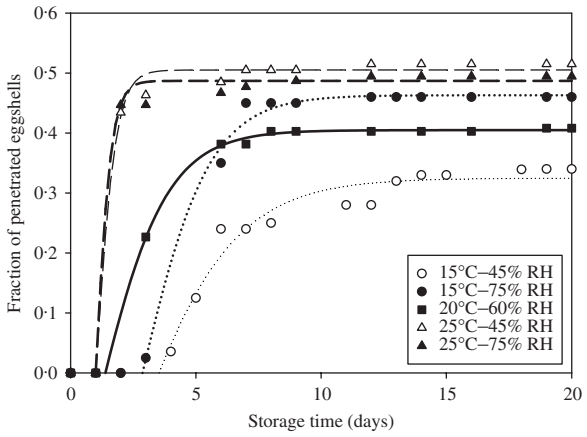

Figure 2. *Cumulative fraction of eggshells penetrated by* Salmonella *as a function of storage time at various storage conditions.*

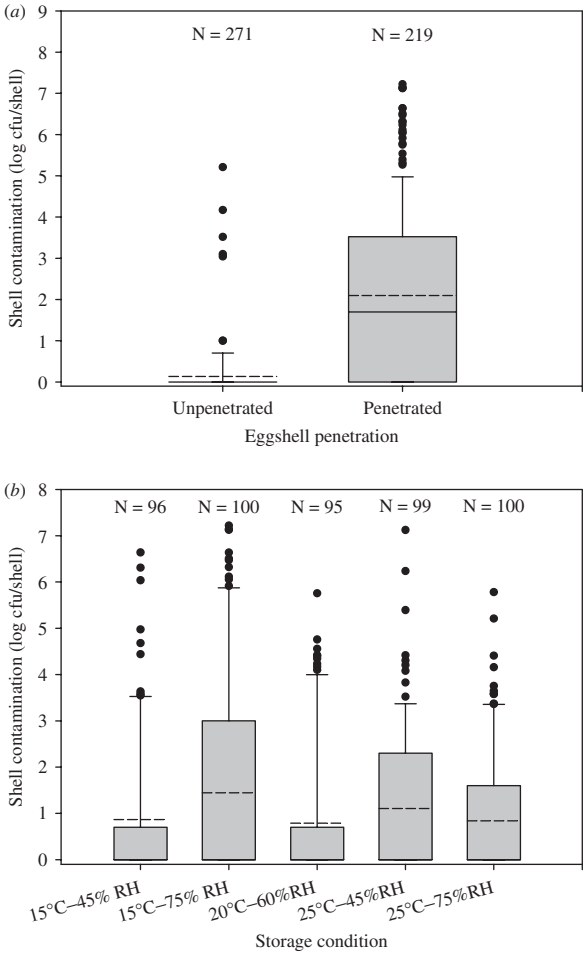

Figure 3. Salmonella *shell contamination of agar-filled eggs at 20 d of storage for (a) unpenetrated versus penetrated eggshells and for (b) various storage conditions. Box plot as in Figure 1.*

Influence of shell contamination on eggshell penetration

Using the agar approach (Figure 3*a*) shell contamination at 20 d of storage was higher ($P < 0{\cdot}0001$) for the shells that were penetrated compared to those that were not penetrated. The higher the contamination at the end of storage, the higher the probability that the eggshell will be penetrated (Table 2). Shell contaminations exceeding 4 log cfu yielded more than a 90% probability for an internally contaminated egg. Shells contaminated with a value of less than 10 cfu had a 50% probability of becoming internally contaminated.

In Figure 3(*b*), shell contamination of agar-filled eggs at 20 d of storage for the various storage conditions is depicted. The median value for each storage condition was 1 cfu/shell. *Salmonella* was not recovered from 59·3% (CI 48·9 to 69·3%), 63·0% (CI 52·8 to 72·4%), 67·4% (CI 57·0 to 76·7%), 52·5% (CI 42·2 to 62·7%) and 70·0% (CI 60·0 to 78·8%) of the shells after 20 d of storage at 15°C–45% RH, 15°C–75% RH, 20°C–60% RH, 25°C–45% RH and 25°C–75% RH, respectively. No significant difference was found in mean value on the shell for the various storage conditions ($P = 0{\cdot}094$).

Salmonella counts on the shell of whole (Figure 1) and agar-filled eggs (Figure 3) combining all storage conditions were compared. The percentage of shells where *Salmonella* could no longer be recovered from was 68·2% (CI 63·3 to 72·8%) for the whole eggs and 62·5% (CI 58·0 to 66·8%) for the agar-filled eggs. Statistical differences were not found.

Table 2. *Influence of* Salmonella *shell contamination at the end of storage on the percentage of penetrated eggshells*

Salmonella shell contamination	Penetrated eggshells (%)	*N*
Not contaminated	19	453
<1 log cfu/shell	51	113
1 to 2 log cfu/shell	68	41
2 to 3 log cfu/shell	86	36
3 to 4 log cfu/shell	90	62
4 to 5 log cfu/shell	95	39
5 to 6 log cfu/shell	94	18
6 to 7 log cfu/shell	92	13
≥7 log cfu/shell	100	5

N = number of eggs sampled.

DISCUSSION

Previous studies by our group (Messens *et al.*, 2005*a*; De Reu *et al.*, in press) indicated that *Salmonella* shell contamination at the end of storage correlated strongly with eggshell penetration. The present study was set up to examine the relationship between egg storage conditions (with corresponding survival/growth of SE on eggshells) and the penetration of SE through

the eggshell. Agar-filled eggs were used to study the actual penetration through the eggshell and its membranes, without interference of the albumen. In other studies, using whole eggs, no differentiation between penetration and survival/growth in albumen was made; for example, Braun *et al.* (1999) have shown penetration to be enhanced at higher temperatures although survival on the eggshell was poorer. De Reu *et al.* (in press) compared eggshell penetration (agar approach) and whole egg contamination (intact egg approach) for SE upon storage at 20°C and 60% RH: 43% of the agar-filled eggs were penetrated and 33% of the whole eggs. In that study, the behaviour of various bacterial species on the eggshell was compared. This showed that although some strains were able to penetrate, they could not pass the albumen barrier.

Prevention of eggshell penetration or cross-contamination from eggshells contaminated with *Salmonella* can be achieved by decontaminating the eggshell. Several methods can be applied: washing eggs (Hutchison *et al.*, 2004), ionising radiation (Farkas, 1998; Davies and Breslin, 2003*b*), UV radiation (Rodriguez-Romo and Yousef, 2005), ozone (Davies and Breslin, 2003*b*; Rodriguez-Romo and Yousef, 2005) and ultrasonication (Cabeza *et al.*, 2005). Currently, these methods are not allowed in the European Union for class A table eggs.

When eggshells are contaminated with *Salmonella*, our study indicates that *Salmonella* remained viable on the shell during storage more often when the temperature was lowered from 25°C to 15°C and especially at a low RH. Enhanced survival at lower temperatures has been previously reported (Rizk *et al.*, 1966; Baker, 1990; Braun *et al.*, 1999; Radkowski, 2002). The RH during storage also plays a crucial role: at 75% RH, bacteria died off rapidly even at low temperature. Simmons *et al.* (1970) have shown that Salmonellae on shell surfaces survive better as either the temperature or RH is lowered farther from the optimum for growth. Bacteria survived longer at 10°C compared to 15 and 23°C, especially when the RH was lowered from 97% to 75%. We have shown that this is still valid when lowering the RH to 45%. *Salmonellae* probably survive longer due to the slower metabolism induced by the disadvantageous conditions on the dry eggshell surface (Radkowski, 2002). Extrapolating our results to practical situations, *Salmonella* organisms on the eggshell will die off rapidly in the laying unit as a result of the high RH. In the egg-packing plant, survival will be higher due to the lower temperature and RH. Davies and Breslin (2003*a*) concluded that contamination in egg-packing plants may be a significant contributory factor to external contamination of shell eggs. In the store during summer, survival will be intermediate; during winter, survival will be low. As observed previously (Rizk *et al.*, 1966), average *Salmonella* counts declined over time. On most shells numbers decreased and *Salmonella* could no longer be recovered from the shell, even with enrichment. Remarkably however on a limited proportion of shells growth of *Salmonella* took place. Although survival rates on the shell differed greatly between the various storage conditions, the proportion of eggshells where growth occurred was not statistically different. In total, *c.*10% of the eggshells carried >4 log SE and some shells were contaminated with as many as >7 log units per shell. Thus, when eggs reach the consumer, the shell of a relatively small proportion of eggs might be contaminated heavily and give rise to cross-contamination in the kitchen.

Although temperature enhanced the rate of appearance of red spots on the agar within the eggs, this does not prove faster initial penetration, because faster growth of SE at higher temperatures was also observed on a control agar plate. On the control plate, pronounced growth was obvious after 1·5, 2 and 3 d at 25, 20 and 15°C, respectively. At the end of storage, a not significantly different proportion of eggs was contaminated, indicating that eggshells are prone to become penetrated at the same frequency at all conditions studied. Elevated temperatures will however enhance the subsequent multiplication of *Salmonella* in the egg contents as shown in other studies (Schoeni *et al.*, 1995; Fleischman *et al.*, 2003).

In conclusion, our study indicates that Salmonellae on shell surfaces survive better as either the temperature (range 10 to 23°C) or RH (range 45 to 75%) is lowered. Growth occurred on a limited proportion of shells resulting in high numbers of SE at the end of storage. Overall, the percentage of penetrated eggshells was not affected by the storage conditions, but strongly affected by the shell contamination at the end of storage. Decontamination of the eggshell is required to prevent eggshell penetration.

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