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The influence of temperature on the behaviour of mixed bacterial contamination of the shell membrane of the hen's egg

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SUMMARY

The inner membrane of the air cell of hens' eggs was inoculated with *Pseudomonas putida*, *Staphylococcus xylosus*, *Enterococcus faecalis*, *Escherichia coli* and *Salmonella enteritidis*. The first mentioned eventually dominated the contamination of the albumen of eggs stored at 4, 15, and 20 °C. The last mentioned did so in eggs stored at 37 °C. The interval between inoculation of the membrane and gross contamination of the albumen was markedly influenced by site of contamination relative to yolk movement.

INTRODUCTION

Much attention has been given recently to the role of the hen's egg in the transmission of *Salmonella enteritidis* PT 4 to the human populations in Europe [1] and America [2].

There are two potential routes of egg contamination with this organism, oviducal and trans-shell. With the former, an egg's contents is contaminated by *S. enteritidis* that have gained access to the ovary or some part of the oviduct [3]. Several surveys of eggs from laying flocks known to be infected with this organism, have shown a very low incidence of contamination [4, 5]. Moreover in general, salmonellas of oviducal origin do not appear to multiply to any appreciable extent until contaminated eggs have been stored for more than 21 days [6]. With trans-shell contamination, it has been surmised that salmonellas on the shell pass through the pores, contaminate the shell membranes and eventually the egg contents [7]. With this route of contamination, the maturity or otherwise of the cuticle enveloping the outer surface of the calcitic shell is important. Circumstantial evidence suggests that the cuticle at and for a short period following oviposition is immature and hence a relatively ineffective barrier to microbial translocation across the shell [8]. Once mature, the cuticle contributes to the water resistance of the shell and increased pressure is necessary to force water and microorganisms through the pores [9]. To date there appears to have been no detailed study of factors affecting salmonella translocation across the shell. In contrast, much attention has been given recently to the second phase of trans-shell contamination, namely the fate of salmonellas injected into eggs [10, 11]. Clay and Board [11] noted that the following factors influenced the rate of development of gross contamination of the albumen and yolk when salmonellas were inoculated on the inner membrane of the air cell: storage temperature, size of inoculum, site of

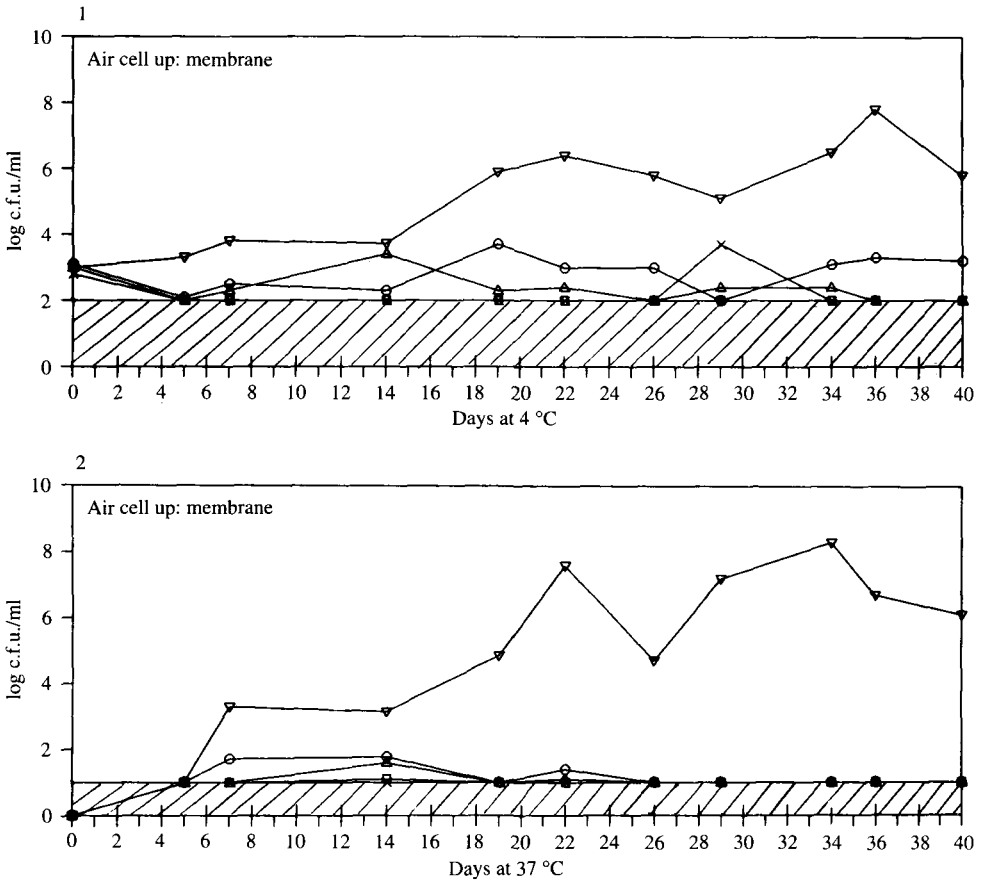

Fig. 1. Growth of a mixed culture of bacteria in hens' eggs. ▽, *Ps. putida*; △, *E. coli*; ⊟, *S. enteritidis*; ⊖, *St. xylosus*; ✳, *Ent. faecalis*; cfu, colony forming units; ▨, not detectable in this range. These results come from one experiment; each point is the average result obtained with four eggs.

contamination relative to yolk movement and the iron status of an inoculum. All studies to date may be considered artificial because pure cultures of salmonellas were used. As far as can be ascertained, trans-shell contamination rarely if ever results in a pure culture being lodged in the shell membranes [12, 13]. Judging from the studies by Humphrey and colleagues [14], who worked with *S. enteritidis*, oviducal contamination leads to a pure culture being deposited in the egg contents. This communication presents the results of an experiment in which salmonellas were included in a mixed inoculum placed on the inner membrane of the air cell of hens' eggs.

MATERIALS AND METHODS

Eggs

Eggs (size 4, approximately 58 g) less than 2 days old were purchased from a local producer/retailer and used immediately. The eggs were candled, the air cell marked by pencil, the shells sterilized with 70% (w/v) ethanol and a hole drilled

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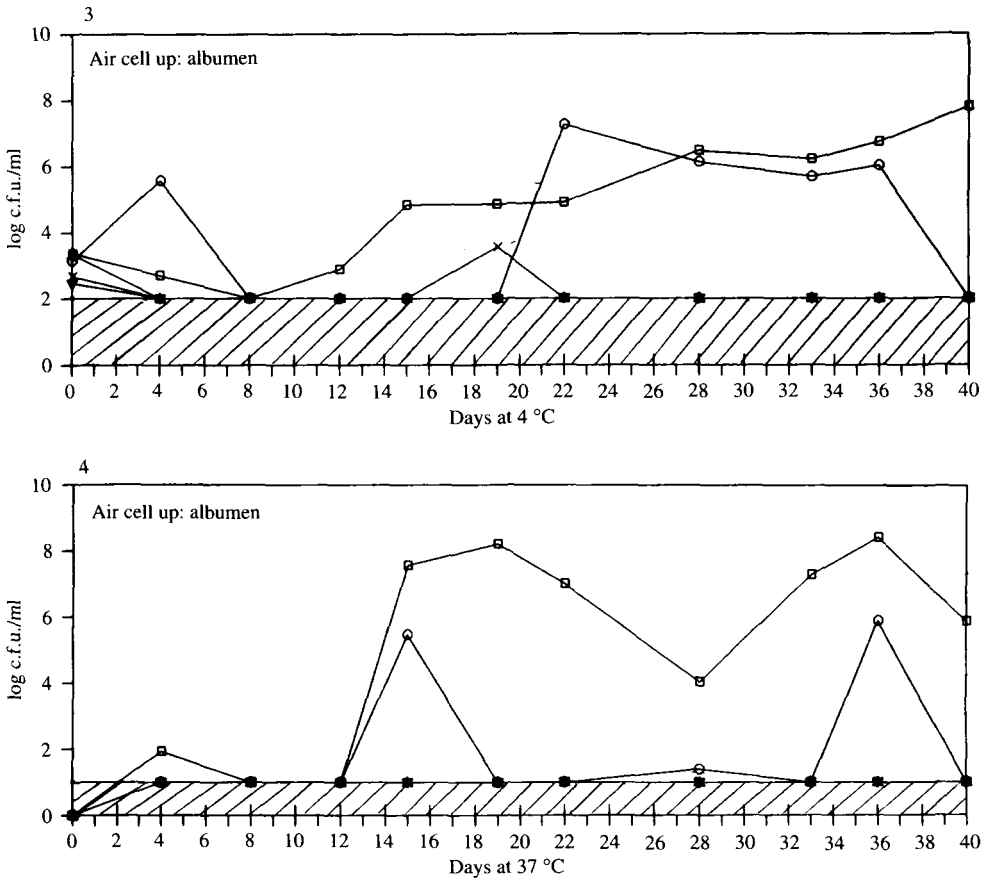

Fig. 1—*Cont.*

in the shell at the centre of the air cell. The inner membrane of the air cell was inoculated with 0·1 ml of a mixed culture (details below). After the hole in the shell had been sealed with paraffin wax, the eggs were incubated, either air cell upwards or downwards, at 4, 15, 20, and 37 °C. At frequent intervals, four eggs from each air cell position and temperature were analysed. The shell was sterilized with 70% (w/v) ethanol, cracked and the contents collected in a sterile Petri dish. The inner membrane of the air cell was excised with a sterile scalpel, placed in 9 ml $\frac{1}{4}$-strength Ringer's solution, held in a Dawe sonicleaner (Dawe Instruments Ltd) for 2 min, mixed on a vortex mixer (whirlimixer, Fison's) for 1 min and decimal dilutions prepared in the same diluent. Albumen was mixed in the Petri dish so that the thick white was broken down and 1 ml was decimally diluted in $\frac{1}{4}$-strength Ringer's solution. Appropriate dilutions of both dilution series were spread (0·1 ml) on the media given below.

Organisms

The following were used: *Pseudomonas putida* (isolated from a rotten egg); *S. enteritidis* PT 4 (isolated from an egg at the PHLS, Exeter, Devon) and *Escherichia coli*, *Enterococcus faecalis* and *Staphylococcus xylosus* all of which were

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isolated from chicken faeces. All organisms were maintained on nutrient agar (Lab M) at 4 °C and transferred monthly. For experimental purposes, the organisms were grown in 50 ml of shaken nutrient broth (Lab M) for 18 h at 25, 37, 37, 37, and 30 °C respectively. The cells were harvested (centrifuged at 2000 g), washed once with 0·85 (w/v) saline. Portions (1 ml) of the washed suspensions were placed in 5 ml $\frac{1}{4}$-strength Ringer's solution, decimal dilutions made, and 0·1 ml of 10^{-5} dilution of cell mixture was used to inoculate eggs.

Media

The following were used to isolate the organisms: *Ps. putida*, CFC medium (Lab M, [15]; incubated at 25 °C for 2 days); *S. enteritidis*, xylose, lysine decarboxylase agar (Lab M; incubated at 37 °C for 24 h); *E. coli*, xylose, lysine decarboxylase agar (Lab M, incubated at 37 °C for 24 h); *Ent. faecalis* kanamycin aesculin agar (Lab M, incubated at 37 °C for 48 h); *St. xylosus*, nutrient agar containing additional 6% (w/v) sodium chloride (incubated at 30 °C for 72 h).

RESULTS

The results obtained with eggs stored air cell uppermost and incubated at 4 or 37 °C are shown in Figure 1. All five of the organisms used in the mixed inoculum, *Ps. putida*, *St. xylosus*, *Ent. faecalis*, *E. coli* and *S. enteritidis*, were isolated from the inner membrane of the air cell of eggs incubated at 4 °C. In contrast only *St. xylosus*, *S. enteritidis* and, on one occasion only, *Ent. faecalis* were recovered from the membrane in eggs stored at 37 °C. At 4 °C there was a progressive increase in the level of contamination of both the air cell membrane and the albumen such that the latter contained 10^5 pseudomonads/ml on day 26. *S. enteritidis* attained populations of this size in albumen by the 15th day of incubation of eggs at 37 °C. At this temperature, *St. xylosus* occurred in large but not dominant numbers on days 15 and 36. The populations of both species increased markedly in the shell membrane also. With eggs incubated air cell downwards at 4 °C, *Ps. putida* attained numerical dominance in the inner shell membrane of the air cell as well as in the albumen but not until the 34th day (cf. 22 days in eggs with air cells uppermost). All five members of the mixed inoculum died out in the membrane of eggs incubated air cell downwards at 37 °C and none of them was isolated from the albumen (Table 1).

With incubation at the intermediate temperatures (15 or 20 °C), the pseudomonad attained numerical dominance in both the inner membrane of the air cell as well as in the albumen of eggs stored air cell uppermost (Table 1). At 15 °C but not at 20 °C, *St. xylosus* and *Ent. faecalis* were present on the air cell membrane but not in the albumen of eggs in which the air cell was uppermost. There was no growth of any of the organisms in either the shell membrane or albumen of eggs stored with their air cells downwards at 15 °C. With eggs held in this position and incubated at 20 °C, *Ps. putida* grew in the shell membranes and caused gross contamination of the albumen. Again there was an appreciable interval between the inoculation of eggs and the onset of gross contamination of the albumen (day 35, cf. 21 days in eggs stored air cell uppermost).

Table 1. *Prevalent organisms in the inner shell membrane of the air cell and albumen of eggs in which contamination of white ⩾ 10^6 organisms/ml*

Temp. (°C)	Air cell location	Day*	Inner membrane of air cell					Albumen				
			Pp	Sx	Ef	Ec	Se	Pp	Sx	Ef	Ec	Se
4	Up	22	+	−	−	−	−	+	−	−	−	−
	Down	34	+	−	−	−	±	+	−	−	−	−
15	Up	28	+	±	±	−	−	+	−	−	−	−
	Down	—	−	−	−	−	−	−	−	−	−	−
20	Up	21	+	−	−	−	−	+	−	−	−	−
	Down	35	+	−	−	−	−	+	−	−	−	−
37	Up	15	−	+	−	−	−	−	±	−	−	±
	Down	—	−	−	−	−	−	−	−	−	−	−

* Day on which count first attained ⩾ 10^6 cfu/ml albumen.

Inner membrane: +, extensive growth of organism; ±, modest growth; −, slight growth.

Albumen: +, > 10^6 cfu/ml; ±, 10^4–10^6 cfu/ml; −, < 10^4 cfu/ml; cfu, colony forming units.

Pp, *Pseudomonas putida*; Sx, *Staphylococcus xylosus*; Ef, *Enterococcus faecalis*; Ec, *Escherichia coli*; Se, *Salmonella enteritidis* PT 4. Each result is an average from four eggs.

DISCUSSION

Many studies have shown that Gram-positive bacteria are the dominant contaminants on the outer surface of egg shells [16] but that Gram-negative ones almost invariably cause rotting of the contents [13]. There are no reasons to suppose that agents causing translocation of contaminants across the shell would select the latter at the expense of the former. This led Tranter and Board [17] to surmise that factors within the egg, especially the low levels of readily available non-protein nitrogenous substances in the albumen, selected the fast growing but nutritionally non-fastidious Gram-negative bacteria. The behaviour of the major members of a consortium of bacteria inoculated into the air cell of eggs has been studied on only one previous occasion. Seviour and Board [18] used the organisms present on naturally contaminated egg shells as an inoculum. They found that Gram-positive bacteria, the dominant organisms in the inoculum, became subordinate to Gram-negative ones within a few days following the inoculation of the inner membrane of the air cell. Several species of the latter grew to a limited extent in this membrane but only one species attained dominance during the gross contamination of the albumen. Thus with storage at 37 °C, coliform organisms attained dominance when the air cell was uppermost. At temperatures of 30 °C or less, pseudomonads became dominant. The marked selective effect of temperature was confirmed in this study also, but with one notable exception, namely that *S. enteritidis* outcompeted *E. coli* in eggs incubated at 37 °C. In the context of the present studies of egg associated salmonellosis, this is an important observation particularly to those who may be contemplating the reintroduction of egg washing. The importance of the site of contamination relative to yolk movement at the onset of gross contamination of the albumen (Fig. 1, Table 1) was noted by Clay and Board [11]. It needs to be stressed however that it had no apparent effect on the selection of organisms. This study has shown that *S. enteritidis* failed to compete with pseudomonads in eggs incubated below 30 °C. It might well be

concluded therefore that should contaminants including salmonella be translocated across the shell, the latter would be unlikely to achieve dominance in eggs held under commercial conditions. As was noted in the introduction, the vast majority of studies have demonstrated a mixed contamination in rotten eggs. Indeed some workers, e.g. Miles and Halnan [12], gave the name adventitious contaminants to those members of a consortium that failed to cause macroscopic changes when inoculated into eggs in pure culture. Board and Board [13] inoculated eggs with pure cultures from rotten eggs and found that the majority produced macroscopic changes. This evidence cannot be taken to mean that no egg will ever contain large numbers of bacteria that fail to cause rotting. Indeed Johns and Bérard [19] identified two such eggs (contaminating organism, 'Achromobacter') in egg breaking plants in Canada. *S. enteritidis* does not cause macroscopic changes in an egg and should it be the sole organism after gaining access to an egg by trans-shell contamination then it would behave like the adventitious contaminants of Miles and Halnan [12]. The present study suggests that this would be a very unusual occurrence and it strengthens the contention of Humphrey and colleagues [14] that naturally contaminated eggs acquire organisms via the oviducal route.

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