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Growth of *Salmonella enteritidis* in artificially contaminated hens' shell eggs

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SUMMARY

The effect of some factors on the growth of *Salmonella enteritidis* phage type 4 in artificially contaminated shell eggs was investigated.

Salmonella enteritidis was found to be resistant to the antimicrobial properties of the albumen. Growth occurred on storage at 25 °C but not at 4 or 10 °C. The rate and extent of infection was influenced by the size of inoculum, the site of contamination relative to yolk movement, and the presence of iron in the inoculum.

INTRODUCTION

The number of reported cases of illness due to *Salmonella enteritidis* – predominantly phage type 4 (PT4) – dramatically increased in the United Kingdom between 1984 and 1988 [1–3]. Common sources of *S. enteritidis* food-poisoning outbreaks, in which attributable foods have been identified, are eggs and poultry [3]. Similar increases have been observed in the northeastern United States and Spain where upwards of 70% of outbreaks have been attributed to shell eggs or egg products [4, 5]. Reasons behind the emergence of this problem are not fully understood. *S. enteritidis* may contaminate the egg either through the chickens' ovaries becoming infected such that the yolk is contaminated before laying – transovarian transmission [6–10], or through transient intestinal infection of laying birds leading to shells becoming contaminated after laying [11, 12]. The present study was concerned with the latter.

The course of microbial infection of shell eggs by the latter route may be considered in stages [13, 14]: (1) penetration of the cuticle and shell; (2) colonization of the underlying membranes, and (3) contamination of the albumen leading eventually to generalized infection of the egg contents. If the infecting organism produces pigments, H_2S, proteases and/or lecithinase, then such infection is associated with addling of the albumen and yolk [15, 16]. The study by Sparks and Board [17] demonstrated the important role of the cuticle in the defence of an egg's contents against bacterial infection. Following contamination of the shell membranes, various factors may affect the course of the infection process, namely initial bacterial load, temperature, physical and antimicrobial attributes of the albumen and the properties of the infecting organism. In particular it has been established, both with albumen *in vitro* and in whole shell

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eggs, that through chelation of iron, ovotransferrin is the major growth inhibitor of Gram-negative bacteria [18]. Its protective action can be overcome by the addition of saturating amounts of iron to the albumen [19–24] or trace amounts at the site of contamination of the shell membranes [25]. In the latter case, the iron remains localized through absorption onto the mantle of the fibres of the shell membranes [26].

To date the majority of studies of infection of eggs have been done with rot-producing bacteria. The present study was concerned with *Salmonella enteritidis* inoculated into the air cell of whole eggs. The effects of the following factors were examined: position of the air cell during incubation at 25, 10 and 4 °C, inoculum size and the addition of iron to an inoculum.

MATERIALS AND METHODS

Eggs

Eggs (size 4, approx. 58 g) less than 2 days old were purchased from a local producer/retailer and stored for less than 2 days before use.

Eggs were assumed to be free from *S. enteritidis*. Eggs from the same source were used in other experimental work in which endogenous salmonellas would have been detected. None was found to be contaminated.

Culture

A culture of *Salmonella enteritidis* PT4, originally isolated from an egg (PHLS, Exeter), was used in all experiments. An overnight shaken culture of *S. enteritidis* in nutrient broth (Lab M) at 37 °C was spun down (2000 $g \times 10$ min), washed with sterile saline, spun down again and resuspended in saline. An appropriate dilution of this cell suspension was made in $\frac{1}{4}$-strength Ringer solution (Lab M) so that 0·1 ml would contain approximately the required inoculum (10, 10^3 or 10^6 organisms).

Shell egg experiments

All eggs were candled to locate the air cell. Cracked eggs were rejected and no eggs had visible faecal contamination of the shell. After surface sterilization (70% ethanol), a small hole was drilled through the shell. A portion (0·1 ml) of a cell suspension was injected into the air cell. The hole was then sealed with molten wax.

Eggs were stored at 4, 10 or 25 °C; in all cases half the eggs were stored with their air cell downwards and half with their air cells uppermost.

At intervals of up to 5 weeks, four eggs in each position were taken from each treatment (inoculum or temperature), surface sterilized (70% ethanol), cracked open into a sterile Petri dish, and the numbers of salmonellas in the albumen determined. In some cases the number of salmonellas in the yolk was also determined.

All viable counts were carried out by spreading 0·1 ml of an appropriate dilution on xylose lysine desoxycholate agar (XLD: Lab M) with overnight incubation at 37 °C.

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The air cell membrane of each egg was excised from the shell and spread onto XLD to check the persistence of viability of the original inoculum.

The albumen of each egg was examined microscopically using a $\times 40$ phase contrast objective.

The pH of the albumen was tested with Whatman indicator paper (full range pH 1–14). Laboratory safety regulations precluded the use of a pH meter with material contaminated with salmonellas.

Presumptive salmonella-positive colonies were confirmed by serology.

The following variations of this basic experimental plan were undertaken:

1. Fresh eggs were inoculated with approximately 10^3 organisms/air cell. Storage was at 4, 10 or 25 °C. After 20 days some eggs stored at 4 or 10 °C were moved to storage at 25 °C.

2. Fresh eggs were inoculated in the air cell with 10, 10^3 or 10^6 organisms. Storage was at 25 °C.

3. Ferrous sulphate ($FeSO_4.7H_2O$; 50 μg/ml) was added to the cell suspension so that 5 μg $FeSO_4$ was injected with 10^3 organisms into the air cell. Storage was at 25 °C.

RESULTS

The majority of eggs with the inner membrane of the air cell seeded with 10^3 salmonellas showed low levels of contamination of the albumen (Fig. 1) in the early stages of storage at 25 °C. Later, amongst those eggs stored with the air cell uppermost, there was a progressive increase in the proportion in which gross contamination ($> 1{\cdot}0 \times 10^6$ organisms/ml) of the albumen developed. This level of contamination occurred in far fewer of the eggs stored air cell downwards.

These findings were supported by another experiment in which 30 eggs (half air cell uppermost and half vice versa) were examined on each of 8 sampling days during storage at 25 °C (Fig. 2).

Linear regression analysis (Fig. 3) shows clearly that eggs stored with contaminated air cell uppermost, where the yolk moved towards the inoculum during storage, had a higher probability of developing gross contamination of the yolk and albumen than those stored air cell downwards.

A similar analysis of all the data obtained on contaminated eggs stored at 25 °C (Fig. 4) identified two groups of eggs: (A) those in which the albumen contained $< 1{\cdot}0 \times 10^4$ salmonellas/ml and fewer in the yolk, and (B) those in which the albumen and yolk each contained $> 1{\cdot}0 \times 10^6$ salmonellas/ml. Eggs of Group (A) were those in which the contamination of the albumen was due to organisms coming from the shell membrane growing to a limited extent if at all. About one third of these eggs had no detectable contamination of the yolk. This suggested that the site of infection was not located close to the yolk – a large proportion of eggs in Group (A) had been stored air cell downwards. Group (B) eggs were those in which some event, most probably induced by the yolk (see Discussion), caused profuse growth of contaminants in the albumen and yolk – a large proportion of these had been stored air cell uppermost.

The number of salmonellas inoculated onto the inner membrane of an air cell affected the progress of infection of the albumen and yolk during storage at 25 °C (Fig. 5). As the inoculum was increased the probability of gross contamination

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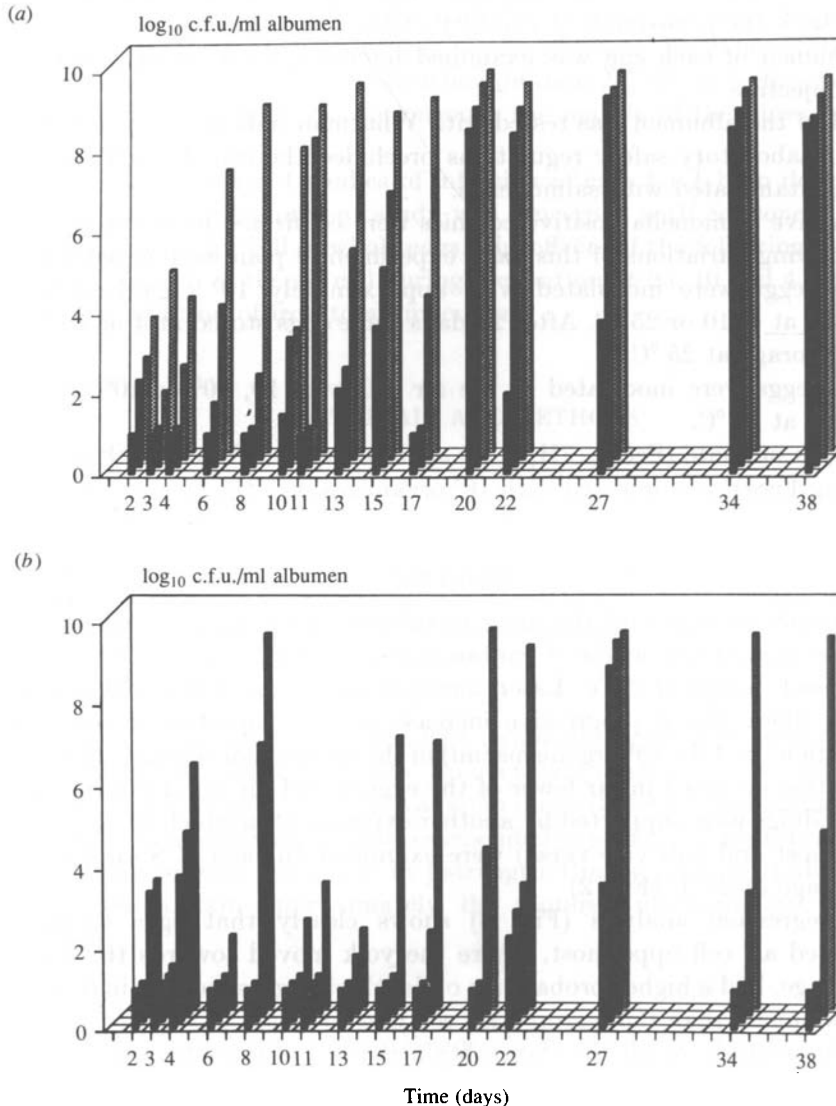

Fig. 1. The growth of *S. enteritidis* in shell eggs artificially contaminated in the air cell and stored at 25 °C. Eggs air cell uppermost (*a*) or downwards (*b*).

($> 10^6$ organisms/ml) of the albumen at an earlier stage of storage (up to 12 days) rose in eggs in which the air cell was uppermost. Almost all eggs stored air cell uppermost developed gross contamination of their contents by the later stages of storage (19–34 days) regardless of the size of the inoculum. A similar though lesser rise in the probability of contamination of the albumen during the early stages of storage (up to 8 days) was also seen in eggs stored air cell downwards. However, in the later stages of storage the occurrence of gross contamination of the contents of these eggs was infrequent and erratic with all inocula.

The course of infection of eggs was affected by the addition of iron to the inoculum. This effect was particularly marked in eggs stored with their air cell

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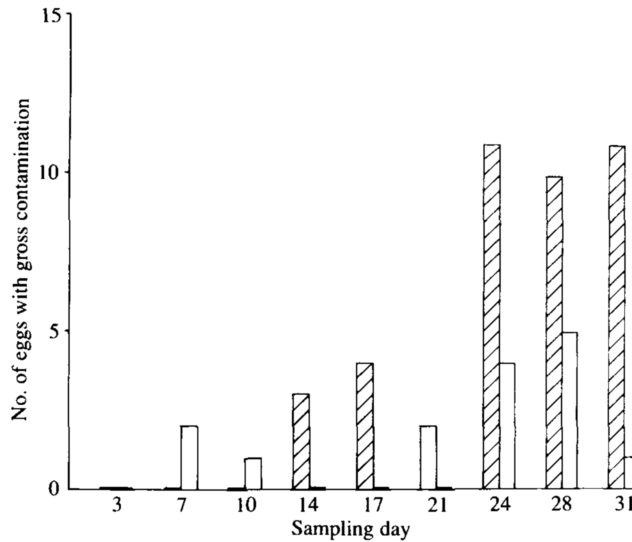

Fig. 2. The incidence of gross contamination (> 10^6 salmonellas/ml albumen) in shell eggs artificially contaminated in the air cell and stored at 25 °C. Open bars, air cell downwards; hatched bars, air cell uppermost (15 of each tested every sampling day).

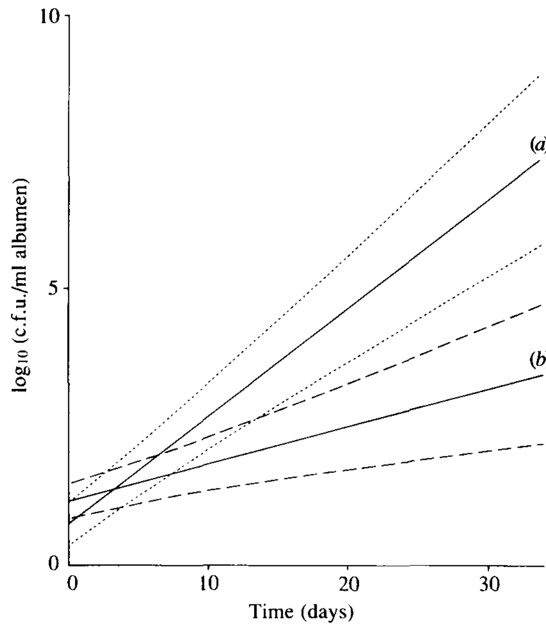

Fig. 3. The growth of *S. enteritidis* in artificially contaminated shell eggs at 25 °C – linear regression analyses with 95% confidence limits, (*a*) air cell uppermost ($r = 0{\cdot}59$); (*b*) air cell downwards ($r = 0{\cdot}31$).

downwards (Fig. 6*b*, *d*). Thus the albumens of significantly more of the eggs treated with iron were grossly contaminated (> $1{\cdot}0 \times 10^6$ organisms/ml) than those of the control eggs.

When eggs inoculated with 10^3 salmonellas in the air cell were stored at 4 °C,

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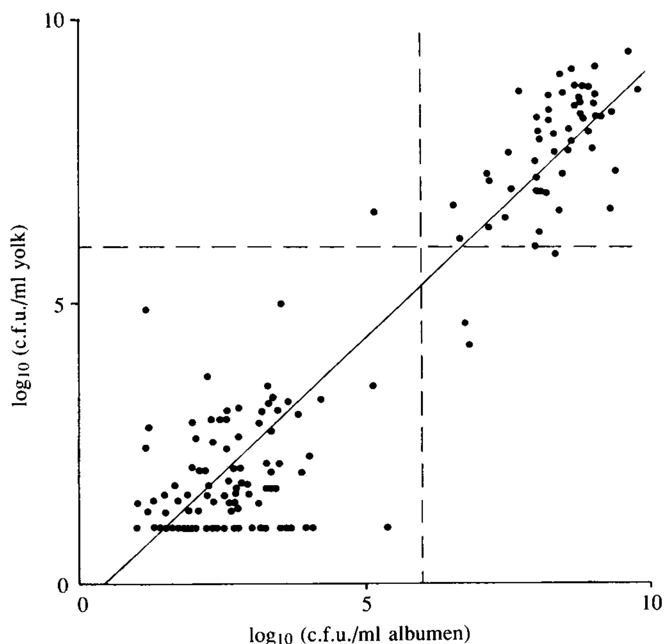

Fig. 4. A comparison of the growth of *S. enteritidis* in albumen and yolk of shell eggs artificially contaminated in the air cell and stored at 25 °C – linear regression analyses ($r = 0{\cdot}95$).

viable organisms were present in the inner membrane of the air cell throughout the 30 days of storage (Fig. 7). In no case was contamination of the albumen demonstrated (limit of detection, 10 organisms/ml). Transfer of eggs from 4 to 25 °C led to generalized infection of the eggs' contents, the incidence and extent of contamination being greatest in eggs stored with their air cells uppermost. Similar results were obtained with storage at 10 °C, and subsequent transfer to 25 °C.

There was no evidence of addling of albumen containing $> 1{\cdot}0 \times 10^6$ salmonellas/ml, although grossly contaminated albumen appeared more turbid than that of uncontaminated albumen. Actively motile cells were invariably seen in fresh preparations of albumen contaminated with $> 1{\cdot}0 \times 10^5$ salmonellas/ml. An acid drift (pH 9·0 to 7·0–8·0) was noted only with albumen containing $> 1{\cdot}0 \times 10^6$ salmonellas/ml. While in general there was no obvious change in the appearance of the yolk of eggs having $> 1{\cdot}0 \times 10^6$ salmonellas/ml, occasionally a yolk had a mottled appearance with a sticky vitelline membrane.

DISCUSSION

This study sought to identify factors that influence the course of infection following translocation of *S. enteritidis* across the shell and contamination of the underlying membranes [27]. These were the direction of yolk movement relative to site of inoculation, the size of inoculum, the presence of iron in an inoculum and the temperature of storage.

The incidence of gross contamination of the albumen was highest in eggs stored with their air cell uppermost. We contend that this trend is associated with the

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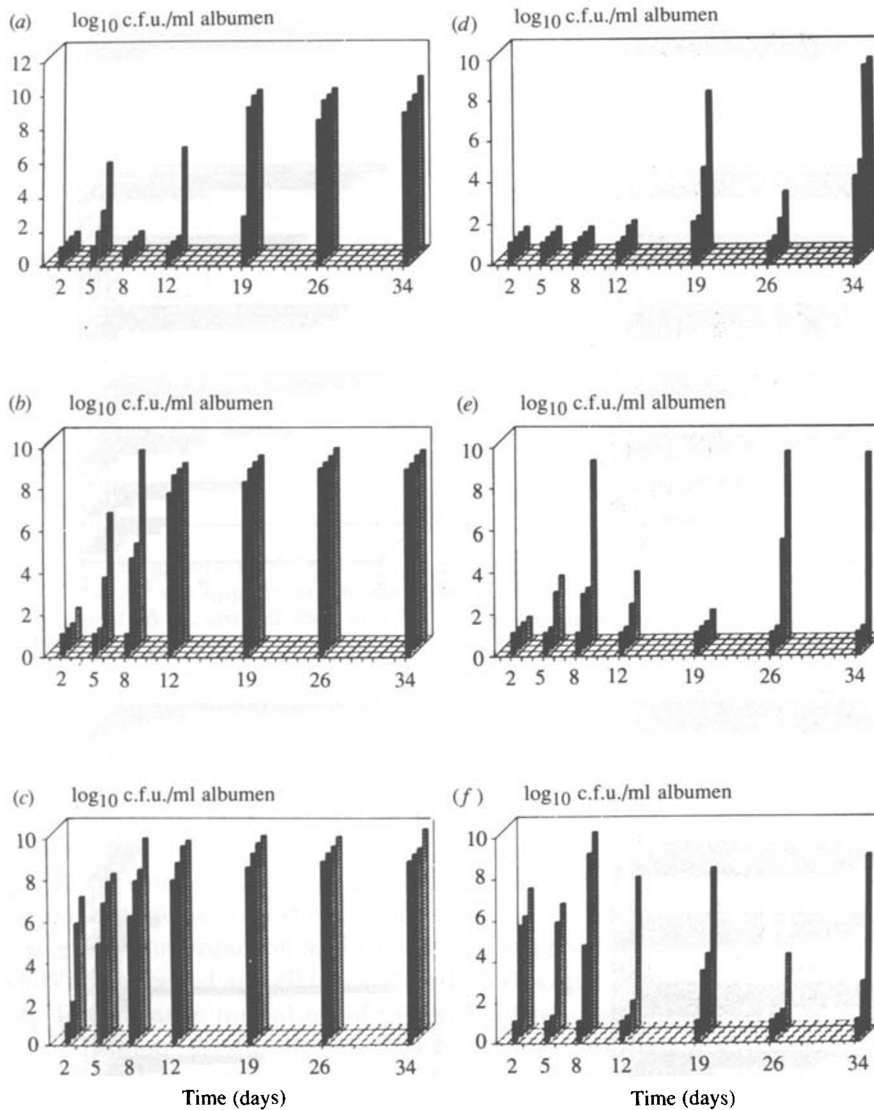

Fig. 5. The effect of inoculum size on the growth of *S. enteritidis* in shell eggs stored at 25 °C. (*a*) Inoculum of 10 organisms; (*b*) inoculum of 10^3 organisms; (*c*) inoculum of 10^6 organisms [(*a–c*) air cell uppermost]. (*d*) Inoculum of 10 organisms; (*e*) inoculum of 10^3 organisms; (*f*) inoculum of 10^6 organisms (*d–f*) air cell downwards.

decay of egg structure. At oviposition the yolk is more dense than the albumen. Indeed it would settle to the bottom of a newly laid egg standing on its pole were it not for the mechanical properties of the albuminous sac [28]. During storage evaporative water loss from the albumen through pores in the shell [29–31] and the absorption of water by the yolk [29] reverses the relative densities of the albumen and yolk. The enlarged yolk begins to float in the albumen. The rate of upward movement will be determined by the extent to which the albuminous sac has decayed. The extent of a yolk's movement will also be determined by the size

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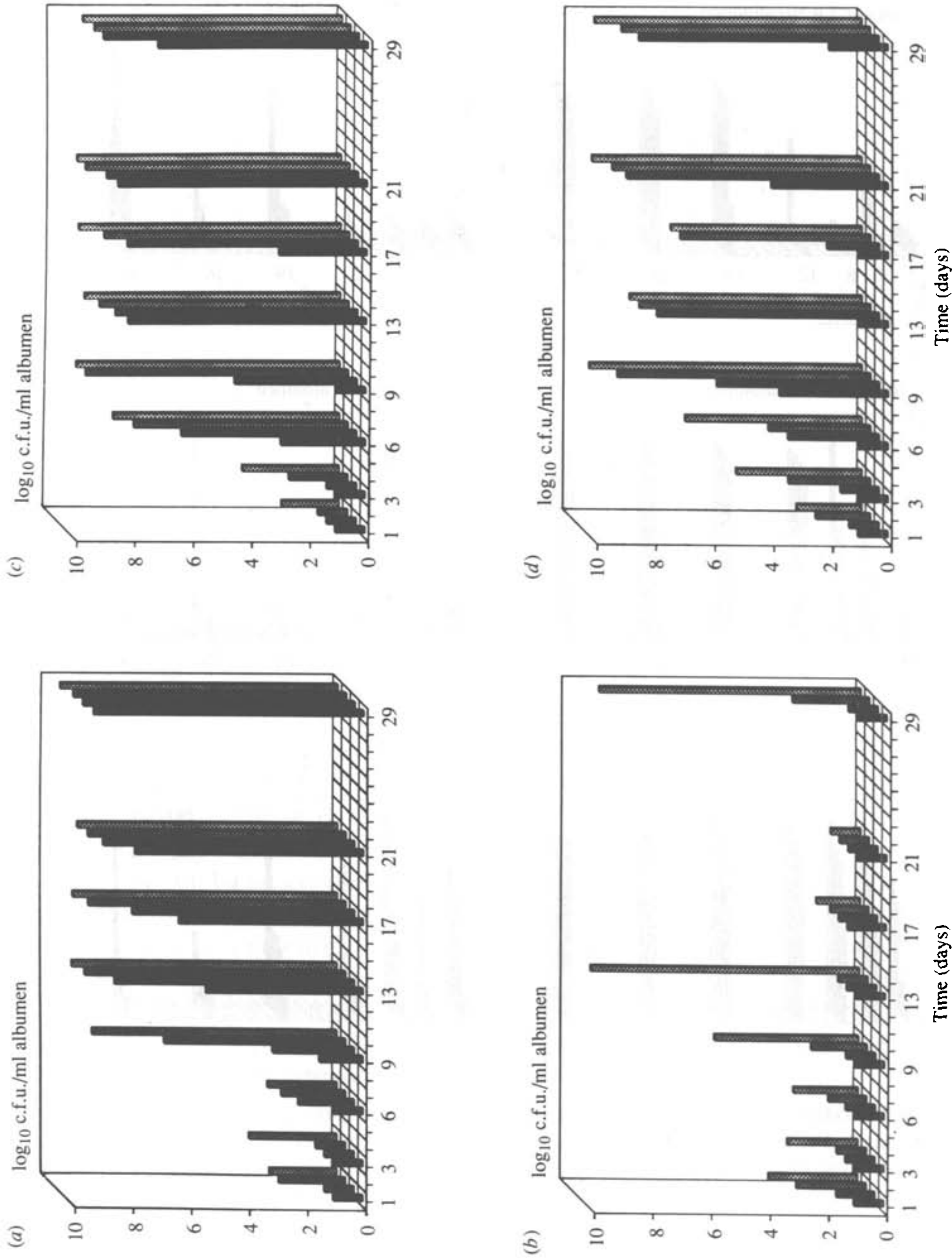

Fig. 6. The effect of the addition of iron (Fe III, 5 μg/membrane) with the inoculum on the growth of *S. enteritidis* in shell eggs stored at 25 °C. (*a*) Air cell uppermost; (*b*) air cell downwards [(*a*, *b*) controls without iron]. (*c*) Air cell uppermost; (*d*) air cell downwards [(*c*, *d*) with iron addition].

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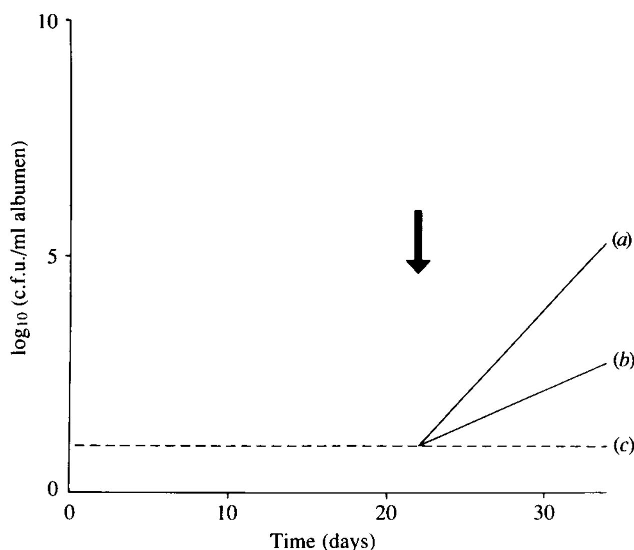

Fig. 7. The growth of *S. enteritidis* in artificially contaminated shell eggs stored at 4 °C. (*a*) Air cell uppermost; (*b*) air cell downwards [(*a*, *b*) arrow indicates switch to storage at 25 °C, after 22 days at 4 °C.] (*c*) Air cell uppermost or downwards, (at 4 °C throughout the test.)

of the air cell which will have increased progressively in volume to accommodate water lost by evaporation [29]. Thus when an egg seeded in the air cell is incubated broad pole uppermost, the contaminated membrane of the air cell will move towards the rising yolk and the distance for contaminants to travel from the site of contamination to the surface of the yolk will progressively diminish [32]. Should transfer of contaminants of the albumen to the yolk not occur, eventually contaminants present in the shell membranes will directly affect the yolk [33]. In either event the organism will exploit the poorly defended nutrients in the yolk [34]. When a seeded air cell is incubated downwards, infection of the yolk will only result from chance migration of organisms from contaminated albumen [14].

The addition of iron to an inoculum was associated with extensive contamination of the albumen of eggs stored at 25 °C. It has been established that iron is retained by the mantles on the fibres of the shell membranes [26] and that only trace amounts [19, 20, 22] are required to induce microbial growth and contamination of the underlying albumen. Iron contamination of the shell membranes is only a problem when eggs are washed with water taken from bore holes rather than municipal supplies [21, 35] and unsatisfactory methods of washing are used. Other observations (Jane Lock, unpublished) have shown that extracts of hens' faeces are as effective as iron in inducing salmonella growth in albumen *in vitro*. Thus should egg washing in the UK be approved in the future, the methods must not cause translocation of micro-organisms and faecal material across the egg shell.

This study has shown also that incubation of eggs at 4 or 10 °C inhibited the gross contamination of the albumen and yolk with salmonellas present in the shell membranes (Fig. 7). Other studies with albumen *in vitro* (Jane Lock, unpublished)

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have shown that several salmonella serotypes fail to grow at 4 °C even when the ovotransferrin has been saturated with iron. These observation suggest that chill storage of eggs could be a part of the protective barrier between the laying flock and the consumer. However, salmonellas retained in the shell membranes do not die out during chill storage. Indeed when temperatures conducive to growth were used to store previously chilled eggs, the contamination of the albumen and yolk followed the same general trend as that in inoculated eggs incubated, for example, at 25 °C. Thus, for its potential to be effectively realized, chill storage would have to be imposed from shortly after lay until immediately before the cooking and consumption of an egg.

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