REVIEW ARTICLE

Mechanisms of egg contamination by *Salmonella* Enteritidis

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Abstract

Salmonella Enteritidis (SE) has been the major cause of the food-borne salmonellosis pandemic in humans over the last 20 years, during which contaminated hen's eggs were the most important vehicle of the infection. Eggs can be contaminated on the outer shell surface and internally. Internal contamination can be the result of penetration through the eggshell or by direct contamination of egg contents before oviposition, originating from infection of the reproductive organs. Once inside the egg, the bacteria need to cope with antimicrobial factors in the albumen and vitelline membrane before migration to the yolk can occur. It would seem that serotype Enteritidis has intrinsic characteristics that allow an epidemiological association with hen eggs that are still undefined. There are indications that SE survives the attacks with the help of antimicrobial molecules during the formation of the egg in the hen's oviduct and inside the egg. This appears to require a unique combination of genes encoding for improved cell wall protection and repairing cellular and molecular damage, among others.

Eggs as the most important source of *Salmonella* Enteritidis (SE) infections in humans

The epidemiology of SE tells the story of a pathogen that has found a biological niche in table eggs. SE has caused the majority of food-borne outbreaks of salmonellosis reported worldwide since the mid-1980s. In the United States, 298 (80%) of the 371 known-source SE outbreaks from 1985 to 1999 were egg-associated (Patrick *et al.*, 2004). In 2006, a total of 165 023 confirmed cases of human salmonellosis were reported in the European Union (EU) via the European Surveillance System (TESSy) (EFSA, 2007a). SE was identified as the cause of infection in 62.5% of the cases, and *Salmonella* Typhimurium in 12.9%. Other serotypes causing human illness are responsible for $< 2\%$ of the human infections. Other serotypes among the top 10 causes of human salmonellosis cases in the EU are Infantis, Virchow, Newport, Hadar, Stanley, Derby, Agona and Kentucky. Eggs and egg products were the most often identified food vehicles in the *Salmonella* outbreaks (Braden, 2006). These findings clearly illustrate the link between eggs and human SE infections. An EU-wide study based on faecal and dust sampling from layer houses revealed that 30.8% of 5310 commercial large-scale laying hen holdings were *Salmonella* positive in 2006 (EFSA, 2007b). SE was the most common serotype in the laying flock environment (52.3%). Thus, almost 50% of the isolates were non-SE and the serotype distribution on layer farms does not match with that found in table eggs. The overall EU prevalence of *Salmonella* in table eggs was 0.8% in 2006, and $> 90\%$ of all egg isolates were SE (EFSA, 2007a). These data should be interpreted with caution because the sampling site is not specified. The remaining 10% of the isolates belonged to different *Salmonella* serotypes, but these were mostly isolated in only one EU member state, indicating that the overall importance of

non-SE serotypes in eggs is negligible. These data suggest that SE has some intrinsic characteristics that allow a specific interaction with either the reproductive organs of laying hens or the egg components.

Generally, there are two possible routes of egg contamination by *Salmonella*. Eggs can be contaminated by penetration through the eggshell from the colonized gut or from contaminated faeces during or after oviposition (horizontal transmission) (Messens *et al.*, 2005a; De Reu *et al.*, 2006). The second possible route is by direct contamination of the yolk, albumen, eggshell membranes or eggshells before oviposition, originating from the infection of reproductive organs with SE (vertical transmission) (Timoney *et al.*, 1989; Keller *et al.*, 1995; Miyamoto *et al.*, 1997; Okamura *et al.*, 2001a, b). Figure 1 shows a schematic representation of the egg pathogenesis. It is not yet clear as to which route is most important for SE to contaminate the egg contents. Although some authors claim horizontal transmission to be the most important way to contaminate eggs (Barrow & Lovell, 1991; Bichler *et al.*, 1996), most authors claim that vertical transmission is the most important route (Gast & Beard, 1990; Miyamoto *et al.*, 1997; Guard-Petter, 2001).

This review provides an overview of host–pathogen interactions in the hen reproductive tract and eggs at the cellular and molecular level. It aims to highlight potential differences between SE and other *Salmonella* serotypes that could allow SE strains to contaminate eggs more successfully than other serotypes.

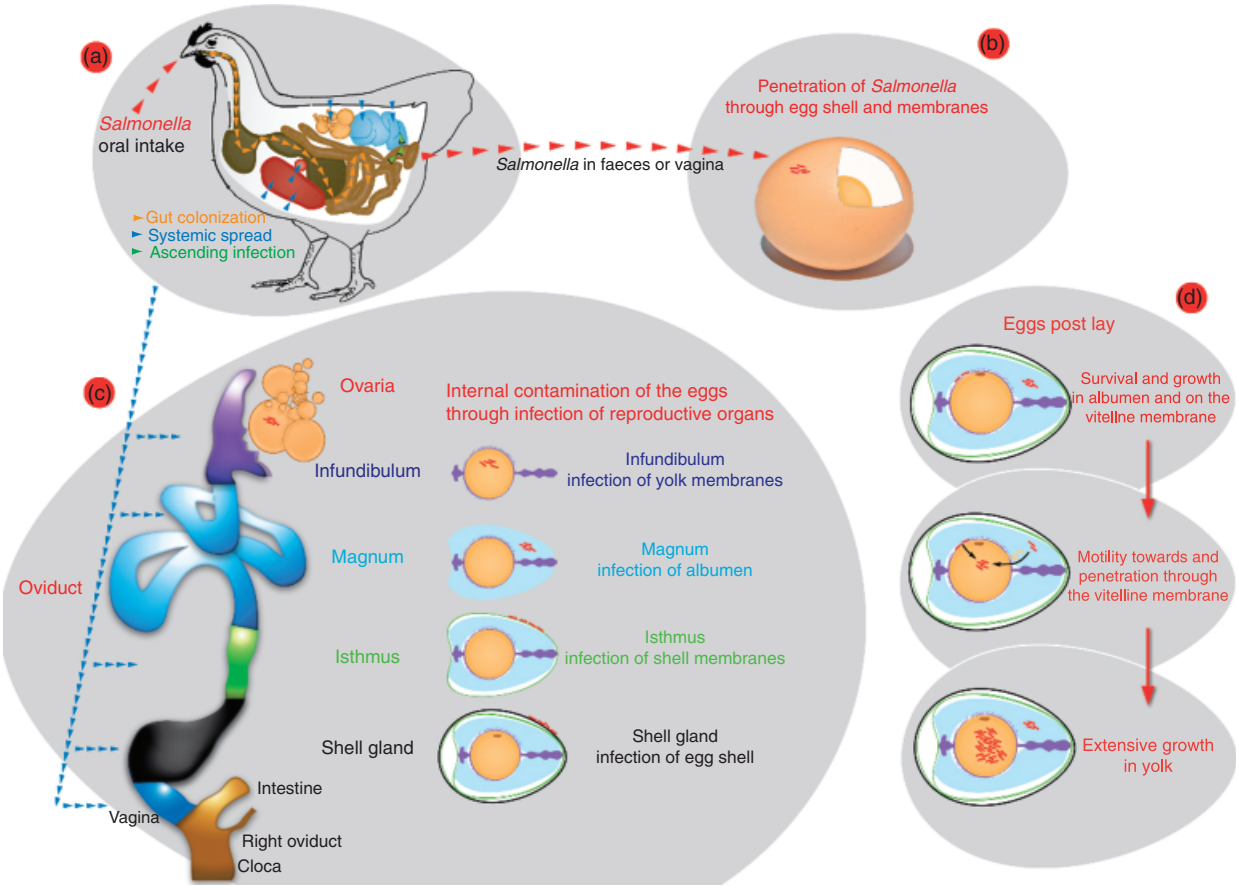

Fig. 1. Pathogenesis of egg contamination by *Salmonella*. (a) *Salmonella* is orally taken up by the hen and enters the intestinal tract. Bacteria colonizing the intestinal lumen are able to invade the intestinal epithelial cells (gut colonization). As a consequence, immune cells, more specifically macrophages, are attracted to the site of invasion and enclose the *Salmonella* bacteria. This allows the bacteria to survive and multiply in the intracellular environment of the macrophage. These infected macrophages migrate to the internal organs such as the reproductive organs (systemic spread). In addition to systemic spread, bacteria can also access the oviduct through ascending infection from the cloaca. (b) One possible route of egg contamination is by *Salmonella* penetration through the eggshell and shell membranes after outer shell contamination. Surface contamination may be the result of either infection of the vagina or faecal contamination. (c) The second possible route is by direct contamination of the yolk, yolk membranes, albumen, shell membranes and egg shell originating from infection of the ovary, infundibulum, magnum, isthmus and shell gland, respectively. (d) *Salmonella* bacteria deposited in the albumen and on the vitelline membrane are able to survive and grow in the antibacterial environment. They are also capable of migrating to and penetrating the vitelline membrane in order to reach the yolk. After reaching this rich environment, they can grow extensively.

Internal egg contamination after penetration of the eggshell

Outer shell contamination

Following oviposition, any contaminated environment in the area of the laid egg, such as the nest box, the hatchery environment or the hatchery truck, can lead to outer shell contamination. The presence of chicken manure and other moist organic materials facilitates the survival and growth of *Salmonella* by providing the required nutrients and a degree of physical protection. When eggs are artificially contaminated on the shell with faeces containing *Salmonella* and subsequently stored at 25 °C, numbers increase by 1–2 logs by day 1 and 4–5 logs by day 3 (Schoeni *et al.*, 1995). Such a growth indicates that faeces can serve as a nutritional reservoir for *Salmonella*. However, *Salmonella* can also survive and grow on the eggshell in the absence of faecal contamination, especially at lower temperatures and a low relative humidity (Messens *et al.*, 2006). *Salmonella* bacteria probably survive for a longer time at a low temperature due to the slower metabolism induced by the disadvantageous conditions on the dry eggshell surface (Radkowski, 2002). The egg surface can also be contaminated within the hen reproductive system after formation of the shell, but this will be discussed further in the text (Humphrey *et al.*, 1991a). Presuming that no differences exist between different *Salmonella* serotypes in the interaction with the outer shell, more prevalent serotypes such as SE are more likely to contaminate egg surfaces. In order to reduce the risk of externally contaminated eggs in the food chain, the need to rapidly remove any faecal contamination should be emphasized. However, intensive control measures in the United States, such as examining eggs for cracks, and washing and disinfecting eggs, have not eliminated egg contamination with SE (Braden, 2006). It is, however, possible that penetration has occurred before examining and washing the eggs.

Factors influencing eggshell and membrane penetration

Bacteria can easily penetrate through a cracked egg shell (Fajardo *et al.*, 1995). The intact egg, however, possesses three physical barriers to bacterial penetration (Fig. 2). These are the cuticle, which is a hydrophobic proteinaceous layer covering the eggshell and the pore openings, the crystalline eggshell and the shell membranes (Ruiz & Lunam, 2002). Shell membranes consist of three different layers, i.e. the inner and the outer membrane, consisting of a network of randomly oriented fibres, and a homogenous third layer of electro-dense material called the limiting membrane, demarcating the membrane at the interface with the albumen (Wong-Liong *et al.*, 1997).

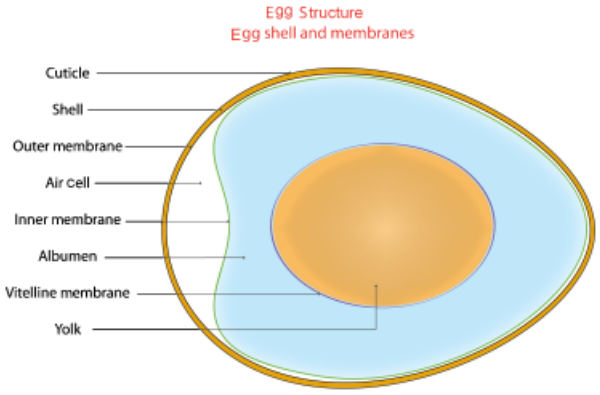

Fig. 2. Schematic representation of the egg structure.

In addition to their function as a physical barrier, the eggshell and shell membranes also act as a chemical barrier. Although antibacterial proteins have been identified mainly in the albumen, proteins with well-known antibacterial properties have also been associated with the eggshell and shell membranes. Lysozyme is abundant in the limiting membrane and is also present in the shell membranes, the matrix and the cuticle of the eggshell (Hincke *et al.*, 2000). Ovotransferrin has also been identified in the eggshell membranes and the basal calcified layer, possibly acting as a bacteriostatic filter (Gautron *et al.*, 2001). Recently, ovocalyxin-36, a novel chicken eggshell and eggshell membrane protein, has been identified (Gautron *et al.*, 2006). An antimicrobial role for ovocalyxin-36 was proposed because its protein sequence is highly similar to lipopolysaccharide-binding proteins, bactericidal/permeability-increasing (BPI) proteins and Plunc family proteins. These proteins are involved in antibacterial defence, and therefore it is believed that ovocalyxin-36 is of particular importance to keep the eggs free from pathogens. Protein extracts derived from the cuticle and the outer eggshell matrix indeed possess antimicrobial properties against both Gram-positive and Gram-negative bacteria (Hincke & Wellman-Labadie, 2007). Three bacterial species, *Pseudomonas aeruginosa*, *Bacillus cereus* and *Staphylococcus aureus*, were found to be inhibited in the presence of soluble eggshell matrix proteins, and it was demonstrated that these proteins might interact and disrupt the membrane integrity of the bacteria (Mine *et al.*, 2003). On the other hand, *Escherichia coli* and SE were weakly inhibited only at an early stage of incubation time (up to 4 h).

In spite of the protective physical and chemical barriers, numerous researchers have demonstrated rapid penetration into the egg by various bacteria, including *Salmonella* (Williams *et al.*, 1968; Humphrey *et al.*, 1989, 1991b). Miyamoto *et al.* (1998a) observed that after exposing freshly laid eggs to a *Salmonella* suspension for 2 h at 25 °C, the inner eggshell and egg contents were contaminated. Several

studies investigated the various factors affecting the probability of bacterial penetration. Both intrinsic and extrinsic factors are highlighted in a review by Messens *et al.* (2005a). The eggshell appears to be more easily penetrated immediately after the egg is laid (Sparks & Board, 1985; Padron, 1990; Miyamoto *et al.*, 1998a). It is suggested that for the first minutes after oviposition, the cuticle is immature and some pores may be open. Moreover, when the egg is exposed to an environment cooler than the chicken body temperature (42 °C), a negative pressure may develop and the bacteria migrate more easily through the eggshell and membranes (Board, 1966; Bruce & Drysdale, 1994). In addition, the cuticle in older eggs becomes dehydrated, resulting in its shrinkage, and the pores become more exposed to bacterial penetration (Mayes & Takeballi, 1983). In recent studies (De Reu *et al.*, 2006; Messens *et al.*, 2007), it was reported that cuticle deposition is important for the prevention of penetration, and in the absence of cuticle deposition, penetration is a frequent event. However, some research groups (Nascimento *et al.*, 1992; Messens *et al.*, 2005b) observed no correlation between cuticle deposition and penetration of *Salmonella* through the eggshell. Additionally, bacterial penetration was found to be independent of the pore number (Nascimento *et al.*, 1992; Messens *et al.*, 2005b; De Reu *et al.*, 2006). As mentioned earlier, temperature is also an important factor affecting the penetration. Fast penetration is observed when a positive temperature differential is created between the egg (warm) and the bacterial suspension (cool) (Mayes & Takeballi, 1983; Bruce & Drysdale, 1994). It is believed that a positive temperature differential, combined with the presence of moisture, provides an ideal opportunity for the bacteria to penetrate the eggshell (Berrang, 1999). The use of different penetration models, differences in the bacterial strains used, differences in the number of bacteria inoculated, the temperature and relative humidity during storage and the egg characteristics (eggshell quality and egg age) may partly explain the conflicting results seen in the studies regarding eggshell penetration, as reviewed by Messens *et al.* (2005a).

Eggshell penetration by different *Salmonella* serotypes and other bacterial species

It has been well demonstrated that penetration of the eggshell and shell membranes is not a unique characteristic of SE and that other *Salmonella* serotypes, and even unrelated bacteria, are capable of passing through these barriers (Sauter & Petersen, 1969, 1974; Mayes & Takeballi, 1983; Jones *et al.*, 2002; De Reu *et al.*, 2006). In a comparative study, the penetration of seven selected bacterial species originally isolated from egg contents was assessed using two different egg penetration models (De Reu *et al.*, 2006). The results indicate that Gram-negative, motile and nonclustering bacteria penetrate the eggshell most frequently. Using an agar model, i.e. filling eggs with agar and dipping in a bacterial suspension, *Pseudomonas* sp. (60%), *Alcaligenes* sp. (58%) and SE (43%) traversed the eggshell most frequently. However, using intact eggs dipped in a bacterial suspension, egg contents were most frequently contaminated by SE (33%), followed by *Carnobacterium* sp. (17.5%) and *Acinetobacter baumannii* (14.8%). The results obtained by the two experimental eggshell penetration assays suggest that shells can be penetrated by various bacterial species, but that SE has mechanisms to survive and/or grow in the internal egg contents, in contrast to the other bacterial species. In a study of naturally infected flocks, numerous *Salmonella* serotypes, such as Enteritidis, Typhimurium and Hadar, were isolated from eggshells, whereas only Enteritidis was isolated from egg contents (Humphrey *et al.*, 1991b). Interestingly, only one egg was positive in both sites, suggesting that internal egg contamination is more likely to occur during formation of the egg rather than by penetration through the shell. Moreover, the relative prevalence of non-Enteritidis serotypes in faecal samples (measured by the overshoe method) of laying hen flocks (50%) is not consistent with the high prevalence of SE in table eggs (90%) (EFSA, 2007a). All these data support the idea that eggshell and egg membrane penetration are not a specific property of SE and that other characteristics of this serotype are related to egg contamination. These could include the ability to colonize the hen reproductive tract and survival and multiplication inside eggs, both of which could contribute to the epidemiological association of SE with eggs.

Contamination of eggs during egg formation

Colonization of the reproductive organs

Several lines of evidence support the view that egg contamination with SE is more likely to take place during the formation of the egg in the reproductive organs than by eggshell penetration. In several studies, SE was isolated from the reproductive tissue of infected birds, in the absence of intestinal colonization (Lister, 1988). Moreover, SE is capable of persistence in reproductive tissues of naturally and experimentally infected hens, even though the animals generate an innate and adaptive immune response to the infection, indicating that the bacteria can reside intracellularly and escape the host defence mechanisms. The deposition of *Salmonella* inside eggs is thus most likely a consequence of reproductive tissue colonization in infected laying hens (Keller *et al.*, 1995; Methner *et al.*, 1995; Gast & Holt, 2000a). Very little is known, however, about the exact site in reproductive tissues where the bacteria reside and the bacterial and host factors that play a role in the association

between the reproductive tissue and *Salmonella*. The oviduct can be subdivided into five functional regions. Starting from the ovary, there are the infundibulum, magnum, isthmus, uterus and vagina. The infundibulum captures the ovulatory follicles, the magnum produces the albumen, the isthmus deposits the eggshell membranes, the uterus forms the eggshell and the vagina is involved in oviposition. *Salmonella* colonizing the oviduct could be incorporated into the albumen, the eggshell membranes or the eggshell itself, depending on the site of colonization (magnum, isthmus and uterus, respectively). Although SE has been isolated from both the yolk and the albumen, according to most authors, the albumen is most frequently contaminated, pointing to the oviduct tissue as the colonization site (Gast & Beard, 1990; Humphrey *et al.*, 1991b; Keller *et al.*, 1995; Miyamoto *et al.*, 1997; De Buck *et al.*, 2004c). However, some studies found the yolk to be primarily contaminated, suggesting the ovary to be the primary colonization site (Bichler *et al.*, 1996; Gast & Holt, 2000a; Gast *et al.*, 2002). One report indicated that several *Salmonella* strains colonized the ovary significantly more often than the oviduct, but were deposited at similar frequencies in the yolk and the albumen (Gast *et al.*, 2007). Because of the very low incidence of egg contamination in natural infections and the fact that it is very labour-intensive to examine large numbers of eggs, not enough studies have been carried out to definitely establish the principal site of contamination. It is thus difficult, based on the contamination site in eggs, to predict the most important colonization site of *Salmonella* in the reproductive tract. However, it would be reasonable to suggest that, given that SE can be isolated from all sites in the hen reproductive tract, that contamination of any part of the egg is possible. An overview of all studies on internal egg contamination through reproductive organ colonization is presented in Table 1.

It is generally believed that colonization of the reproductive organs is a consequence of systemic spread of *Salmonella* from the intestine (Vazquez-Torres *et al.*, 1999). Invasion in the intestinal epithelial cells triggers infiltration of immune cells, mainly macrophages, resulting in the uptake of bacteria by these cells. Because of its capability to survive and replicate in the immune cells, bacteria carried in the macrophages are spread within the host, resulting in colonization of the reproductive organs (Keller *et al.*, 1995; Miyamoto *et al.*, 1997; Okamura *et al.*, 2001a, b; Gast *et al.*, 2007; Gantois *et al.*, 2008c). *Salmonella* pathogenicity island-2 (SPI-2) is essential in the ability to spread within the host and to cause a systemic infection (Jones *et al.*, 2001). Using a deletion mutant in the regulator of SPI-2 (*ssrA*), it was shown that after intravenous infection of laying hens, the bacterial numbers of the *ssrA* mutant were significantly lower in the oviducts and the ovaries as compared with the wild-type strain. These reduced *ssrA* colony counts in the reproductive organs point to a role for SPI-2 in the spread or the colonization of the reproductive tract tissues (Bohez *et al.*, 2008).

Colonization of the reproductive organs has also been shown to be a consequence of systemic spread after airborne infections (Baskerville *et al.*, 1992; Leach *et al.*, 1999). It was even observed that the contamination rate of eggs was much higher following an aerosol challenge of the laying hens than following an oral challenge (Leach *et al.*, 1999).

Colonization of the ovary

The extensive permeability of the vascular endothelia observed in the ovary may contribute to the high colonization rate at this site (Griffin *et al.*, 1984). In the majority of experimental studies in laying hens, a higher frequency of ovary colonization is reported, compared with the frequency of recovery from the oviduct (De Buck *et al.*, 2004b; Gantois *et al.*, 2006; Gast *et al.*, 2007). Therefore, it is strongly believed that SE must interact with the cellular components of the preovulatory follicles. It was indeed shown that SE can attach to developing and mature follicular granulosa cells exhibiting different attachment patterns (Thiagarajan *et al.*, 1994). Higher bacterial numbers in the membranes of the preovulatory follicles than in the yolk itself suggest that during transovarian transmission, SE remains attached to the egg vitelline membranes. A previous study has also suggested that yolk contamination is more often associated with the vitelline membrane than with the interior yolk contents (Gast & Beard, 1990; Gast & Holt, 2000a). It has been noticed that *in vitro* attachment of SE to granulosa cells may involve binding to fibronectin (Thiagarajan *et al.*, 1996a). Furthermore, a major role of the type 1 fimbriae in the attachment process was suggested because the *in vitro* attachment of SE to granulosa cells was inhibited by preincubation of the cells with purified fimbrial preparation (Thiagarajan *et al.*, 1996a). There are also indications that *Salmonella* can invade and multiply in granulosa cells (Thiagarajan *et al.*, 1996a). Howard *et al.* (2005) compared the ability of *Salmonella* to invade ovarian follicles at different stages of follicular maturity *in vitro*: the small white follicles (immature) were more susceptible to *Salmonella* invasion than the more mature small and large yellow ones. These authors believe that the penetration of immature follicles has practical implications because it can lead to contamination of eggs after maturation and can cause continuous transovarian infection of eggs throughout the reproductive cycle. This statement is, however, questionable because not all small white follicles will mature and because the extensive growth of *Salmonella* in the nutrient-rich follicles will most likely lead to their degeneration (Kinde *et al.*, 2000).

Table 1. Overview of studies carried out to analyse the internal egg contamination through infection of the reproductive organs

	Method			Result	
References	Inoculation route	Strain	Inoculation dose (log_{10} CFU mL^{-1})	General result	Egg contamination rate
Timoney *et al*. (1989)	Oral	SE PT4	6	The relatively high frequency of internal egg contamination clearly demonstrates the potential for egg transmission of SE	Yolk: 9.6% Egg white: 3.6%
Shivaprasad *et al*. (1990)	Oral, intravenous, cloacal	SE PT8	8.3–8.6	SE was only cultured from the yolk and egg white of a small number of eggs until 11 days postinfection	Yolk: 0.4% Egg white: 1.5%
Gast & Beard (1990)	Oral, contact transmission	SE PT13a	9	Although a high contamination rate of egg white and yolk was observed, *Salmonella* could not be recovered from any yolk content sample, suggesting that *Salmonella* is contaminating the vitelline membrane	Yolk: 18.5% (first week) Egg white: 20% (first week) Yolk contents: 0%
Humphrey *et al*. (1991a)	Oral	SE PT4	3, 6, 8	There was no relationship between the contamination of the egg contents and antibody status, faecal excretion or the dose administered	Total egg content: Inoculum 3: 3.5% Inoculum 6: 0% Inoculum 8: 0%
Thiagarajan *et al*. (1994)	Oral	SE PT8, SE PT28	Not mentioned	SE can colonize the preovulatory follicles at different stages of development. It is therefore suggested that SE remains attached to the vitelline membrane instead of contaminating the yolk content	Preovulatory follicle (16 birds): Membrane: 10 positive samples Yolk content: four positive samples Laid eggs: Yolk: eight positive yolks Egg white: three positive egg whites
Keller *et al*. (1995)	Oral	SE	8	The contamination rate of forming eggs is much higher than the contamination rate of laid eggs, indicating that antibacterial factors within the egg may control the pathogen before the egg is laid	Forming eggs: 27.1–31.4% Freshly laid eggs: 0–0.6%
Methner *et al*. (1995)	Oral	SE	10	No correlation was found between the contamination of the eggshell and that of the egg content	Yolk: 0% Egg white: 0.4%
Bichler *et al*. (1996)	Oral	SE	10	The hens produced SE-positive eggs at high frequencies in the first week postinfection.	In the first week postinfection: Eggshell washing: 26.5% Egg content: 2.9% Egg white: 43% Yolk: 41%
Keller *et al*. (1997)	Oral	SE, *Salmonella* Typhimurium	8	SE and *Salmonella* Typhimurium may be equal in their potential to colonize the tissues of the reproductive tract and forming eggs, but only SE was isolated from egg contents after oviposition	Total egg content: *Salmonella* Typhimurium: 0%
Miyamoto *et al*. (1997)	Intravenous, intravaginal, cloacal	SE PT4	7	Intravaginal and cloacal inoculation resulted in the colonization of only the lower oviduct whereas intravenous infection resulted in colonization of the entire oviduct	Total egg content/shell: Intravenous: 11.5%/7.7% Intravaginal: 9.6%/12% Cloacal: 0%/4.6%
Williams *et al*. (1998)	Oral	*Salmonella* Typhimurium DT104	7	These experiments have demonstrated that DT104 can contaminate the egg contents after oral infection	Total egg content: 2.1%
Miyamoto *et al*. (1998b)	Intravaginal, cloacal	SE PT4	7	After intravaginal inoculation, SE was recovered from the uterus and after cloacal inoculation SE was recovered from the vagina, indicating that SE only ascends to the lower parts of the oviduct	Total egg content/shell Intravaginal: 5%/15% Cloacal: 0%/0%
Gast & Holt (1998)	Oral	SE PT13	7	This study has shown that infection of 1-day-old chicks can lead to frequent intestinal colonization and occasional egg contamination when these birds mature	Total egg content: 0.44%

Table 1. Continued.

	Method			Result	
References	Inoculation route	Strain	Inoculation dose (log_{10} CFU mL^{-1})	General result	Egg contamination rate
Leach *et al*. (1999)	Oral, aerosol	*Salmonella* Typhimurium DT104	7, 2–4	The egg contamination rate in aerosol-infected birds was much higher compared with orally infected birds	Total egg content: Oral: 1.7% Aerosol: 14–25%
Gast & Holt (2000a)	Oral	Two SE PT4 and one SE PT13a	9	For all three isolates, the incidence of yolk contamination was significantly higher than the incidence of egg white contamination and no significant difference was observed between the SE strains	Yolk: 2.5% Egg white: 0.5%
Kinde *et al*. (2000)	Oral, intravenous	SE PT4	9, 6	In the orally infected birds, 43% of the reproductive organs were positive, compared with 83% in the intravenously infected birds	Total egg content: 2.6%
Okamura & Holt (2001a, b)	Intravaginal	SE, *Salmonella* serotypes Typhimurium, Infantis, Hadar, Heidelberg and Montevideo	6	This study suggests that SE has a specific advantage over the other *Salmonella* serotypes by its capacity to colonize the vaginal tissues of hens	Egg content/shell SE: 7.5%/25% *Salmonella* Typhimurium: 3.1%/1.6% *Salmonella* Infantis: 0%/4% *Salmonella* Hadar: 0%/4.9% *Salmonella* Heidelberg: 0%/4.5% *Salmonella* Montevideo: 0%/1.9%
Okamura *et al*. (2001a, b)	Intravenous	SE, *Salmonella* serotypes Typhimurium, Infantis, Hadar, Heidelberg and Montevideo	6	This study suggests that SE is the predominant serovar to colonize the reproductive organs of laying hens among the six serotypes tested	Yolk/egg white SE: yolk: 6.9%/2.3% Other *Salmonella* serotypes: 0%/0%
Wigley *et al*. (2001)	Oral	*Salmonella* Pullorum	9	One-day-old chicks orally infected with *Salmonella* Pullorum produced contaminated eggs frequently during the period of sexual maturity as a consequence of reproductive tract colonization	Total egg content: 6.5%
Gast & Holt (2001)	Oral	SE PT13a	9	Deposition of SE within egg yolks appears to occur infrequently and SE is mostly deposited on the vitelline membrane	Total yolk: 4.3% Yolk content: 0.5%
Gast *et al*. (2002)	Oral, aerosol, intravenous	SE PT13a	9, 9 and 5–7	No significant differences were observed in egg contamination among the three inoculation routes	Yolk: 4–7% Egg white: 0–2%
Gast *et al*. (2003)	Oral	SE PT13a wild type (WT), passaged SE PT13a (spleen, liver) passaged SE PT13a (oviduct and ovary)	9	Passaged SE strains recovered from ovaries and oviducts induced a significantly higher incidence of egg contamination than the WT SE strain	Total egg content: SE PT13a WT: 8.27% Passaged SE PT13a (spleen, liver): 10.41% Passaged SE PT13a (oviduct and ovary): 17%
Gast *et al*. (2004)	Oral	SE, *Salmonella* Heidelberg	9	There was no significant difference in reproductive tract colonization between the two serotypes, but *Salmonella* Heidelberg was recovered from the eggs at lower frequencies than SE	Total egg content: SE: 7.0% *Salmonella* Heidelberg: 1.1–4.5%
De Buck *et al*. (2004c)	Intravenous	SE PT4	7	The infected birds produced the highest frequency of contaminated eggs in the first week postinfection	Shell: 17.4% Yolk: 20.3% Egg white: 4.3%
Gast *et al*. (2005b)	Oral	SE, 2 *Salmonella* Heidelberg strains and passaged	9	The *Salmonella*-passaged strains caused a significantly higher frequency of egg contamination than did the WT strains.	Total egg content: SE WT: 5% SE passaged strain: 8.84%

Table 1. Continued.

References	Method: Inoculation route	Method: Strain	Method: Inoculation dose (log_{10} CFU mL^{-1})	Result: General result	Result: Egg contamination rate
		variants of each WT strain		Furthermore, no correlation was found between the duration of faecal shedding and the production of contaminated eggs	*Salmonella* Heidelberg WT 1: 1.63% *Salmonella* Heidelberg passaged strain 1: 4.95% *Salmonella* Heidelberg WT 2: 3.14% *Salmonella* Heidelberg passaged strain 2: 5%
Gast *et al*. (2007)	Oral	SE PT13a, SE PT14b, *Salmonella* Heidelberg	9	The frequency of ovarian colonization was significantly higher than the frequency of recovery from the oviduct for all three *Salmonella* strains, but no corresponding difference was observed between the incidence of deposition in yolk or egg white. The incidence of egg contamination with SE was higher than that of *Salmonella* Heidelberg	SE: Yolk: 5.5% Egg white: 4.1% *Salmonella* Heidelberg: Yolk: 1.5% Egg white: 1.8%
Gantois *et al*. (2008c)	Intravenous	2 SE strains, *Salmonella* serotypes Typhimurium, -Heidelberg, Virchow, Hadar	8	The SE strains showed a higher colonization of the reproductive organs in comparison with the *Salmonella* serotypes Heidelberg, Virchow and Hadar. No significant difference was observed between the SE strains and the *Salmonella* Typhimurium strain	Total egg content (positive eggs/total eggs): SE1: 4/5 SE2: 3/5 *Salmonella* Typhimurium: 2/5 *Salmonella* Heidelberg: 0/6 *Salmonella* Virchow: 1/16 *Salmonella* Hadar: 0/16

The fact that *Salmonella* can interact with the cellular components of preovulatory follicles raises the question as to whether serotype Enteritidis harbours some intrinsic characteristics allowing it to specifically interact with these cells and, as a consequence, be transmitted to eggs. In a study by Okamura *et al*. (2001a, b), it was shown that among six different *Salmonella* serotypes, Enteritidis colonized ovaries and preovulatory follicles at significantly higher levels than five other serotypes after intravenous inoculation. Because samples in this study were only taken at 4 and 7 days postinfection, and bacteria were still persistent in the peripheral blood, it cannot be concluded, however, that SE displays a stronger interaction with follicles than other serotypes. Similar results were obtained by Gantois *et al*. (2008c) in an intravenous infection model, demonstrating a higher affinity of the serotype Enteritidis for the ovary compared with other *Salmonella* serotypes (Hadar, Virchow and Infantis), except for Typhimurium. The fact that SE and *Salmonella* Typhimurium may be equally capable of colonizing the ovary is in accordance with the data obtained by Keller *et al*. (1997). Studies comparing invasion of the serotypes Enteritidis and Typhimurium in ovarian follicles *in vitro* yielded conflicting results (Howard *et al*., 2005; Mizumoto *et al*., 2005). Based on the fact that systemic spread is a characteristic of most *Salmonella* serotypes, it is believed that ovarian colonization is not a specific trait allowing the serotype Enteritidis to contaminate eggs. However, the possibility that SE has a specific ability to interact and invade the preovulatory follicles cannot be ruled out. A large-scale study using multiple strains from different *Salmonella* serotypes should be carried out in order to provide more information regarding the serotype specificity of ovarian colonization and persistence. High levels of nutrients are available to bacteria invading ovarian follicles. Therefore, it is to be expected that this should lead to extensive replication of the bacteria, almost inevitably resulting in follicular degeneration. Because this is not a common phenomenon in naturally infected laying hens, as the laying percentage is usually not reduced, follicle colonization is not believed to be an important source of egg contamination, although this is under debate.

Colonization of the oviduct

Although several studies reported the vitelline membrane as the most common site of *Salmonella* contamination (Bichler *et al*., 1996; Gast & Holt, 2000a; Gast *et al*., 2002), other reports point to albumen as the principal site of contamination in eggs (Shivaprasad *et al*., 1990; Humphrey *et al*., 1991b; Keller *et al*., 1995), indicating that SE is colonizing oviduct tissues. Miyamoto *et al*. (1997) observed that developing eggs in a highly contaminated oviduct are likely to be *Salmonella* positive. Colonization of the reproductive tract can be the result of an ascending infection from the

cloaca (Reiber *et al.*, 1995; Miyamoto *et al.*, 1997), a descending infection from the ovary (Keller *et al.*, 1995) and/or a systemic spread of *Salmonella*. Depending on the site of contamination, i.e. the vagina, isthmus and magnum, *Salmonella* could be incorporated into the eggshell, the eggshell membranes or the albumen.

Vaginal colonization

Several authors have focused on the role of the vagina in the production of SE-contaminated eggs (Barrow & Lovell, 1991; Keller *et al.*, 1995; Reiber *et al.*, 1995; Miyamoto *et al.*, 1999; Okamura *et al.*, 2001a, b; Mizumoto *et al.*, 2005). It is believed that intravaginal infection tends to ascend only to the lower parts of the oviduct because *Salmonella* is rarely recovered from the ovary and the upper oviduct in intravaginally inoculated hens (Miyamoto *et al.*, 1997, 1998b; Okamura *et al.*, 2001a, b). These studies obtained high egg contamination rates after intravaginal infection, indicating a high risk of contamination (primarily eggshell contamination) as the egg passes through a heavily colonized vagina. When the egg is laid, penetration through the eggshell can occur, due to suction of the organisms into eggs under the negative pressure caused by cooling of the egg (Schoeni *et al.*, 1995; Miyamoto *et al.*, 1998a). In spite of the fact that it is difficult to distinguish between contamination during formation of the egg or after oviposition, internal egg contamination after vaginal colonization most likely occurs after penetration of the eggshell and not by internal contamination following ascending infection of the upper oviduct, although this cannot be ruled out. In a comparative study with six different *Salmonella* serotypes, significantly higher numbers of SE were recovered from the vagina in comparison with strains belonging to other serotypes after intravaginal inoculation (Miyamoto *et al.*, 1998a). The authors suggested a higher ability of the serotype Enteritidis to attach to the vaginal epithelium. It was also noticed that the rank order of the *Salmonella* invasiveness in vaginal epithelium was dependent on the lipopolysaccharide type, namely lipopolysaccharide type O9 (SE) > lipopolysaccharide type O4 (*Salmonella* Typhimurium, *Salmonella* Heidelberg and *Salmonella* Agona) > lipopolysaccharide type O7 (*Salmonella* Montevideo and *Salmonella* Infantis) and lipopolysaccharide type O8 (*Salmonella* Hadar) (Mizumoto *et al.*, 2005).

Isthmus and magnum colonization

It is clear that different segments of the oviduct can be colonized by SE. Using different infection models, the tubular glands of the isthmus were identified as the predominant colonization site of SE in the oviduct by De Buck *et al.* (2004a). Colonization of the isthmus can result in contaminated eggshell membranes. These observations are in accordance with other experimental studies (Bichler *et al.*, 1996; Miyamoto *et al.*, 1997; Okamura *et al.*, 2001a, b). In principle, eggshell membrane contamination can also be a consequence of penetration of *Salmonella* bacteria after deposition on the shell during the passage through the vagina rather than direct contamination of the eggshell membranes during passage through the isthmus. In addition, when culturing the eggshell and egg contents separately, some albumen sticks to the eggshell, making the interpretations of the shell membranes as the site of egg contamination even more complex.

Numerous studies suggest that SE most frequently migrates into eggs through the upper oviduct in association with the albumen (Gast & Beard, 1990; Hoop & Pospischil, 1993; Humphrey & Whitehead, 1993; Schoeni *et al.*, 1995). Detection of SE associated with secretory cells of the upper and lower magnum by immunohistochemical staining is in agreement with the hypothesis that the pathogen may contaminate forming eggs through the albumen (Hoop & Pospischil, 1993; Keller *et al.*, 1995; De Buck *et al.*, 2004a). Recently, the abilities to invade and proliferate in isthmus and magnum oviduct cells of different *Salmonella* serotypes were assessed using a tubular gland cell primary culture model. All serotypes tested were equally able to invade and proliferate in the glandular epithelial cells, suggesting that invasion and proliferation in oviduct cells is most likely not a unique characteristic of the serotype Enteritidis (Gantois *et al.*, 2008c). In the study of Gantois *et al.* (2008c), it was also shown that a *Salmonella* serotype Enteritidis and Typhimurium strain colonized the oviduct to higher levels than strains belonging to the serotypes Heidelberg, Virchow and Hadar, even if all serotypes invaded oviduct cells *in vitro*. This is in accordance with a previous intravenous infection study by Okamura *et al.* (2001a, b), demonstrating that of six serotypes, only Enteritidis and Typhimurium were able to colonize the reproductive organs at days 4 and 7 postinoculation. One-day-old chicks that were orally infected with the chicken-adapted *Salmonella* Pullorum produced a high amount of contaminated eggs (6.5%) during the period of sexual maturity as a consequence of reproductive organ colonization (Wigley *et al.*, 2001). Isolates of SE and *Salmonella* Pullorum, together with isolates of *Salmonella* Gallinarum and *Salmonella* Dublin, form a related strain cluster that share the same lipopolysaccharide-based O-antigen structure (O-1, 9, 12, characteristic of serogroup D). Comparative genome analysis of SE and *Salmonella* Gallinarum indicated that these serotypes are highly related and that *Salmonella* Gallinarum may be a direct descendant of SE (Thomson *et al.*, 2008). It can be speculated that these two serotypes harbour the same characteristics, allowing them to efficiently contaminate eggs, but this is not clear.

Reproductive tract colonization: a matter of controversy

It is difficult to make comparisons between different experimental studies attempting to determine the preferred site of colonization or the strongest colonizing and persisting serotype. Indeed, experimental infection studies use different strains, inoculation methods, infection doses and laboratory techniques for bacteriological analysis. Individual strains of *Salmonella* (within and across serotype boundaries) can differ considerably in their ability to contaminate eggs (Gast & Holt, 2000a, 2001). Four *Salmonella* Heidelberg strains colonized the ovaries and oviducts of inoculated hens at frequencies similar to SE, but were found significantly less often inside eggs (Gast *et al.*, 2004). Phenotypic attributes, such as the ability to produce high-molecular-mass lipopolysaccharide and the ability to grow to a high cell density, have been linked to an enhanced capability of egg contamination by SE (Guard-Petter *et al.*, 1997; Guard-Petter, 1998). Recently, a set of small nucleotide polymorphisms (SNPs), which differ in two SE strains that vary in egg contamination, were identified (Guard-Bouldin, 2006). In addition, a high-throughput phenotype microarray assaying the growth of bacteria in response to 1920 different culture conditions revealed that these two strains show dramatic differences in amino acid and nucleic acid metabolism, which is most likely correlated to the SNPs (Morales *et al.*, 2005). Furthermore, it was shown that serial passage through the reproductive organs also enhances egg contamination (Gast *et al.*, 2003, 2005a). This indicates that the selective pressure in the reproductive tissues may promote the induction of specific bacterial properties, resulting in an elevated egg contamination. Numerous studies have also been performed to study the effect of the inoculation route on the production of contaminated eggs (Miyamoto *et al.*, 1997; Gast *et al.*, 2002). While Gast *et al.* (2002) reported that oral, aerosol and intravenous inoculations led to similar frequencies of egg contamination, Miyamoto *et al.* (1997) observed a higher contamination rate when birds were inoculated intravenously and intravaginally. Moreover, it should be taken into account that using different laboratory techniques to isolate the bacteria from eggs may have an impact on the outcome of the experiments. In some studies, the yolk samples are cultured together with the vitelline membrane and thus some albumen, while other studies extract yolk contents and thus do not culture the vitelline membrane. Furthermore, extending the incubation time from 24 to 48 h can increase the isolation rate of SE from eggs significantly (Humphrey & Whitehead, 1992), meaning that studies that do not take account of this may have underestimated the prevalence of egg contamination. The use of different pre-enrichment and enrichment media can also result in different outcomes (Humphrey & Whitehead, 1992). For isolation of *Salmonella* from whole eggs, it was found that the Rappaport Vassiliadis broth was superior to Selenite broth as a selective medium (Humphrey & Whitehead, 1992). Additionally, the outcomes of *Salmonella* infections may also be influenced by host susceptibility characteristics, such as the breed or the line of chickens (Beaumont *et al.*, 1994, 1999; Keller *et al.*, 1995; Kinde *et al.*, 2000). Some studies reported that brown-egg layers are more susceptible than white-egg layers (Keller *et al.*, 1995; Kinde *et al.*, 2000), and on comparing four different lines of chickens, it was found that one line was more susceptible to SE than others (Protais *et al.*, 1996). All these aspects indicate that care should be taken when interpreting data obtained from experimental infections.

Virulence factors associated with oviduct colonization

In order to gain a better understanding of the molecular mechanisms allowing the serotype Enteritidis to interact with the hen's reproductive tract and to adapt to this particular ecological niche, a genome-wide screen was carried out by Gantois *et al.* (2008b) to identify genes expressed in the oviduct, using *in vivo* expression technology. This study identified the genes involved in cell wall integrity, regulation of fimbrial operons, amino acid and nucleic acid metabolism, stress response and motility as being highly induced during colonization of the reproductive tract. This indicates that the oviduct is a stressful and damaging environment for *Salmonella* bacteria, but it also indicates that the bacteria can counteract this by stress-induced protective and reparative responses, enabling the bacteria to survive in the hostile environment and/or escape the host defence reactions.

Other SE factors that play a role in oviduct infections are fimbriae (De Buck *et al.*, 2003, 2004b; Li *et al.*, 2003). Li *et al.* (2003) were the first to identify binding sites for fimbriated SE in the chicken oviduct. The binding of type 1 fimbriae to glycosphingolipids and gangliosides from the oviduct mucosa is not uniform along this organ, and is mainly in the infundibulum. De Buck *et al.* (2003) clearly demonstrated that SE isolates are able to adhere to immobilized secretions of the oviduct. These authors showed that the receptor of adhesion is also localized inside the tubular gland cells of the isthmus and adhesion is blocked by the addition of mannose, indicating that the adhesion is mediated by type 1 fimbriae. Conversely, a study using SE isolates differing in their fimbrial expression found no difference in infection of the reproductive organs and eggs, making their role equivocal (Thiagarajan *et al.*, 1996b). Nevertheless, a screening for promoters induced in the albumen showed that SE *fimZ* is highly induced during incubation at 42 °C. This may mean that when *Salmonella* resides extracellularly in the oviduct lumen, in the presence of albumen, the transcription of type

1 fimbriae will be activated, resulting in bacterial attachment to the secretory glandular cells (own unpublished data).

There is mounting evidence that lipopolysaccharide is also of particular importance for SE persistence in the reproductive tract tissues. Lipopolysaccharide is a major component of the outer membrane of Gram-negative bacteria and a prime target for recognition by the innate immune system. At least two different functions have been attributed to lipopolysaccharides with respect to persistent reproductive tract infection. It has been suggested that the composition of lipopolysaccharides is important in determining the survival of SE in avian macrophages and these cells may be a site where SE resides in the oviduct (He *et al.*, 2006). Different levels of attachment of different *Salmonella* serotypes to chicken vaginal explants possibly also involve a role of the lipopolysaccharide structure (Mizumoto *et al.*, 2005). Furthermore, it has been shown that high-molecular-weight lipopolysaccharide in SE is correlated with increased egg contamination (Guard-Petter *et al.*, 1997). Although the exact role of high-molecular-weight lipopolysaccharide is not yet known, its presence has been correlated with an unusual pathology of the reproductive tract, although this was not reflected in higher egg contamination (Parker *et al.*, 2002).

The type 3 secretion systems-1 and -2 (T3SS-1 and T3SS-2) may also play a role in egg contamination. T3SS-1 is mainly associated with bacterial invasion of the intestinal epithelium via the concerted action of effector proteins (Zhou *et al.*, 1999), while T3SS-2 is responsible for the establishment of systemic infection by promoting the intracellular survival of *Salmonella* in macrophages, as mentioned earlier. Li *et al.* (2008) were the first to confirm the pathogenic role of T3SS-1 and T3SS-2 effectors in SE invasion and intracellular survival in chicken oviduct epithelial cells. It is believed that invasion and survival in tubular gland cells of the oviduct is not specific for serotype Enteritidis (Gantois *et al.*, 2008c). Most likely, functions exerted by T3SS-1 and T3SS-2 are also required by *Salmonella* serotypes other than Enteritidis to invade and survive inside chicken oviduct epithelial cells (Jones *et al.*, 2002). Furthermore, a recent study suggested that inactivation of *ssrA*, a regulator of T3SS-2, rendered SE unable to colonize the chicken reproductive tract successfully (Bohez *et al.*, 2008).

Meanwhile, it has become clear that the process of oviduct colonization is complex and depends on many factors including fimbriae, flagellae, lipopolysaccharide, cell wall structure and stress tolerance. Although most, if not all, bacterial factors, shown to play a role in reproductive tract colonization, are not specific to the serotype Enteritidis, a unique regulation of these known virulence factors in the reproductive tract environment could be one plausible explanation for the epidemiological association with hen's eggs. This, however, has not been shown yet. It was demonstrated that repeated *in vivo* passages through the reproductive tissues of chickens increase the ability of an SE strain to induce internal egg contamination, whereas serial passage through the liver and the spleen did not affect the ability of the strain to cause egg contamination (Gast *et al.*, 2003). This is an indication that interaction of SE with the reproductive tissues may either induce or select for the expression of microbial properties important for egg contamination. The complementarity between phenotypic traits with relevance for colonization and survival in different tissues may allow SE to traverse the complex series of events between the introduction of infection and the deposition inside eggs (Gast *et al.*, 2002).

The interaction between *Salmonella* and the forming egg

Transfer of *Salmonella* from the hen to the egg

The survival of SE in forming eggs has been considered crucial for internal egg contamination (Keller *et al.*, 1995, 1997). *Salmonella* colonizing reproductive organs can potentially be incorporated into the forming egg, provided the contamination of the egg contents does not lead to an abortive egg formation and provided the bacteria are not killed by the albumen.

Yolk contamination can occur due to ovary colonization by *Salmonella*. Degeneration of follicles in the ovary has often been observed after experimental *Salmonella* infections, most likely caused by extensive growth in the nutrient-rich yolk at chicken body temperature, 42 °C (Kinde *et al.*, 2000). Interestingly, extensive growth in whole eggs when stored at room temperature (20–25 °C) does not lead to changes in the colour, smell and consistency of the egg contents (Humphrey & Whitehead, 1993), suggesting that the process of yolk degeneration is dependent on physiological factors, such as the temperature. Degeneration of the ovarian follicles would result in a decline in the production cycle and thus no production of eggs containing contaminated yolks. The extent to which the yolk contents become positive after infection of the ovary is, however, not clear. After intravenous inoculation, SE cells are confined to the interstitial tissues and not to the yolk contained in the large follicles (Barrow & Lovell, 1991). Moreover, experimental studies have suggested that *Salmonella* are far more likely to be deposited on the outside of the vitelline membrane rather than inside the nutrient-rich yolk during ovary colonization (Gast & Holt, 2001). Inoculation of *Salmonella* onto the vitelline membrane in an *in vitro* egg contamination model demonstrated that some strains were capable of penetrating into the yolk contents at a low frequency during 24 h of incubation at 30 °C (Gast *et al.*, 2005b), but a similar study reported no positive yolk contents samples after incubation for 24 h at 42 °C (Guan *et al.*, 2006). Moreover, SE multiplication on the exterior vitelline membrane both preceded and exceeded multiplication resulting

from penetration into the yolk contents during 36 h of incubation at 30 °C (Gast *et al.*, 2008). This suggests a low invasion of yolk contents by *Salmonella* during egg formation. These data support the possibility of incorporation into the egg by carriage on the vitelline membrane.

Albumen or shell membrane contamination would occur when *Salmonella* colonizes the upper oviduct. According to Keller *et al.* (1995), the infection of the forming egg occurs at this site, before eggshell deposition. Indeed, after oral infection with SE, about one-third of the forming eggs were positive compared with 0.6% of the freshly laid eggs (Keller *et al.*, 1995). This reduction clearly suggests that antibacterial factors within the albumen can exert a degree of control of SE in forming eggs. During the *c.* 26 h required for the formation of an egg, the ovum spends *c.* 5 h in the magnum, where it is surrounded by the albumen, followed by the addition of two shell membranes in the isthmus. The remaining 21 h are required for shell deposition in the uterus, after which the completed egg is moved through the vagina to pass through the cloaca as it is laid (Solomon, 1997). Survival in the forming egg could be a possible reason for selective isolation of the serotype Enteritidis in laid eggs, provided that this serotype harbours intrinsic or induced factors related to albumen resistance. Oral infection of laying hens with three different SE and Typhimurium strains revealed that both serotypes are equally able to colonize tissues of the reproductive tract and forming eggs in the oviduct before oviposition. However, only SE, but not *Salmonella* Typhimurium, was isolated from egg contents after oviposition (Keller *et al.*, 1997), suggesting survival strategies of SE inside the forming eggs. Nevertheless, *Salmonella* Typhimurium DT104 was shown to contaminate the egg contents after oral infection of laying hens (Williams *et al.*, 1998).

Antimicrobial components in the albumen and vitelline membrane

The reproductive tract produces antimicrobial components that are incorporated into the albumen, and that are growth restricting for *Salmonella*. The most well known are lysozyme and ovotransferrin. Lysozyme is a muramidase affecting the cell wall of Gram-positive bacteria (Hughey & Johnson, 1987), but that has also been shown to form pores in the cell wall of Gram-negative bacteria (Gast *et al.*, 2005a). Ovotransferrin possesses two distinct mechanisms of bacteriostatic action against bacteria. The first is iron chelation, which creates an iron-deficient environment for bacteria (Mayes & Takeballi, 1983). The second is a direct interaction with the membrane and induction of damage to biological functions of the bacterial cytoplasmic membrane (Ibrahim *et al.*, 1998, 2000).

Another group of antimicrobial proteins are those showing proteinase-inhibiting activity. They include ovomucoid, ovoinhibitors (serine protease inhibitors), cystatin (a cysteine protease inhibitor) and ovostatin (Stevens, 1991). Their function lies in inhibiting tryptic digestion of egg proteins by bacteria and thus protection of the antimicrobial activity of albumen proteins.

A recent study identified 11 types of gallinacins (β-defensins) expressed in the segments of the oviduct (Abdel Mageed *et al.*, 2008). Defensins are antimicrobial peptides that play significant roles in innate immunity (Sugiarto & Yu, 2004; Higgs *et al.*, 2005). The greatest expression of gallinacins was seen in the infundibulum and the vagina (Ohashi *et al.*, 2005). Recently, Yoshimura *et al.* (2006) reported that the expression of gallinacin-1, -2 and -3 was increased within 24 h in response to SE infection or in response to purified lipopolysaccharides in cultured vaginal cells. The study by Abdel Mageed *et al.* (2008) confirmed that gallinacin-3 expression was enhanced by lipopolysaccharide *in vivo*. *Escherichia coli* lipopolysaccharide injection in laying hens also induced gallinacin expression in the ovarian follicles (Subedi *et al.*, 2007). β-Defensin-11 was identified to be present in the chicken albumen as well (Mann, 2007). Remarkably, the albumen contains many proteins that are connected in some way to lipopolysaccharide binding or modification. One such albumen component is similar to the mammalian acyloxyacyl hydrolase, known to cleave acyl chains from bacterial lipopolysaccharide. Furthermore, proteins containing BPI domains have been identified. These usually occur in proteins binding and neutralizing lipopolysaccharide and thus eventually mediating the destruction of bacteria (Elsbach & Weiss, 1998). Such domains also occur in Tenp, a protein recently identified as an albumen component (Guérin-Dubiard *et al.*, 2006), and in the eggshell-specific protein ovocalyxin-36 (Gautron *et al.*, 2006). Recently, Silphaduang *et al.* (2006) were the first to report the presence of histones H1 and H2B as antimicrobial proteins in the avian reproductive system, but their functional significance in the chicken reproductive tract remains obscure. In the human placenta, histones H2A and H2B show a dose-dependent inhibition of the endotoxin activity of lipopolysaccharide by binding to and therefore blocking both the core and the lipid A moieties (Kim *et al.*, 2002).

It is not known to what degree these antibacterial components affect different *Salmonella* serotypes. It is, however, striking that the function of most albumen proteins is linked to lipopolysaccharide binding. Given that the O-antigen structure of lipopolysaccharide is a major determinant of serotype specificity, it may be that the lipopolysaccharide structure plays a major role in the SE survival in forming eggs *in vivo* and that the lipopolysaccharide chemotype will affect the degree of binding with antimicrobial components and thus bacterial survival.

Besides the albumen, the vitelline membrane can also become contaminated (Bichler *et al.*, 1996; Gast & Holt, 2000a; Gast *et al.*, 2002). Recently, a proteomic analysis of

the chicken egg vitelline membrane was carried out (Mann, 2008). Most of the components of the vitelline membrane that were known previously from other egg compartments, such as lysozyme, ovalbumin, ovotransferrin, ovomucin and lysozyme, also constitute *c.* 60% of the dry weight of the outer vitelline membrane. One outer vitelline membrane protein was identified as β-defensin 11.

Immunoglobulins are considered to belong to the antimicrobial defence system of avian eggs. Antibodies to *Salmonella* have been detected in the egg albumen and yolk from naturally and experimentally infected chickens (Schiemann & Montgomery, 1991; Desmidt *et al.*, 1996). It has been suggested that antibodies transferred to the yolk after hyperimmunization of laying hens have no influence on the multiplication of *Salmonella* in the yolk (Takase *et al.*, 1999; Gürtler & Fehlhaber, 2004). In contrast, Holt *et al.* (1996) described a significant difference in the growth behaviour of SE under the influence of antibodies. These authors, however, performed their experiments by inoculating *Salmonella* in a mixture of albumen and yolk, while the two previous studies were based on inoculations in separated yolk. Thus, the possibility exists that the antimicrobial components of the albumen had an additional inhibitory effect on *Salmonella*. The exact antimicrobial role of immunoglobulins in avian eggs thus remains to be defined.

Salmonella virulence factors affecting SE survival in the forming egg

The important role of lipopolysaccharides in conferring protection against the bactericidal component albumen was recently confirmed by Gantois *et al.* (2008a). Applying *in vivo* expression technology, the *rfbH* gene, involved in lipopolysaccharide O-antigen synthesis, was found to be transcriptionally induced during growth in whole eggs at room temperature. After inoculation of a *Salmonella* Δ*rfbH* strain in albumen at 42 °C, immediate killing was observed while the wild-type strain was able to survive in albumen during 24 h. Moreover, the Δ*rfbH* mutant was also unable to grow in whole eggs at room temperature. Lu *et al.* (2003) suggested that *yafD* and *xthA* play an essential role in the repair of DNA damage caused by the albumen and hence confer an advantage to SE to survive in forming chicken eggs. In a recent paper using transposon mutagenesis, it was found that the majority of genes associated with SE survival in albumen at 37 °C are involved in either cell wall structure/function or nucleic and amino acid metabolism (Clavijo *et al.*, 2006). Two mutants had insertions in genes unique to SE. One is homologous to a restriction endonuclease and the other is the *pef* operon encoding a fimbrial biosynthesis gene. Both genes were transformed into a *Salmonella* Typhimurium strain, but only the former conferred an enhanced survival in albumen. The same study also demonstrated that survival in the albumen at 37 °C was higher for serotype Enteritidis compared with Typhimurium and *E. coli*. It is striking that many antimicrobial proteins in albumen bind to lipopolysaccharides while cell wall and lipopolysaccharide biosynthetic genes of *Salmonella* seem to play an important role in albumen survival.

Survival of different *Salmonella* serotypes in the forming egg

Some research groups have compared the survival capabilities of strains belonging to different serotypes in albumen at different incubation temperatures, yielding conflicting results. In one study, a similar survival for SE strains and *Salmonella* Typhimurium strains at 37 and 42 °C was shown (Guan *et al.*, 2006). These findings are in contrast with earlier studies, demonstrating an enhanced survival in the albumen at 37 °C for the serotype Enteritidis (Lu *et al.*, 2003; Clavijo *et al.*, 2006). In the latter studies, strains were inoculated in the albumen of 1-week-old eggs, while in the study by Guan *et al.* (2006), the albumen of freshly laid eggs was used. It is assumed that fresh albumen (pH = 8.16) enhances growth compared with stored albumen (pH = 9.26), and this is most likely caused by the lower pH of the former (Messens *et al.*, 2004). However, the results presented in a paper by Humphrey & Whitehead (1993) suggest that storage has little direct impact on the albumen with respect to the growth of SE. The lysozyme and ovotransferrin concentrations in the albumen increased with the hen's age throughout the laying period, which is reflected in an increased bacteriostatic effect of the albumen on SE at the mid and the final laying period, possibly influencing the data (Sellier *et al.*, 2007).

It is striking that studies comparing strains belonging to different serotypes in their ability to survive in albumen at chicken body temperature are missing. Recently, the bactericidal effect of albumen at chicken body temperature was examined for five different *Salmonella* serotypes (Gantois *et al.*, 2008c). Remarkably, the strains belonging to the serotypes Enteritidis, Typhimurium and Heidelberg were able to survive in the hostile environment of the albumen for 24 h, while the strains belonging to the serotypes Virchow and Hadar were very susceptible to the albumen, and after 24 h, almost all bacteria were killed. This could explain why *Salmonella* Virchow and *Salmonella* Hadar are almost never associated with eggs. However, not enough isolates were compared to draw general conclusions from this study.

Growth of SE in eggs post-lay

Growth patterns of SE in eggs

The risk of human infections following consumption of *Salmonella*-contaminated eggs depends on the bacterial

numbers present. SE can grow in the contents of naturally contaminated eggs at room temperature (Humphrey & Whitehead, 1993). Cogan *et al.* (2001) observed growth after 8 days at 20 °C in 7% of whole eggs inoculated in the albumen near the shell with as few as 2 CFU. It is clear that this implies a serious threat to human health because extensive growth in eggs does not lead to changes in the colour, smell and consistency of the egg contents (Humphrey & Whitehead, 1993). After experimental and natural infections, some authors point to the albumen as being most frequently contaminated (Gast & Beard, 1990; Humphrey *et al.*, 1991b) , while others point to the vitelline membrane as the most common contamination site (Bichler *et al.*, 1996; Gast & Holt, 2000a, 2001; Gast *et al.*, 2007). The albumen is growth restricting for *Salmonella* because it contains multiple antimicrobial components, inducing bacterial cell wall and DNA damage (see Growth patterns of SE in eggs). At temperatures < 10 °C, *Salmonella* bacteria are unable to grow in the albumen (Braun & Fehlhaber, 1995; Schoeni *et al.*, 1995). At room temperature, data are conflicting and it is difficult to compare the various studies because the inoculation size, strains, incubation temperatures and period, age of eggs and many other factors vary (Humphrey & Whitehead, 1993; Braun & Fehlhaber, 1995; Schoeni *et al.*, 1995; Gast & Holt, 2000b). Recent data showed that, at 20 °C, upon inoculation with 39 CFU mL^{-1} albumen, both SE and non-SE strains are able to grow in separated fresh albumen samples up to $> 10^6$ CFU mL^{-1} (Clavijo *et al.*, 2006) and, on extending the incubation time, the number of samples with pronounced growth increased further . Numerous other studies also observed the growth of SE in egg albumen at room temperature (Braun & Fehlhaber, 1995; Schoeni *et al.*, 1995; Duboccage *et al.*, 2001), indicating that the *Salmonella* bacteria harbour intrinsic characteristics to counteract the attacks of the antimicrobial components present in the egg albumen. Inoculation of bacteria in the egg albumen of whole eggs resulted in faster growth than separated egg albumen and also high numbers of *Salmonella* bacteria were detected in the yolk, indicating migration towards the yolk (Cogan *et al.*, 2001; Messens *et al.*, 2004).

It is believed that *Salmonella* cells that are deposited in the albumen are able to migrate to and penetrate through the vitelline membrane in the egg post-lay, in order to reach the yolk and thus gain access to a pool of nutrients that are necessary for its survival and growth. Rapid and extensive multiplication of SE in the nutrient-rich egg yolks at 25 °C has been reported (Gast & Holt, 2000b). This was confirmed in a recent study showing that all strains multiplied rapidly in yolk contents and reached *c.* 9.0 log cells mL^{-1} after 24 h of incubation at 37 or 42 °C (Guan *et al.*, 2006). Data from contaminated eggs from either naturally (Humphrey & Whitehead, 1992) or artificially (Gast & Beard, 1992) infected hens suggest that there is a delay before yolk penetration and fast growth occurs in yolk, in eggs stored at room temperature. This is believed to be because the vitelline membrane in fresh eggs inhibits yolk invasion by *Salmonella*. Gradually, the integrity of the vitelline membrane will become lost during storage, resulting in leakage of nutrients into the albumen. This is considered to allow the bacteria to migrate to the vitelline membrane and multiply and invade the yolk (Humphrey & Whitehead, 1993).

Experimentally infected laying hens also often deposit SE on the vitelline membrane (Bichler *et al.*, 1996; Gast & Holt, 2000a, 2001; Gast *et al.*, 2007). A recent study has suggested that substantial growth occurred in association with the vitelline membrane before penetration through the membrane (Gast *et al.*, 2008a). Contamination of the nutrient-rich yolk content is uncommon (Gast & Holt, 2001; Gast *et al.*, 2003), but if it occurs, it leads to rapid bacterial multiplication (Gast & Holt, 2000b; Guan *et al.*, 2006). There is considerable controversy on the major deposition site in eggs. Most likely, the use of different isolation techniques has a major impact on the outcome of the experiments. Therefore, the use of imaging such as 3D localization with bioluminescent or fluorescent *Salmonella* bacteria could help to unravel the preferred deposition site in eggs (Chen *et al.*, 1996).

Studies on the growth of *Salmonella* in eggs have usually been performed after direct artificial infection of the eggs because the production of *Salmonella*-positive eggs is low after either natural (Humphrey *et al.*, 1989; Humphrey & Whitehead, 1992) or experimental (Gast & Beard, 1992) infection of the hens. The *Salmonella* growth profiles seen in naturally contaminated eggs are different from those seen in eggs contaminated artificially. The latter suggests that growth is rapid in most eggs and that yolk invasion is common (Braun & Fehlhaber, 1995; Chen *et al.*, 1996). The experimental studies, however, used either high contamination levels (10^4 cells per egg) (Chen *et al.*, 1996), most likely not representative for naturally contaminated eggs, or buffered peptone water for the SE solution to be injected, which enhances bacterial growth in the albumen (Chen *et al.*, 1996). Data obtained from artificial egg contamination models should thus be interpreted with caution. A model for artificial egg contamination mimicking the natural situation was developed by Cogan *et al.* (2001). Using low numbers of bacterial cells in a low-nutrient, low-iron suspension as inoculum, a low level of growth was detected in eggs, comparable to that seen in naturally contaminated eggs. When few *Salmonella* bacteria are deposited in the albumen, very little bacterial multiplication occurs and SE can persist there at supportive temperatures (Lock & Board, 1992; Hammack *et al.*, 1993; Gast & Holt, 2000b). Humphrey & Whitehead (1993) observed that in artificially contaminated eggs, the inoculum increased *c.* 10-fold

during the first 24 h postinoculation, as confirmed by Gast & Holt (2000b). The initial growth phase may involve the bacterium using its iron reserves, which appear to be sufficient to support about four generations. When the iron reserves are exhausted, cells enter a lag phase, where, in the majority of eggs, there is little or no change in the numbers of *Salmonella* organisms. It has been postulated that there may be leakage of nutrients from the yolk, leading to a bacterial attraction towards the yolk, some weeks after storage. Cogan and colleagues (Baron *et al.*, 1997) provide evidence indicating that the bacteria will then (after weeks of storage) attach to and penetrate through the vitelline membrane and gain access to the yolk contents in order to grow (Baron *et al.*, 1997).

Salmonella virulence factors related to growth in whole eggs post-lay

It is logical to assume that all *Salmonella* factors that play a role in albumen survival or growth in forming eggs will also play a role in whole egg survival post-lay.

Using *in vivo* expression technology, it was demonstrated that expression of SE *rfbH*, a lipopolysaccharide O-antigen biosynthesis gene, was strongly induced in eggs at room temperature (Gantois *et al.*, 2008a). The *rfbH* gene was shown to be crucial for growth in eggs at room temperature. This again demonstrates the importance of lipopolysaccharide in survival in the albumen. This may be important in the first weeks of egg storage.

During storage, the vitelline membrane gradually deteriorates, resulting in the release of nutrients into the albumen, possibly attracting bacteria that can penetrate the vitelline membrane and multiply in the nutrient-rich yolk. It is tempting to speculate that leakage out of the yolk into the albumen would generate a gradient of amino acids, sugars or other yolk components, triggering a chemotactic movement towards the vitelline membrane. This hypothesis is consistent with the fact that SE grows rapidly in eggs only after *c.* 28 days of storage at room temperature (Gantois *et al.*, 2008a). Flagella are thought to be necessary components for bacterial migration towards the vitelline membrane in whole eggs (Baron *et al.*, 1997). Nonmotile mutants, such as *fliC* and *motAB* mutants, were unable to move through the albumen towards the yolk; hence, proliferation did not take place (Baron *et al.*, 1997). Moreover, the nonmotile serotypes Pullorum and Gallinarum were also not capable of growing in egg contents. Motility is indeed a significant factor for chemotactic bacteria to move towards higher concentrations of attractants and to avoid higher concentrations of repellents by sensing temporal changes in chemoeffector concentrations (Cogan *et al.*, 2004). Chemotaxis in *E. coli* is the best-studied signal-transduction network of any living organism. It allows *E. coli* to sense amino acids, sugars, dipeptides and even redox, temperature and pH changes. In response to these chemical changes, a signal cascade of methylation/demethylation and phosphorylation/dephosphorylation is switched on. Binding of chemoeffectors to the transmembrane receptors triggers the Che operon, which transmits the signals to flagellar motors. The net result of this signal cascade is a change in the direction of flagellar motor rotation and thus induction of motility towards or away from a certain trigger. *Salmonella* harbours a similar chemotaxis system reacting to chemoeffector stimuli (Bourret *et al.*, 1989; Sourjik, 2004).

The vitelline membrane comprises a collagenous matrix overlaid with a layer of glycoproteins (Mariconda *et al.*, 2006). It is believed that curli fimbriae (Sef17) mediate bacterial adherence to these glycoproteins such as fibronectin (Bellairs *et al.*, 1963). Yolk invasion and thus multiplication of a curli-deficient strain, an *agfA* mutant, occurred significantly less than that in the wild-type SE strain (Lock & Board, 1992). It is, therefore, suggested that curli fimbriae are needed to attach to the vitelline membrane, in order to facilitate yolk invasion and multiplication (Baron *et al.*, 1997). The expression of curli fimbriae has been investigated under poor (stationary phase) and rich (exponential phase) nutrient conditions for 15 different *Salmonella* strains in a high-pH and iron-restricted medium at 20 °C (Baron *et al.*, 1997). A correlation has been found between the expression of curli fimbriae during the late exponential phase and a high frequency of growth in eggs at room temperature. This suggests that when bacteria move closer to the yolk, they will start to grow exponentially, and thus, strains that show better growth in eggs are those that are able to express curli fimbriae under growth, rather than starvation. The fact that genes encoding curli fimbriae appear to be ubiquitous within the genus *Salmonella* (Collinson *et al.*, 1991) does not mean that all *Salmonella* serotypes are equally effective at multiplying in eggs because the expression of curli fimbriae can be regulated differently depending on the environmental triggers and the bacterial growth phase.

Growth in eggs post-lay by different *Salmonella* serotypes

Numerous research reports have compared growth in eggs between different *Salmonella* serotypes using various artificial egg contamination models. Messens *et al.* (2004) showed that, at 20 °C, after inoculation with 39 CFU mL^{-1} in the separated albumen, both SE and non-Enteritidis serotypes were able to grow up to $> 10^6$ CFU mL^{-1}. These findings were in contrast to those of Lock and Board (Hammack *et al.*, 1993). In the latter study, the SE strains showed slow growth in the albumen at 20 and 30 °C, while the majority of the strains belonging to other serotypes did

not multiply, but only survived in the albumen. Data obtained from growth studies in albumen should be interpreted with caution because most studies report variable and inconsistent results for independent repeats of the experiments (Lock & Board, 1992; Hammack *et al.*, 1993; Messens *et al.*, 2004). Nevertheless, according to most studies, the capacity to persist and grow in the albumen at non-hen body temperatures is not a specific trait of serotype Enteritidis.

Penetration through the vitelline membrane provides an opportunity for extensive bacterial multiplication inside the yolk. Penetration of SE through the vitelline membrane has been reported in several *in vitro* egg contamination experiments (Humphrey & Whitehead, 1993; Gast & Holt, 2000b). Recently, a number of studies have compared penetration of other *Salmonella* serotypes using the *in vitro* contamination model described by Gast and colleagues (Doran *et al.*, 1993; Gast *et al.*, 2005b; Guan *et al.*, 2006; Murase *et al.*, 2006; Gantois *et al.*, 2008c). In this model, the yolk and the albumen are separated and then the yolk is inoculated with *c.* 100 CFU of *Salmonella* onto the exterior surface of the vitelline membrane, after which the albumen of one single egg is gently poured onto the yolk. All reports agree that serotypes, other than Enteritidis, are able to invade the vitelline membrane and multiply in egg yolk. Furthermore, the multiplication of different *Salmonella* serotypes was assessed by inoculating very small numbers of *Salmonella* cells in fresh eggs according to the egg infection model described by Cogan and colleagues (Cogan *et al.*, 2001; Gantois *et al.*, 2008c). Except for the serotype Typhimurium, no significant difference was observed between the serotype Enteritidis and the strains belonging to the serotypes Heidelberg, Virchow and Hadar, suggesting that the multiplication strategies inside eggs at room temperature are not unique for the serotype Enteritidis. The *Salmonella* Typhimurium strain displayed the lowest frequency of yolk invasion (Gantois *et al.*, 2008c). However, this finding is in strong contrast with a finding by Cogan and colleagues (Baron *et al.*, 1997), showing the highest frequency of yolk invasion for the serotype Typhimurium. Despite the variability seen within and between experiments and the fact that even within the serotype Enteritidis different growth patterns have been observed in eggs post-lay (Baron *et al.*, 1997), the epidemiological association of SE with eggs is assumed not to be caused by specific multiplication strategies in eggs post-lay because most *Salmonella* serotypes are equally effective in multiplying in eggs post-lay.

Conclusion

As opposed to mammals, the chicken embryo does not develop in the safe environment of the womb, continuously protected by the dam's immune system. Hence, it is not surprising that the egg has an impressive arsenal of antimicrobial protective mechanisms, including both nonspecific physical barriers and highly efficacious microbiocidal molecules. Although it is possible to infect eggs with various bacterial species under the artificial conditions of a laboratory experimental set-up, under natural conditions, this is a rare event. When it occurs, it usually causes so much damage that the egg will be easily identified as being infected. SE is unique in the way that it can pass into the egg and multiply inside it without inducing noticeable changes. Combining this exceptional trait with the pathogenicity for the human intestinal tract allowed this serotype of *Salmonella* to cause a pandemic that has lasted for more than a quarter of a century. Only now are we beginning to understand the mechanism by which SE contaminates chicken eggs much more successfully than any other *Salmonella* serotype. Evidence is accumulating that contamination of the eggs is not by penetration through the shell, but by passage from the hen's intestinal tract to the reproductive tract and from there incorporation into the forming egg on the vitelline membrane, in the egg white or the shell membranes. It turns out that many different *Salmonella* serotypes can pass from the intestine of the chicken into its blood stream. Even passage from the blood stream into the hen's reproductive tract is not a unique characteristic of SE. Apparently specific to SE, however, is its capacity to survive the attacks by antimicrobial molecules during the formation of the egg in the hen's oviduct. This appears to require a combination of genes or gene expression patterns encoding for improved cell wall protection and damage repair, among others. The exact reason for the epidemiological association of SE with eggs is, however, still undefined.

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