Bacteriological contamination and infection of shell eggs in the production chain

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Bacteriological contamination and infection of shell eggs in the production chain

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Bacteriologische besmetting en infectie van schaaleieren in de productieketen

Illustratie: Jarne en Jitze De Reu©

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Woord vooraf

Het was geen eenvoudige taak maar wel een belangrijke uitdaging om naast de dagelijkse routine werkzaamheden op het voormalige DVK-CLO, nu ILVO – Technologie en Voeding dit doctoraal proefschrift tot een goed einde te brengen. Dit was enkel mogelijk dankzij de directe en indirecte hulp van diverse personen. Een woordje van dank is hier dan ook op zijn plaats.

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4 juni 2006

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CURRICULUM VITEA I

Abbreviation list

Abbreviation list

ANOVA	Analysis of variance
BHI	Brain heart infusion
BPW	Buffered peptone water
CFU	Colony forming unit
EC	European Communities
EU	European Union
ISM	Inner shell membrane
k_{dyn}	Dynamic stiffness
kve	Kolonie vormende eenheden
NA	Nutrient agar
n.a.	Not applicable
n.d.	Not determined
n.p.	Not present
OSM	Outer shell membrane
PBS	Phosphate buffered saline
PCA	Plate count agar
ppm	Parts per million
ref.	Refrigerated
RH	Relative (atmospheric) humidity
sp. gr.	Specific gravity
16S rDNA	16S ribosomal deoxyribonucleic acid
stdev	Standard deviation
TSA	Tryptone soya agar
TTC	2,3,5 Triphenyl tetrazolium chloride
UK	United Kingdom
US	United States (of America)
UV	Ultraviolet
YE	Yeast extract

Thesis objectives

Thesis objectives

Consumers and food business operators are more and more aware of food safety issues. Microbiological contamination of eggs has important implications. For shell eggs, internal contamination may occur, leading to spoilage and in the case of a pathogen to human disease. Eggs are one of the main sources of contamination cited in relation to human salmonellosis, with *Salmonella* Enteritidis being the most frequently isolated *Salmonella* serovar. There are two possible routes of bacterial infection of shell eggs: either vertically or horizontally. In the vertical transmission the egg content is directly contaminated as a result of bacterial infection of the reproductive organs, *i.e.* ovaries or oviduct tissue. In the horizontal transmission the micro-organisms penetrate through the eggshell. Some studies suggest that most contamination is due to horizontal transmission (Barrow and Lovell 1991). The number of bacteria present on the surface of the shell, the bacterial identity, eggshell quality and extrinsic factors may be important factors influencing microbial ingress by the horizontal route. In this study different aspects of these four factors are mainly examined.

The first aim of this study was to assess the general bacterial contamination of the eggshell of consumption eggs. In chapter 2 a concept for sampling of eggs in the production chain was evaluated and a methodology to recover and count the bacterial eggshell contamination was optimized. As from 2012 conventional cage housing for laying hens will be prohibited in the European Union, the impact of the alternatives such as furnished cages and the non-cage alternative aviary system on the initial bacterial eggshell contamination was studied (chapter 3). The developed concept and methodology from chapter 2 was used. The objective of chapter 4 was to use the protocol of chapter 2 to detect critical points for bacterial eggshell contamination in the production chain of different commercial housing systems. The evolution of the eggshell contamination in the chain and during storage was studied. The efficacy of a commercial UV disinfection system for decontamination of the eggshell and egg conveyor belts was studied in chapter 5.

This thesis also aims to study factors influencing the bacterial penetration and survival in the egg content. The importance of different eggshell characteristics in the defence against microbial ingress into the egg contents by horizontal transmission was studied in chapter 6. Bacterial eggshell penetration and whole egg contamination was correlated with various

eggshell characteristics. A second major objective of chapter 6 was to study the influence of the bacterial identity and the number of organisms present on the surface of the eggshell on the eggshell penetration and the egg content contamination. During storage eggs are sometimes cooled for a period. Eggs held at lower temperature have condensate on the shell when moved into a warmer environment. The last objective of this study (chapter 7) was to study the influence of eggshell condensation on the bacterial eggshell penetration and on the whole egg contamination with *Salmonella* Enteritidis.

Chapter 1

Bacteriological contamination and infection of shell eggs in the production chain: a review

CHAPTER 1: Bacteriological contamination and infection of shell eggs in the production chain: a review

Abstract

The review is discussing the formation and the components of the egg, the mechanisms of microbial contamination of intact eggs, the type of contaminating flora of eggs with special attention for *Salmonella*, the egg production chain with the different housing systems for laying hens, and some aspects on egg washing.

1 FORMATION OF THE HEN'S EGG

The egg of the laying hen is the end product of a complicated series of processes which are outlined by Solomon (1991) and Johnson (2000). The first step is the ovulation of the yolk (with associated ovum) from the left ovary into the left oviduct (see Figure 1.1). The right ovary and oviduct do not develop in the commercial laying hen. (Roberts 2004)

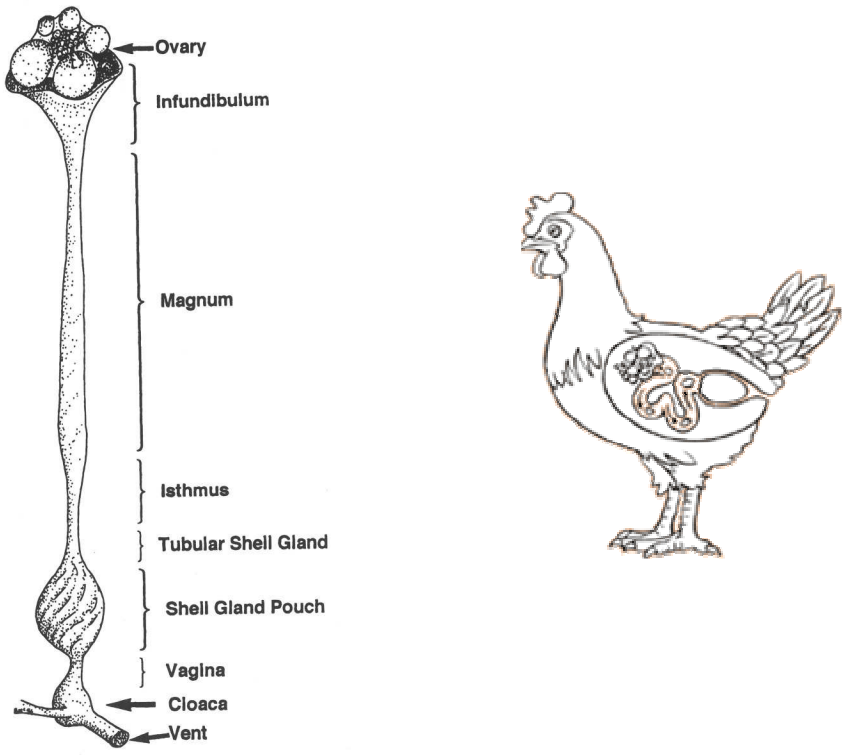

Figure 1.1: The left ovary and oviduct (infundibulum → vagina) of the laying hen (Roberts 2004).

The oviduct consists of six regions (Solomon 1991; Roberts and Brackpool 1994). From the ovary to the cloaca they are: 1) the infundibulum or funnel which receives the oocyte after it has been shed by the ovary; 2) the magnum or albumen-secreting region; 3) the isthmus which forms the shell membranes; 4) the tubular shell gland where the calcification of the shell begins; 5) the shell gland pouch (uterus) where the bulk of shell growth occurs and 6) the vagina. The time frame of the whole process is outlined in Figure 1.2.

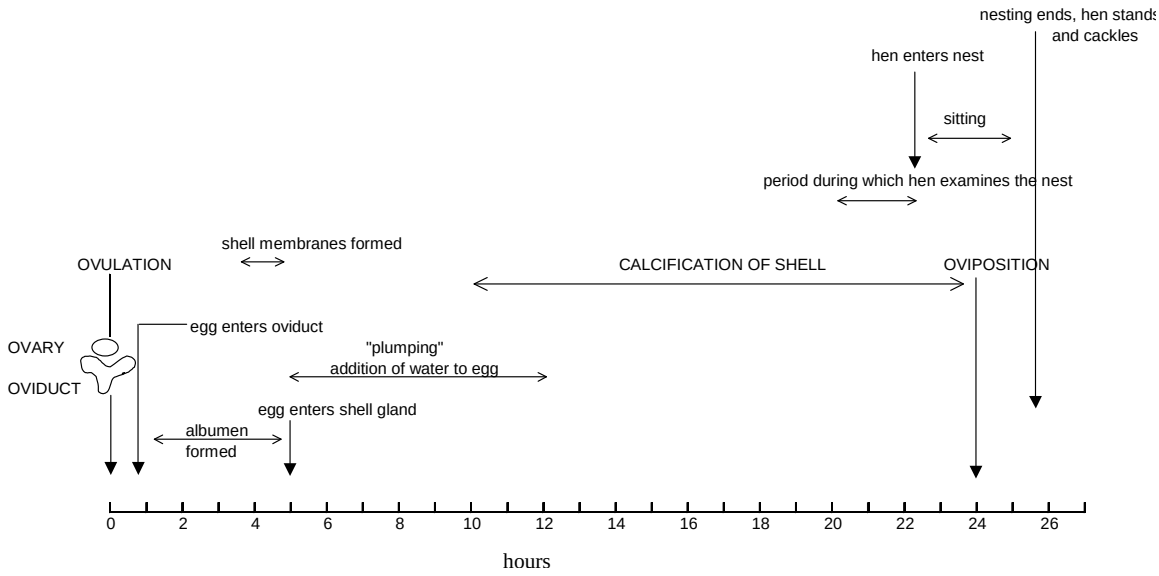

Figure 1.2: Time frame of the egg laying process (Gilbert 1971).

1.1 Formation of the yolk

Ovogenesis, *i.e.* yolk development, begins 10 to 12 days preceding ovulation (Solomon 1991). Yolk components are formed in the liver and transported via the blood to the ovary. The ovary of hens in active production contains three types of follicles where the yolk can be deposited (Kan and Petz 2000):

- very small follicles, in the slow phase of development, which can take months or even years. These are also called the white follicles as no (coloured) oxy-carotenoids are deposited.
- the follicles in the intermediate phase of growth (lasting some 60 days).
- follicles in the rapid growth phase, which lasts approximately 10 days. The follicle weight increases during this time from some 1 gram to about 20 grams and deposition occurs in concentric layers one after each other.

As one follicle ovulates approximately every 24 h, roughly ten follicles are present in different stages of the rapid growth. Figure 1.3 shows the ovary with follicles as can be found in hens in active production and the separate yolks or follicles.

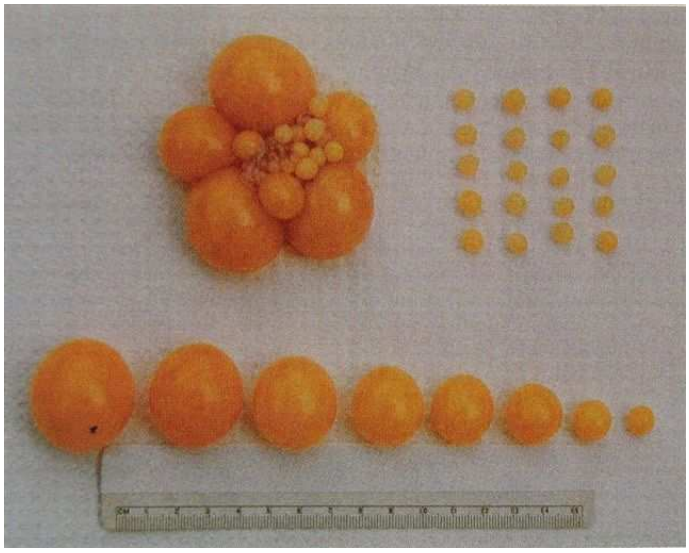

Figure 1.3: Ovary with follicles, follicles in the intermediate growth phase and follicles in the rapid growth phase (Donoghue and Myers 2000)

1.2 Formation of the albumen

In the highly glandular magnum (normaly about 33 cm long) the majority of the albumen is formed. Formation of the proteins takes 1 – 2 days and deposition of egg white around the yolk occurs at some 2 - 3 h after ovulation. The albumen in the magnum is in a concentrated form and represents only half of the volume of albumen present in a freshly laid egg. Additional fluid (water along with glucose and electrolytes) is added to the albumen, mainly in the shell gland pouch, to produce the final volume of the albumen. The ovum moves through the magnum via peristaltic action. (Roberts and Brackpool 1994)

1.3 Formation of the shell membrane

In the isthmus there is a rapid (approximately 2 h) development of the inner and outer shell membranes around the albumen. Shell membranes are formed at some 3 - 4 h after ovulation. The isthmus is narrower than the magnum, has a thick circular layer of muscle and is approximately 10 cm long. (Roberts and Brackpool 1994)

1.4 Formation of the shell

In the tubular shell gland the initial transfer of calcium salts onto the membrane fibres takes place. A firm bond is established which prepares the way for the main phase of true shell formation. The egg passes into the shell gland pouch where two processes occur simultaneously. There is a slow calcification for approximately the first 4 h with the main event to occur being the uptake of water, some salts and glucose into the albumen probably from the tubular glands. This is known as ‘plumping’ and begins to stretch the shell membranes. This distension separates and exposes the mammillary cones, and is thought to be also the stimulus for the rapid phase of calcification to begin. The bulk of the true shell formation now takes place and the egg spends about 20 h in total in the shell gland pouch, including pumping time. (Roberts and Brackpool 1994)

2 EGGSHELL AND EGG CONTENT: STRUCTURE, COMPOSITION AND ANTIMICROBIAL DEFENCE

The main components of the hen’s (avian) egg are: the eggshell, the shell membranes, the albumen or egg white and the yolk. Figure 1.4 gives a schematic drawing of the egg and its components.

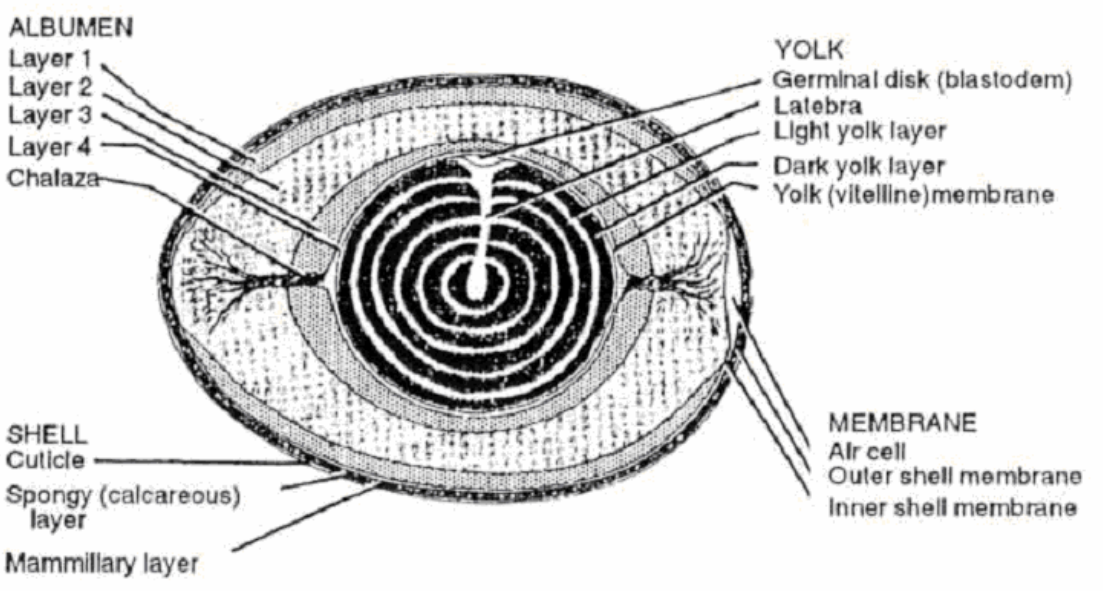

Figure 1.4: Schematic drawing of the egg and its components (Stadelman 1995b).

2.1 The cuticle

The shells of domestic hens have a marked resistance to water, due to a natural protein-like film designated cuticle, which covers the outer surface of the shell and plugs to varying extents the pore canals (Cooke and Balch 1970) (Figure 1.4 and 1.5). The thickness of the hydrophobic cuticle varies from 0.5 to 12.8 µm over the surface of the same egg (Simons 1971). A cuticle-less egg is not an unusual phenomenon (Sparks 1985). The thickness of the cuticle can vary with age, strain and environment (Simons 1971). The cuticle is a protein and carbohydrate complex and has a vesicular structure with irregular spaces between the vesicles of 0.5 to 2.8 µm diameter (Simons and Wiertz 1963). The weight of the cuticle is 0.2% of the entire egg weight (Simons 1971) and consists of 85 – 87% protein, 3.5 – 4.4% carbohydrate, 2.5 – 3.5% fat and 3.5% ash (Wedral *et al.* 1974; Roberts and Brackpool 1994).

Functions concerning bacterial penetration

The cuticle is the primary barrier against bacterial penetration (Board and Halls 1973). Sparks and Board (1985) showed that the physical state of the cuticle alters immediately after the egg has been laid and can have an important bearing on the egg's susceptibility to microbial infection at this particular stage. When the egg is freshly laid the cuticle appears 'wet' but then takes on a 'dry' appearance after approximately 3 min. Bacterial penetration studies indicated a higher incidence of contamination across shell with 'wet' cuticle in comparison with a 'dry' cuticle. Electron microscopy studies of the 'wet' shell revealed a frothy, open, granular appearance to the cuticle whereas the 'dry' cuticle has a tight mature structure resulting in less penetration through the pores. Drysdale (1985) found a significantly higher bacterial contamination in eggs which had a poor cuticle (40%) compared to eggs with a medium or good quality cuticle (26%). Alls *et al.* (1964) found that cuticle removal increased bacterial contamination from 20% to 60%. The first-line defence of the cuticular layer was on the other hand questioned by Nascimento *et al.* (1992) and Messens *et al.* (2005a). Cuticle deposition declines with flock age (Sparks and Board 1984; Drysdale 1985) and may be a factor in explaining why eggs produced from older flocks are more sensitive to eggshell penetration.

2.2 The pores

The shell of the hen's egg is permeated by a variable number of pores ranging from 7 000 to 17 000 (Tyler 1953), with the greatest number occurring at the equator and blunt pole of the

egg. Messens *et al.* (2005a) found number of pores varied between no pores observed (on 120 mm^2 of shell) and 9 360 pores. The diameters of the pores are in the range of 6 - 23 µm at the inner end and 15 - 65 µm at the mouth (Tyler 1956). Not all pores extend through the entire depth of the shell.

Functions concerning bacterial penetration

Attempts have been made by several workers to correlate eggshell porosity with bacterial penetration with varying results. Kraft *et al.* (1958) and Fromm and Monroe (1960) supported a correlation, while Reinke and Baker (1966), Nascimento *et al.* (1992) and Messens *et al.* (2005a) refuted these earlier findings.

2.3 The shell

Immediately beneath the cuticular layer, vertical orientated calcite crystals from a narrow band, the surface crystal layer. This narrow band overlies the polycrystalline columns of the palisade which form the bulk of the true shell (Figure 1.5). During the growth period the former interlock. The earlier they fuse, the greater is the effective thickness of the shell (Bain 1991). The elemental composition of the eggshell is for 98% calcium (Romanoff and Romanoff 1949). In common with other calcified tissues, an organic matrix is present. When the eggshell is decalcified, a delicate web of shell matrix proteins remains. At ultra structural level it has a fibrous appearance interspersed with numerous vesicular holes. The mammillary layer of the shell makes contact with the shell membranes (Figure 1.5). The initial bonding between the shell membranes and the first crystals to precipitate is critical to the formation of the succeeding layers; indeed when a crack occurs, it does so in the first instance at the level of the mammillary layer (Bain 1990). Shell thickness ranges between 0.30 and 0.52 mm (Messens *et al.* 2005a). To date the following protein components from the organic matrix have been isolated: ovocleidin, ovocalyxin, ovotransferrin, ovalbumin, osteopontin, lysozyme and clusterin (Hincke *et al.* 2000).

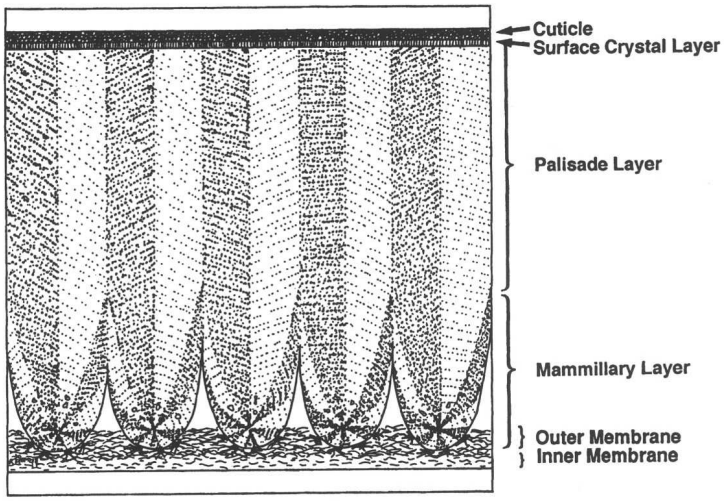

Figure 1.5: Diagrammatic side view of an avian eggshell, showing the various layers and the connection between the shell membranes and the inorganic shell material at the mammillary layer (Roberts and Brackpool 1994).

Functions concerning bacterial penetration

The most important contribution of the shell is to provide a mechanical protection (Board and Tranter 1995). Ernst *et al.* (1998) found a significant increase in egg content contamination with *Salmonella* Enteritidis due to cracked eggs (hair-cracks); 2,8% intact eggs were contaminated versus 77% cracked eggs. Eggs were inoculated with 10^6 CFU *Salmonella* Enteritidis/eggshell. Between 8 and 10% of the eggs laid for the table industry suffer damage to the shell during routine handling (Hamilton *et al.* 1979).
Kraft *et al.* (1958), Williams et al (1968) and Messens *et al.* (2005a) found no relationship between shell thickness and the likelihood of *Salmonella* Enteritidis to penetrate the eggshell. Hincke *et al.* (2000) found lysozyme and the shell gland specific protein ovocalyxin, both present in the shell, are also implicated in the bacterial defence.

2.4 The membranes

The paired shell membranes are *approx* 70 µm thick (Simons and Wiertz 1963) and held firmly together, except at the blunt end of the egg, where they separate to enclose the air space (Figure 1.4 and 1.5). The inner shell membrane (ISM) lies immediately over the albumen, and the outer shell membrane (OSM) is attached to the true shell (Mayes and Takeballi 1983). The fibres of the membranes are on average 0.8 - 1 µm thick, and each has a keratin core

surrounded by a mucopolysaccharide mantle (Romanoff and Romanoff 1949). According to Roberts and Brackpool (1994) the composition of the membrane fibres is still not fully understood. However, the shell membrane protein contains the cross-linking amino acids desmosine and isodesmosine and are different from the other fibrous proteins such as keratin, connectin, collagen or microfibrillar protein. The membranes consisting of a network of branched fibres have pores of approximately 1 µm diameter (Tung and Richards 1972).

Functions concerning bacterial penetration

In relation to bacterial penetration, the shell membranes act as a filter, being more impenetrable to bacteria than the shell (Garibaldi and Stokes 1958). Lifshitz *et al.* (1964) reported that the ISM was the most effective barrier in preventing bacterial penetration of the egg content, the shell ranked second and the OSM was the least important. The ISM is reported to be more porous than the OSM (Mayes and Takeballi 1983), which is surprising in view of the reputation of the former as a more effective barrier to translocation of bacteria (Vadehra and Baker 1972). According to Garibaldi and Stokes (1958) and Lifshitz *et al.* (1964) the OSM has lager interstices.

2.5 The albumen

The albumen or egg white is made up of four distinct layers: outer thin white, viscous or thick white (albuminous sac), inner thin white and a chalaziferous layer (Figure 1.4). The proportions of the various layers have been found to vary widely depending on the breed, environmental conditions (climate), size of the egg and rate of egg production. Moisture content decreases from the outer to the inner albumen layers and ranges from 89% to 84%. Table 1.1 summarizes the mean composition of albumen, yolk and whole egg. (Li-Chan *et al.* 1995)

Table 1.1: Composition of albumen, yolk and whole egg (Li-Chan *et al.* 1995).

Egg component	Protein (%)	Lipid (%)	Carbohydrate (%)	Ash (%)
Albumen	9.7-10.6	0.03	0.4-0.9	0.5-0.6
Yolk	15.7-16.6	31.8-35.5	0.2-1.0	1.1
Whole egg	12.8-13.4	10.5-11.8	0.3-1.0	0.8-1.0

Antimicrobial defence

The albumen makes two contributions to the antimicrobial defence of the egg; mechanical and chemical (Board and Tranter 1995).

There are two components involved in the mechanical defence: 1) the viscosity of the albumen ensures that the micro-organisms remain localized and 2) the combined action of the chalazae and albuminous sac of fresh eggs contributes to the central location of the yolk, thus maintaining it at the greatest distance from the shell membranes (Board and Tranter 1995).

The albumen has a range of chemical components with antimicrobial properties; see Table 1.2. Moreover the alkaline state of the albumen has a deleterious effect on bacterial growth and accentuates the chelating potential of ovotransferrin. The pH of albumen from a recently laid egg is between 7.6 and 8.5; during storage the pH of albumen increases at a temperature-dependent rate to a maximum value of about 9.7. (Li-Chan *et al.* 1995)

Table 1.2: Properties of the main antimicrobial proteins of hen albumen.

Protein	Fraction of the proteins from the albumen (%)	Characteristics
Ovotransferrin	12	Chelating metal ions (particularly Fe^{3+}, but also Cu^{3+}, Mn^{2+}, Co^{2+}, Cd^{2+}, Zn^{2+} and Ni^{2+})
Ovomucoid	11	Inhibition of trypsin
Lysozyme	3.4	a) Hydrolysis of β(1-4)glycosidic bonds in bacterial cell wall peptidoglycan – acting specifically on the polymer n-acetyl glucosamine n-acetyl muramic acid, splitting the link between them b) Flocculation of bacterial cells c) Formation of oligosaccharides from bacterial cell wall tetrasaccharides by transglycosylation
Ovoinhibitor	1.4	Inhibition of several proteases
Ovoflavoprotein	0.8	Chelating riboflavin (or vit. G or vit. B_2); rending it unavailable to bacteria that require it
Avidin	0.05	Chelating biotin; rending it unavailable to bacteria that require it

(modified from Board and Tranter (1995) and Board *et al.* (1994))

2.6 The yolk

The vitelline membrane surrounding the yolk is made up of two main layers: 1) the inner layer formed in the ovary, and 2) the outer layer deposited in the oviduct (Li-Chan *et al.* 1995). Fromm (1967) noted that the outer surface of the vitelline membrane in fresh eggs is composed of fibres connected to the chalaziferous layer. The strength of the membrane decreases as egg ages (Fromm 1964).

Antimicrobial defence

The yolk is a growth-friendly environment for micro-organisms. Gast and Holt (2001) inoculated the vitelline membrane directly with *Salmonella* Enteritidis and found 6% positive yolk interiors after 6h incubation at 25°C and up to 100% positive after 24h. At lower temperatures, the membrane was less frequently, but still significantly, penetrated.

3 *SALMONELLA* AND HUMAN *SALMONELLA* INFECTION

3.1 Characteristics, taxonomy and nomenclature of *Salmonella*

Salmonellae were first described at the end of the nineteenth century and named after Salmon who isolated in 1886 the organism now known as *Salmonella* choleraesuis from pigs. Salmonellae are Gram-negative rods, measuring 0.7 - 1.5 by 2.0 - 5.0 µm, belonging to the family of *Enterobacteriaceae*. They are generally motile with peritrichous flagella, facultative anaerobic, ferment glucose mostly with the formation of gas and reduce nitrate to nitrite. Following characteristics are mostly used for identification: urea not hydrolysed, lysine decarboxylation and hydrogen sulphide production from thiosulphate on triple-sugar iron agar. (Grimont *et al.* 2000)

Table 1.3 gives minimal, optimal and maximal temperature, pH and a_w values for the growth of salmonellae. The growth rate of salmonellae is substantially reduced at <15°C, while the growth of most salmonellae is prevented at <7°C. Salmonellae are sensitive to heat, the average *D* value (min) at a_w>0.95 and pH = 7 is 0.03 at 70°C (Mossel *et al.* 1995).

Table 1.3: Limits of growth of salmonellae when other conditions (e.g. temperature, pH a_w) are near optimum (ICMSF 1996)

Conditions	Minimum	Optimum	Maximum
Temperature (°C)	5.2*	35-43	46.2
pH	3.8	7-7.5	9.5
a_w	0.94	0.99	>0.99

* most serotypes fail to grow at <7°C

The genus *Salmonella* encompasses a large taxonomic group with over 2463 recognized serovars (Heyndrickx *et al.* 2005). The taxonomy and nomenclature of *Salmonella* have been the subject of debate since Le Minor and Popoff (1987) proposed changes in the 1980s (Tindall *et al.* 2005). Historically, serovars of *Salmonella* were considered as species and, for this reason, the serovar names were italicized. In the early 1970s, nucleotide sequence relatedness and other molecular analyses demonstrated that typical salmonellae were closely related and might be considered as single species (Crosa *et al.* 1973). Therefore it no longer seemed justified to consider serovar names as species names. *Salmonella enteritidis* becomes *Salmonella enterica* subsp. *enterica* serovar Enteritidis or simply *Salmonella* serovar Enteritidis or *Salmonella* Enteritidis. The current widely accepted *Salmonella* nomenclature is summarized in Figure 1.6 (Heyndrickx *et al.* 2005; Tindall *et al.* 2005).

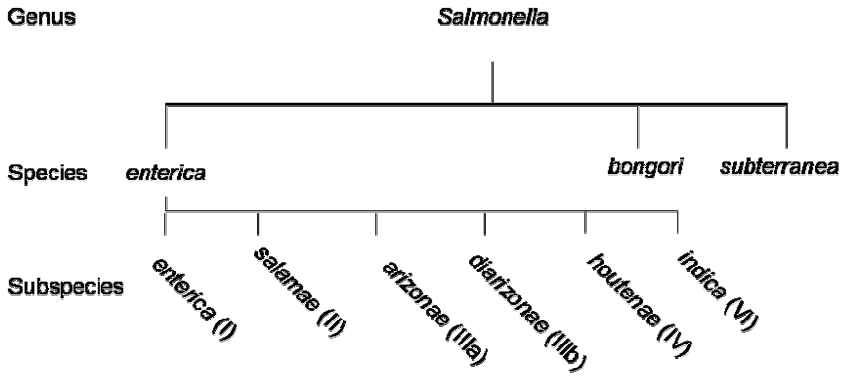

Figure 1.6: Current preferred *Salmonella* nomenclature (Heyndrickx *et al.* 2005; Tindall *et al.* 2005).

Salmonella strains can be classified according to the association with host animal species. *Salmonella* serotypes which are exclusively associated with one particular host species are referred to as being host-restricted. Examples are human *Salmonella* Typhi, fowl *Salmonella* Gallinarum and poultry *Salmonella* Pullorum. All these host-restricted serotypes produce systemic infection with different clinical signs. Serotypes which are prevalent in one particular host species but which can also cause disease in other host species, for example

Dublin and Choleraesuis, will be referred to as host-adapted serotypes. Ubiquitous serotypes, for example Enteritidis and Typhimurium, although capable of causing systemic disease in a wide range of host animals, usually induce a self-limiting gastroenteritis in a broad range of unrelated host species, and these serotypes will be referred to as un-restricted serotypes. (Uzzau *et al.* 2000)

This literature review will be focussed on *Salmonella* Enteritidis and the role of eggs and poultry as a source of *Salmonella* infection in humans.

3.2 Incidence of *Salmonella* Enteritidis infections in humans in Belgium and other countries

The increase in *Salmonella* infections in Belgium from 1986 to 1999 was mainly due to the increase of *Salmonella* Enteritidis (Anon. 2004b). In the recent period, 6 398 or 63.5% (2002), 9 201 or 71.4% (2003) and 6 075 or 63.7 % (2004) of the *Salmonella* isolates in human were *Salmonella* Enteritidis. In 2005 human *Salmonella* isolates decreased significantly from 9 500 in 2004 till 4 872; in analogy *Salmonella* Enteritidis strains decreased till 2 208 or 45.3% of the isolates (Figure 1.7).

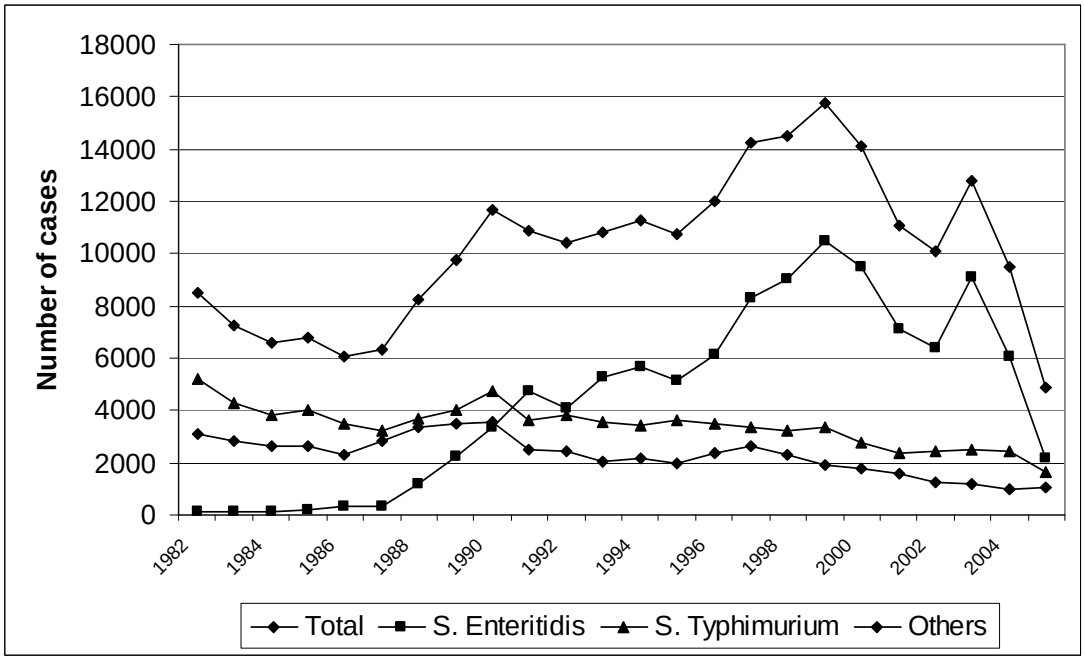

Figure 1.7: *Samonella* isolates in Belgium from human source (Anon. 2004b) + (personal communications J.M.-Collard, Institute of Public Health, Brussels).

During 1997, *Salmonella* Enteritidis accounted for 85% of all cases of human salmonellosis in Europe (Guard-Petter 2001). Although the reported cases of *Salmonella* in England and

Wales declined with 50% from 1997 till 2001; still 65% of the 16 465 *Salmonella* cases of 2001 were *Salmonella* Enteritidis infections (Cogan and Humphrey 2003). In Germany *Salmonella* Enteritidis was the predominating serotype (65%) followed by *Salmonella* Typimurium (23%) of the reported cases of non-typhoidal salmonelloses with known serotype in 2001 (Werber *et al.* 2005).

In the United States (US), approx 40 000 cases of human salmonellosis are reported annually, and an estimate of the real number of cases is about 1.4 million (non-typhoidal salmonellosis) (Mead *et al.* 1999). The most common isolates in the US are Enteritidis (23%) and Typhimurium (22%) (Humphrey 2000).

3.3 Symptoms of non-typhoidal salmonellosis in humans

Salmonellosis is a potential serious infection and in the United Kingdom (UK) there are approximately 70 *Salmonella*-associated deaths each year. As with most other enteric infections, the very young, the elderly and those who are immune-compromised or who have underlying diseases are more at risk for infection (Humphrey 2000). The incubation period varies from a few hours to 72 h and the duration of the illness varies from 4 - 10 days. Symptoms of non-typhoidal infection (*e.g. Salmonella* Enteritidis and *Salmonella* Typhimurium) commonly observed are diarrhoea, headache, abdominal pain, nausea, chills, fever, and vomiting (Poppe 1999). In patients with underlying disease, septicaemia is not uncommon and, in healthy subjects, there may be a wide range of consequences, including pericarditis, neurological and neuromuscular diseases, reactive arthritis, ankylosing spondylitis, and osteomyelitis (Poppe 1999). Damage to the mucous membrane of the intestine and colon may occur, which lead to malabsorption and nutrient loss (Baird-Parker 1990). Severe dehydration, bloody diarrhoea and haematogenous spread of *Salmonella* Enteritidis to bone, the meninges, and soft tissues have occurred in infants (Cross *et al.* 1989). The carrier stage can last for weeks to months. Antibiotic treatment is likely to prolong the carrier state and therefore not recommended in cases with no complications (Aserkoff and Bennet 1969). In Table 1.4 the incidences of the symptoms from a large egg-associated outbreak in 1989 in the UK of *Salmonella* Enteritidis (PT4) are summarised (Stevens *et al.* 1989).

Table 1.4: Symptoms from an egg-associated *Salmonella* Enteritidis infection (Stevens *et al.* 1989).

Symptom	% of cases	Symptom	% of cases
Diarrhoea	87	Muscle pain	64
Abdominal pain	84	Vomiting	24
Feeling feverish	75	Headache	21
Nausea	65	Blood in stools	6

3.4 General pathogenesis of human *Salmonella* infections

The usual route for *Salmonella* infections is by means of the oral route. As such, the organisms are faced with an impressive array of non-specific host defences, such as the acidic environment of the stomach, intestinal mucus, and the normal gut microflora. In the small intestine, especially in the ileum, the bacterium is able of adhering to and invading into the intestinal epithelium via the M cells, causing an inflammatory response with recruitment of neutrophils (in mammals), heterophils (in birds) and macrophages. This phase will be referred to as the enteric phase of infection. Whereas the granulocytes quickly kill the bacteria, a limited number of *Salmonella* bacteria, ingested by macrophages, survive intracellular. Intracellular survival and even multiplication inside host macrophages enables the bacterium to spread to and persist within the host internal organs. This phase will be referred to as the systemic phase of infection. In the course of an infection with a host-restricted serovar in its respective host, the systemic phase is often most prominent. In infections with these serovars, enteritis is generally limited or absent and the reticulo-endothelial system (RES) is rapidly colonised. This results in a septicaemia condition, typhoid fever. Infections with host-adapted serovars, for example Choleraesuis and Dublin, are characterized by both an obvious enteric and systemic phase. The un-restricted serovars most often cause enteritis, although the systemic phase with septicaemia may also occur. (Uzzau *et al.* 2000; Pasmans 2002)

The key stages of the pathogenesis are summarized in Figure 1.8; these include colonization and invasion in the intestine, dissemination of the *Salmonella* throughout the body, and replication and survival of *Salmonella* within professional phagocytes (Uzzau *et al.* 2000).

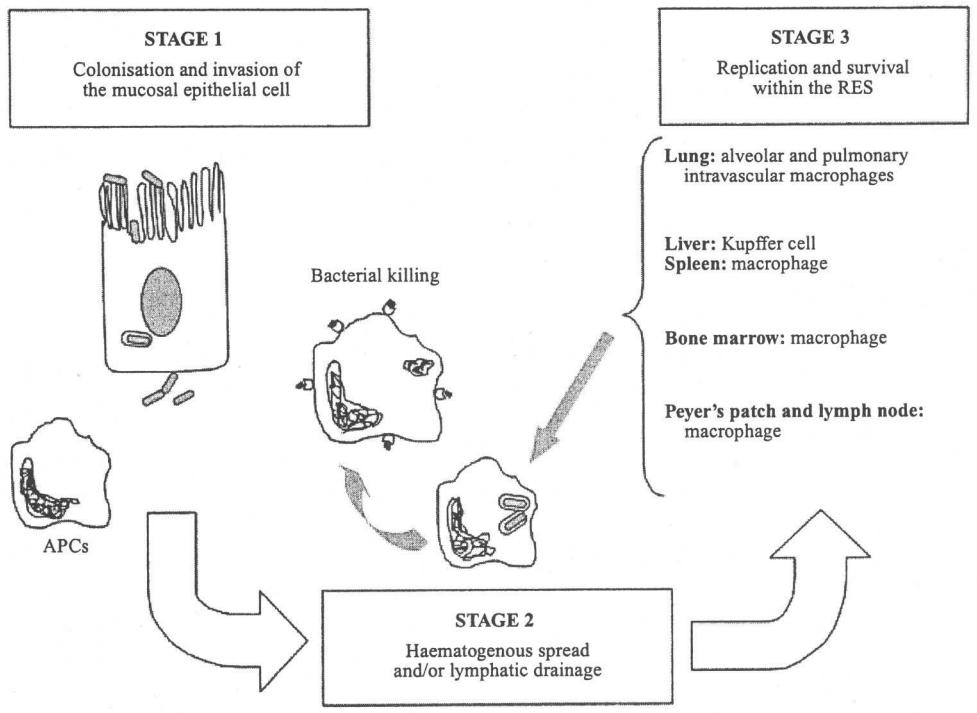

Figure 1.8: Principal steps in *Salmonella* pathogenesis with potential for involvement of host restriction and adaptation (APC, antigen presenting cell, the final 'activated macrophage' is shown coated with processed salmonella antigen) (Uzzau *et al.* 2000).

3.5 Types of food involved

In Europe in the period 1993 - 1998, the incriminated food was identified in 1409 outbreaks caused by *Salmonella* Enteritidis and 188 outbreaks caused by *Salmonella* Typhimurium (Anon. 2001). At least 76% of the *Salmonella* Enteritidis outbreaks reported were related to the consumption of (cooked) eggs, egg products and foods containing eggs (cakes and ice cream) (Table 1.5).

Table 1.5: Type of food identified in the outbreaks in Europe caused by *Salmonella* Enteritidis and by *Salmonella* Typhimurium (Anon. 2001).

Type of food	**Percentage caused by**	
	Salmonella Enteritidis	*Salmonella* Typhimurium
Eggs and egg products	68	39
Cakes and ice cream	8	2
Meat and meat products	4	33
Mixed foods	4	2
Poultry and poultry products	3	10
Milk and milk products	3	2
Fish and shellfish	2	3
Other	8	9
Total (%)	100	100

3.6 *Salmonella* in poultry and laying hens

During the last 10 - 20 years, *Salmonella* Enteritidis has replaced Salmonella Typhimurium as commonest serotype in poultry worldwide (Poppe 2000). In the UK the percentage of *Salmonella* Enteritidis isolates from poultry rose from 3.3% in 1985 to almost 50% of all *Salmonella* isolates in 1989 (McIlroy and McCracken 1990). Despite the reduction in the isolation rates of *Salmonella* Enteritidis from poultry from 1993 - 1995 in the UK, it was still the most isolated serotype (Poppe 2000). The percentage of isolates belonging to the serotype Enteritidis increased in the Netherlands from about 5.5% in 1986 till 15% in 1992 and about 20% in 2000, being the most predominant serotype in poultry (Van Duijkeren *et al.* 2002).
Denmark, Finland, Norway, Sweden and Ireland, running a control program for several years, have documented a low prevalence of *Salmonella* Enteritidis and *Salmonella* Typhymirium, as well as other *Salmonella* serovars in layer breeder and layer flocks (Anon. 2000). All layer breeder flocks were negative in 2000. A few layer flocks infected with *Salmonella* were detected in Finland (0.1%) and Sweden (0.4%). In Finland, the positive layer flock was infected with *Salmonella* Typhimurium; in Sweden the four positive flocks were infected with *Salmonella* Livingstone and *Salmonella* Yoruba. In Denmark and Ireland 3.7% and 4.5% of the layer flocks were *Salmonella* positive respectively; *Salmonella* Enteritidis being the dominating serotype. France reported all layer breeder flocks negative in 2000 and 0.8% of the laying hen flocks being infected with *Salmonella* Enteritidis. Notwithstanding the low infection rates of 0.5% for *Salmonella* Enteritidis and 0.3% for *Salmonella* Typhimurium of

the breeder flocks (layer and broiler) in the Netherlands in 2000; 20% of the layer flocks and 16% of the broiler flocks were infected with salmonellae on production level (Anon. 2000).
In the meat production line, in Finland, Sweden and Norway all broiler breeders were *Salmonella* negative in 2000. Among the Danish broiler breeders 0.7% of the flocks were infected with *Salmonella*; Enteritidis and Typhimurium were isolated in these flocks. The *Salmonella* infection rate for broiler flocks was 2.1%. In Finland and Sweden respectively 1% and 0.1% of the broiler flocks were *Salmonella* positive; the serotypes Enteritidis and Typhimurium were not isolated. In Ireland, 18.7% of samples from the broiler breeders were detected *Salmonella* positive at one sampling occasion, however other serotypes than Enteritidis and Typhimurium were isolated. In the UK, in the meat production line of 2000, *Salmonella* Enteritidis and *Salmonella* Typhimurium was not detected in any breeder flock. In the broiler flocks mainly other serotypes than Enteritidis and Typhimurium were isolated (Anon. 2000). In a study of Heyndrickx *et al.* (2002), 10 of 18 investigated Flemish broiler flocks were *Salmonella* positive; most flocks were positive for multiple serotypes.
Poultry is also still a main reservoir for *Salmonella* in Belgium. In 2004, 688 *Salmonella* strains from poultry were isolated; which is 39.9% of all isolates from animals. Of all poultry isolates, serotype Enteritidis was the most predominant (22.4%), as in former years, followed by Infantis (12.5%) and Virchow (8.1%). Almost 68% of layer isolates were serotype Enteritidis (Anon. 2004e). The surveillance system of laying hens before slaughter showed in 2003 and 2004 respectively, 15% and 27% of the flocks *Salmonella* positive (Anon. 2003a; Anon. 2004a). The surveillance system for broilers showed a *Salmonella* prevalence of 7% in 2003 and in 2004 (Anon. 2003a; Anon. 2004a). In 2004 the layer isolates of Flanders were for 69% Enteritidis; Typhimurium was not isolated (Anon. 2004c). Multiple serotypes were isolated from broiler flocks; *Salmonella* Enteritidis and *Salmonella* Typhimurium was isolated respectively in 6% and 10% of the cases. In Flanders; due to the forced vaccination (see also paragraph 3.7) of the breeder flocks (broiler and layer) since 1997, the *Salmonella* Enteritidis contamination decreased till 0% in 2004 (Figure 1.9).

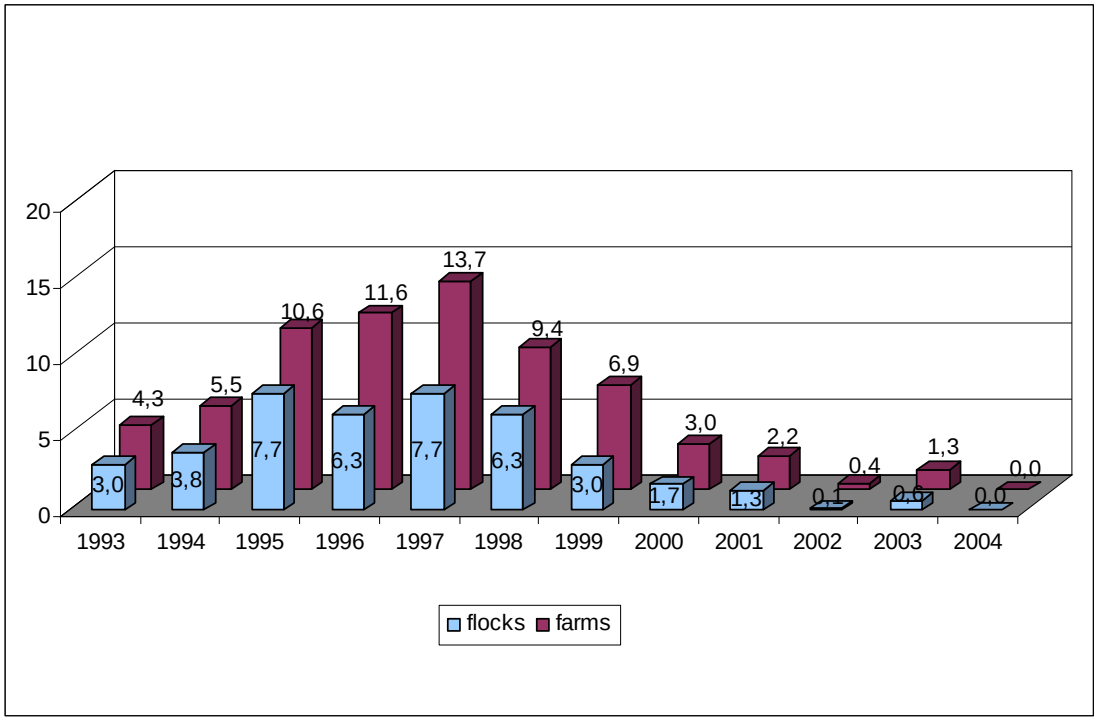

Figure 1.9: Percentage *Salmonella* Enteritidis positive breeder flocks and breeder farms in Flanders in 2004 (Anon. 2004c).

3.7 Control of *Salmonella* in laying hens

The association between infection of layers, eggs contamination and human food poisoning by *Salmonella* Enteritidis was an important reasoning to develop control programs to reduce laying hen infections. This can be achieved by reducing the infection pressure in the environment of the hen and by increasing the resistance of the hen against infections. Vaccination with dead and live *Salmonella* bacteria is probably the most widely used control measure. Other control strategies to control *Salmonella* infection in laying hens aim at preventing intestinal colonization based on the use of prebiotics, synbiotics and other feed additives (Van Immerseel *et al.* 2002). The World Veterinary Poultry Association recommends three successive vaccinations with *Salmonella* Enteritidis, at respectively the hen ages of 1 day, 6 weeks and finally from 16 weeks and 2 weeks before movement of the breeding flocks (broilers and layers). For laying hen flocks, vaccination with *Salmonella* Enteritidis is recommended respectively at the hen ages of 1 day, 6 weeks and finally 2 weeks before movement from the rearing farm to the laying farm (Anon. 2004f).

The HACCP programs for shell eggs are mainly focused on the *Salmonella* Enteritidis prevention. Davison *et al.* (1997) identified three major critical control points for *Salmonella* Enteritidis contamination; cleaning and disinfection between flocks, control of rodents and

using *Salmonella* Enteritidis clean pullet chicks. The vaccination schedules must guarantee this. Monitoring pullet hens, laying hens and environmental samples for *Salmonella* Enteritidis is necessary to control the status of the laying flock. As a part of a field-based study of the distribution and persistence of *Salmonella* infection on commercial egg-laying farms, Davies and Breslin (2003) sampled egg-packaging areas of 12 farms in the UK infected with *Salmonella* Enteritidis. Contamination was common, with salmonellae being found in 23.1% of floor swab samples, 30.8% of grading tables, 23.1% of conveyor belts or rollers and 23.8% of candlers. After cleaning and disinfection of packaging plants of 4 farms, contamination was still found on 6.9% of samples from grading tables, 16.0% holding/sorting tables, 12.6% conveyors or rollers, 16.7% of vacuum egg lifters, 21.4% of floor surface samples and 5% of egg store floor surfaces. Sterilized eggs passing through five contaminated farm packaging plants showed a contamination rate of at least 16/5948 (0.3%) egg passages. The study showed that the contamination in egg-packaging plants is a factor to external contamination of the eggshell and improved methods of cleaning and disinfecting egg-handling equipment is still required.

4 MECHANISMS OF MICROBIAL CONTAMINATION OF INTACT EGGS

There are two possible routes of bacterial infection of shell eggs: either vertically or horizontally.

4.1 Transovarian or vertical transmission

In the transovarian route (vertical transmission), the yolk (very infrequently the yolk itself), the albumen and/or the membranes are directly contaminated as a result of bacterial infection of the reproductive organs, *i.e.* ovaries or oviduct tissue, before the eggs are covered by the shell (Messens *et al.* 2005b). Vertical transmission can originate from infection of the ovaries of a laying hen via systemic infection, or from an ascending infection from the contaminated cloaca to the vagina and lower regions of the oviduct (Keller *et al.* 1995; Miyamoto *et al.* 1997). As *Salmonella* and in particular *Salmonella* Enteritidis is the most important potential human pathogen in eggs, the vertical transmission of the pathogen was studied by many researchers. Colonization of the intestinal tract with *Salmonella* commonly occurs after the consumption of contaminated feed (Williams 1981). *Salmonella* Enteritidis is the dominant

serotype isolated from egg contents; while the phage type 4 is the most important strain (Perales and Audicana 1988; Humphrey 1989; Humphrey *et al.* 1989; Mawer *et al.* 1989). No relation has been found between *Salmonella* Enteritidis contamination of the eggshell and that of the egg content (Humphrey *et al.* 1989; Humphrey *et al.* 1991b; Methner *et al.* 1995). This may suggest that contamination of egg contents is more likely to take place in the reproductive organs than by eggshell penetration. While a range of serotypes have been isolated from eggshells, *Salmonella* Enteritidis has been isolated primarily from the contents of intact eggs (Saeed 1998). According to Cogan and Humphrey (2003) vertical transmission of *Salmonella* Enteritidis is more common because this serovar possesses SEF14 fimbriae, which may be involved in the reproductive tissue colonisation.

It was generally believed that the majority of vertical *Salmonella* Enteritidis contaminations occurred in the albumen (Humphrey *et al.* 1991b). Recently there has been also evidence for contamination of the yolk (Gast *et al.* 2002), particularly its membrane (Gast and Holt 2001). The principal site of infection would appear to be the upper oviduct (Humphrey 1994a). Membrane and eggshell are produced in the lower part of the reproductive tract. These compartments of the egg may also be contaminated during egg development. Contamination of membranes and eggshells by *Salmonella* Enteritidis have been reported to occur also frequently (Humphrey *et al.* 1989; Humphrey *et al.* 1991b); in some studies they are even reported as the most infected components (Miyamoto *et al.* 1997; Okamura *et al.* 2001). However, since *Salmonella* bacteria can penetrate eggshells, it is difficult to distinguish between contamination during formation of the egg or after oviposition.

4.2 Horizontal transmission

In the horizontal transmission the micro-organisms penetrate through the eggshell. The egg passes through the highly contaminated cloaca area at the moment of lay; this is often illustrated by visible faecal contamination on the shell. Following oviposition, the shell acquires contamination from all surfaces with which it makes contact (Board and Tranter 1995). While being wet and entering an environment with a temperature of approximately 20°C below the hen's body temperature, the egg will cool immediately. The egg content will contract and a negative pressure establishes inside the egg, thereby moving contaminants through the shell (Padron 1990). However, the egg presents a complex series of defensive barriers to the contaminating organisms (see also paragraph 2.4 and 2.5) and although

microbes may successfully penetrate the shell of the egg, further development may be arrested or delayed (Bruce and Drysdale 1994).

With salmonellae other than *Salmonella* Enteritidis, horizontal transmission is probably the most important route according to Humphrey (1994b). In the UK, Mawer *et al*. (1989) reported that none of 360 eggs from a small free-range flock implicated in a school-associated outbreak of salmonellosis was shell positive for PT4 even through the organism was isolated from egg contents. There is no indication that *Salmonella* Enteritidis can move more effective through eggshells and the underlying membranes than other competing faecal organisms (Humphrey 1994b).

4.3 Extrinsic factors affecting horizontal transmission

Temperature differential

One of the main factors governing microbial contamination of eggs is the temperature differential at the moment of lay. From the point of lay, as the warm egg cools, a negative pressure (the egg content contracts) is created down the pores which may result in drawing contaminating bacteria of the shell through the pores (Bruce and Drysdale 1994).

Moisture

Moisture is needed to allow penetration according to some authors (Bruce and Drysdale 1994; Berrang *et al.* 1999). It is well established that penetration will be greatly enhanced in circumstances where in addition to moisture a positive temperature differential is present which causes the contents to contract and draw any water present trough the open pores (Board and Halls 1973; Berrang *et al.* 1999). According to Padron (1990) the presence of water on the shell enhances *Salmonella* Typhimurium, but its presence is not essential for penetration. When eggs are removed from refrigerated storage and placed at room temperature, they may “sweat” due to condensation of water droplets on the egg surface (Bruce and Drysdale 1994). In an old study of Fromm and Margolf (1958) bacterial contamination of albumen and yolk was more likely to occur in eggs that were allowed to sweat, while Ernst *et al.* (1998) found that eggs which were allowed to sweat were not more contaminated than the control group (see also chapter 7).

Presence of bacterial contamination and faeces

As it is accepted that the egg is most susceptible to penetration at the point of lay, it follows that the microbiological status of the environment into which the newly laid eggs are deposited has a major influence on the incidence of contamination in eggs. There is ample evidence that eggs laid into a heavily contaminated environment suffer more bacterial spoilage than those laid in a clean environment (Bruce and Drysdale 1994).

Several reports exist in which researchers have recorded the level of contamination on shells of eggs produced under different conditions (Haines 1938; Harry 1963; Board *et al.* 1964; Quarles *et al.* 1970). The level of contamination ranges from 10^3 - 10^5 CFU aerobic bacteria per egg in clean conditions to 10^7 - 10^8 CFU in dirty conditions. More recent literature is focussed on the influence of the housing system on the bacterial eggshell contamination (Cepero *et al.* 2000; Protais *et al.* 2003a; Protais *et al.* 2003b; Protais *et al.* 2003c; Mallet *et al.* 2004). This literature is discussed in detail in chapter 3.

Early investigations by research workers who have tried to study bacterial penetration of eggs by deliberately contaminating the nesting materials with organisms known to induce spoilage or reduce hatchability have produced some noteworthy results. Haines and Moran (1940) and Drysdale (1985) failed to induce spoilage in experiments were eggs were naturally laid into nests containing respectively straw sprayed with a strain of *Pseudomonas* and wood shavings sprayed with *Bacillus cereus*. Drysdale (1985) subsequently subjected eggs to a much more severe challenge by incorporating into the nest litter a mixture of fresh poultry faeces, soiled deep litter and shavings sprayed with *Proteus vulgaris* and *Proteus mirabilis*. This highly contaminated mixture resulted in a bacterial challenge of more than 10^9 CFU/g nest box litter compared to control nests challenged with < 10^6 CFU/g. After being incubated, the egg contents of eggs failing to hatch were examined for the presence of *Proteus* spp; 8% eggs (8/100) from the treated nests were contaminated with *Proteus* spp., and none of the eggs (0/100) from the control nests. The experiment was repeated with shavings sprayed with *Proteus* cultures but without faeces and shavings sprayed with *Proteus* and a moisture level adjusted to that recorded in the nest litter containing faeces and deep litter. In both cases less eggs from the treated eggs were contaminated compared to the first experiment (see Table 1.6).

Table 1.6: Incidence of *Proteus* contamination in eggs laid in highly contaminated nest boxes compared with clean nest boxes (Drysdale 1985).

Treatment	% of eggs contaminated with *Proteus* spp.	
	Treatment pen	Control pen
1. *Proteus* spp. + faeces	8	0
2. *Proteus* spp.	1	0
3. *Proteus* spp. + high moisture	2	0

These results indicate that surprisingly few eggs become contaminated even under conditions which would have been expected to induce serious contamination problems. Nevertheless the presence of faecal material and deep litter waste appears to increase contamination which cannot be attributed solely to increased moisture levels. The results of Graves and Mac Laury (1962) using a mixture of *P. vulgaris*, *Pseudomonas aeruginosa* and *Streptococcus faecalis* were comparable to those of Drysdale (1985). There are a number of possible explanations for this occurrence; faecal or other soiling material may contain substances which reduce the surface tension of any moisture present which increases the rate of bacterial penetration or alternatively, the faeces or other soiling material may contribute some chemical, *e.g.* iron, which interferes with the natural defence mechanisms of the egg, thereby allowing bacteria to establish more easily the egg once penetration has taken place (Bruce and Drysdale 1994).

4.4 Egg infection and chemotaxis

Due to the presence of inhibitory substances in the albumen (see paragraph 2.5) bacteria will grow poorly or not at all in the albumen. The vast majority of the studies on the course of infection with rot-producing or *Salmonella* at ambient temperatures show a lag of 10 - 20 days between infection of the shell membranes with bacteria suspended in water containing <1 - 2 ppm Fe(III) and overt signs of infection of the egg contents (Board *et al.* 1994). Up till 16 studies (mainly old) are summarized in Board *et al.* (1994). Brooks (1960) suggested that the shell membranes were initially an unfavourable niche for microbial growth but some undefined changes in their structures around day 13 of storage led to an improvement and the onset of bacterial growth within the membranes and in the underlying albumen. Board (1965) concluded that, with eggs artificially infected and stored at ambient temperature, the lag period was terminated when the yolk moved upwards and made contact with the infected shell membrane. In other words the loss of highly organized (compartmentalized) structure of the egg contents negated the antimicrobial defence of the albumen. Observations of Lock (1992)

demonstrated that the yolk plays an important role in the infection process. Additionally, the possible role of chemotaxis in this process was indicated. Using *Pseudomonas putida* and UV light to monitor, their results led them to conclude that the following stages resulted in a generalized infection of an egg's content (egg poured out into a Petri dish) (Figure 1.10):

1. Organisms from the site of infection invade the outer thin albumen
2. Some of these pass through the albuminous sac and gain access to the inner thin white
3. Some of the initial invaders of the inner thin white begin to grow probably as a consequence of obtaining essential nutrients from the yolk
4. Within a short time, the whole of the inner thin white is heavily contaminated
5. Gross contamination passes outwards into the albuminous sac where it appears to be temporarily constrained before finally moving out into the outer thin white

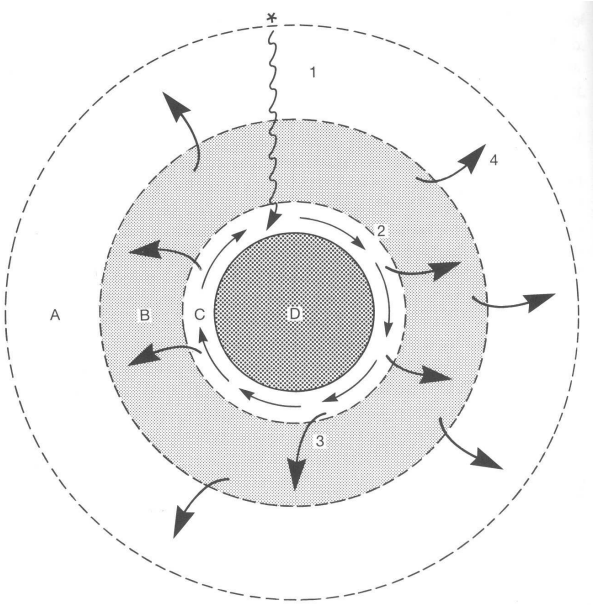

Figure 1.10: Sequence of events leading to generalized infection of egg contents with *Pseudomonas putida*. Contents of a freshly laid egg were poured into a square (10 x 10 cm) Petri dish and inoculated in the outer thin albumen (asterisk) with pseudomonads in a plug of water agar. The numbered arrows refer to steps in the process (see also text for details). A = outer thin white; B = albuminous sac; C = inner thin white; D = yolk.

According to Humphrey *et al.* (1991b) growth of *Salmonella* in albumen could only occurs when egg's age exceeds 21 days if held at 20°C. They postulated that upon storage, either nutrients or some factors negating the inhibitory properties of the albumen leak out from the yolk, because of alterations in the structure of the yolk membrane. Later studies were published that support the earlier findings of poor growth of *Salmonella* Enteritidis (Gast and Holt 2001) and *Salmonella* Typhimurium (Hu *et al.* 2001) in albumen. However, rapid and substantial multiplication occurred when bacteria had access to yolk nutrients (Gast and Holt

2000). Some studies, however, highlighted another view on the behaviour of *Salmonella* in separated albumen from fresh eggs. Schoeni *et al.* (1995) found that *Salmonella* Enteritidis, *Salmonella* Typhimurium and *Salmonella* Heidelberg increased ≥ 3 log units upon one day at 25°C in albumen. Braun and Fehlhaber (1995) found that four out of ten strains of *Salmonella* Enteritidis were able to grow at 20°C in albumen. The researchers also found that *Salmonella* Enteritidis can migrate from the albumen to the yolk in less than 1 day for 17% of the eggs inoculated with 10 cells/ml albumen and subsequent storage at 20°C. After 4 weeks, 72% of the egg yolks were contaminated. Similar results were reported by Baker (1990), who observed contaminated yolks, albeit not frequently, during storage at 8°C. It has to be noted that Braun and Fehlhaber (1995) used buffered peptone water for the *Salmonella* Enteritidis solution to be injected, which enhances bacterial growth in albumen. Cogan *et al.* (2001) observed that the higher the inoculum size of *Salmonella* Enteritidis in either the albumen of whole eggs or into separated albumen, the higher the amount of samples showing a pronounced growth. After 8 days at 20°C, growth was observed in 7% of whole eggs inoculated in the albumen near the shell with as few as two cells. The fraction of contaminated eggs increased up to 50% when the initial inoculum level was increased to 2 500 cells.

Some authors studied the effect of egg storage prior to inoculation. Humphrey and Whiteheat (1993) found *Salmonella* Enteritidis did not grow well in albumen at 20°C, when the albumen had been removed from fresh eggs and in albumen away or near the yolk of eggs that had been stored at 20°C for 6 weeks. When the albumen remaining around the intact yolk was inoculated, growth at 20°C took place more quickly when eggs were stored prior to inoculation for > 3 weeks at 20°C. Messens *et al.* (2004) studied the growth of *Salmonella* in fresh or stored (3 weeks) albumen either in the shell egg or separated from the yolk. The serovar Enteritidis did not behave differently than the other serovars indicating that the association between human *Salmonella* Enteritidis infections and eggs is not due to its growth behaviour in albumen. A pronounced growth occurred more frequently and up to higher level in fresh albumen than in albumen stored prior to inoculation. This was at least partly explained by a pH effect. Since the growth in the separated albumen was similar when the albumen had been stored prior to inoculation in the absence or presence of yolk, the researchers had no indication that nutrients or factors negating the inhibitory properties of the albumen leak out from the yolk during storage.

Temperature and time play an important role in the proliferation of micro-organisms. In the majority of eggs Humphrey and Whiteheat (1993) did not found a rapid growth of *Salmonella*

Enteritidis when eggs were held at 20°C for 3 weeks. When eggs were stored under conditions where temperatures fluctuate between 18 and 30°C, to simulate those that might be found in kitchens, in the majority of eggs examined, after 6 – 10 days, rapid growth was possible. These results reinforced the importance of the proper storage of eggs. Gast and Beard (1992), Humphrey (1994a) and Schoeni *et al.* (1995) concluded that storage temperature dramatically affect the growth of *Salmonella* Enteritidis in shell eggs and reported that at storage temperatures lower than 7°C, *Salmonella* Enteritidis grew sporatic or not. According to Catalano and Knabel (1994) the time at which eggs reach 7°C is also very crucial. They found that slowely chilled eggs were more prone to penetration by *Salmonella* Enteritidis than rapidly chilled eggs. The study of Messens *et al.* (2004) also concluded that cooling practices are recommended shortly after lay to prevent *Salmonella* from growing in eggs. There has been much debate on the advisability of holding eggs under refrigeration in retail outlets; it can present practical difficulties.

Finally, Grijspeerdt *et al.* (2004) developed an individual-based model (IbM) to describe the growth and migration of *Salmonella* Enteritidis in hen's eggs (Figure 1.11). The impact of factors as chemotaxis, growth rate, initial contamination numbers and bacterial swimming speed was assessed by a sensitivity analysis. Their results show that chemotaxis towards the yolk would have a strong effect on the time needed to reach the vitelline membrane. The simulation results illustrate the need for more detailed knowledge on the subject of bacterial migration in hen's eggs.

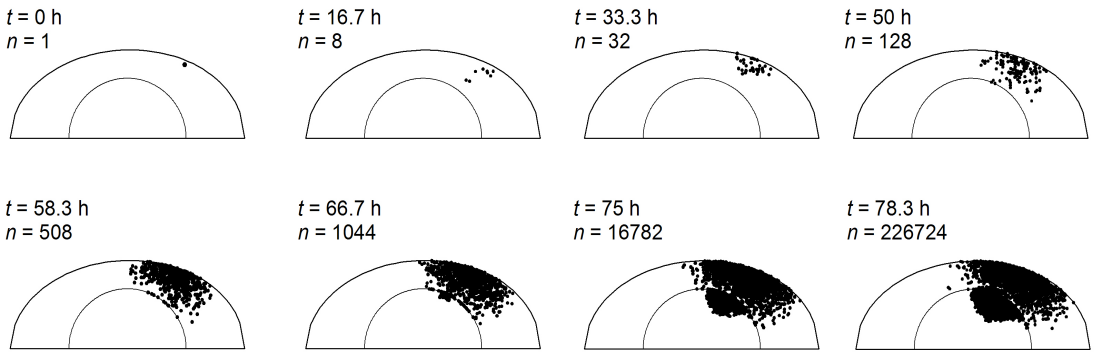

Figure 1.11: *Salmonella* Enteritidis migration and growth starting from one initial cell. Indicated are the simulation time (*t*) in hours, and the total number of cells (*n*) (Grijspeerdt *et al.* 2004).

5 TYPE OF CONTAMINATING MICROFLORA ON THE EGGSHELL AND IN THE EGG CONTENT

5.1 General type of contaminating microflora

Mostly old literature is available on the type of microbial flora which challenges the eggshell and egg content. A number of early workers have reported on the microflora present on eggshells, most studies were focussed on hatching eggs. These observations have been summarized and compared with the types of bacteria isolated from spoiled eggs (Table 1.7) (Mayes and Takeballi 1983).

Table 1.7: Comparison of the microflora on the surface of the egg and within spoiled eggs (Mayes and Takeballi 1983).

Type of organism	Frequency of occurrence[a]	
	On the shell	In rotten eggs
Micrococcus	+++	+
Achromobacter	++	+
Aerobacter	++	-
Alcaligenes	++	+++
Arthrobacter	++	+
Bacillus	++	+
Cytophaga	++	+
Escherichia	++	+++
Flavobacterium	++	+
Pseudomonas	++	+++
Staphylococcus	++	-
Aeromonas	+	++
Proteus	+	+++
Sarcina	+	-
Serratia	+	-
Streptococcus	+	+

[a] The more plus signs, the more frequent the occurrence

Mayes and Takeballi (1983) have also noted that although the microflora found on the eggshell varies quantitatively and qualitatively in different geographical areas, the spoilage flora in eggs trends to be similar irrespectively of geographical area or husbandry methods, indicating that the intrinsic defence mechanisms of the egg influence the selection of spoilage types. Probably because of their tolerance of dry conditions, the microflora of the eggshell is

dominated by Gram-positive bacteria which may originate from dust, soil or faeces (Board and Tranter 1995). Rotten eggs normally contain a mixed infection of Gram-negative and a few Gram-positive organisms. Some of the most common contaminants are members of the genera *Alcaligenes*, *Pseudomonas*, *Escherichia*, *Proteus* and *Aeromonas* (Mayes and Takeballi 1983; Board and Tranter 1995) (see also Table 1.7). This indicates that Gram-negative bacteria are well equipped to overcome the antimicrobial defences of the egg. According to Board and Tranter (1995), the internal properties of eggs favour survival and growth of contaminating organisms which are Gram-negative, have a relatively simple nutrition requirement and have the ability to develop at low temperatures. Comparing the microbial flora in hatching eggs from different birds; Seviour and Board (1972) and Bruce and Johnson (1978) showed that micrococci constituted the main part of the flora in hen's eggs; *Enterobacteriaceae, Staphylococcus* spp. and *Streptococcus* spp. are also an important part.

More recent reports on genera and species present on the eggshell and associated with the egg content are available from egg washing experiments. Unwashed eggs randomly selected from an accumulator were analysed for the presence of yeast and moulds, *Enterobacteriaceae* and pseudomonads (Jones *et al.* 2004). An average yeast and mould concentration of 1.5 log CFU/ml (10 ml rinsing solution) was found on eggshells (n = 36) at the day of collection. Low concentrations of *Enterobacteriaceae* were detected; the highest concentration detected was 0.6 log CFU/ml. For pseudonomads no clear data are mentioned for unwashed eggs at the day of collection; only 16 of approx 380 unwashed eggs whether or not stored up till 10 weeks were positive (generally less than 1 log CFU/ml). Yeast and mould concentration in the contents of unwashed shell eggs was on average 0.1 log CFU/ml at the day of collection (n = 9; pools of 3 eggs). The average bacterial concentration with total aerobic flora was *approx* 1 log CFU/ml (n = 9 pools). No samples of pooled egg contents were positive for *Enterobacteriaceae*. Pseudomonads were found in 8 of the approx 100 egg contents of unwashed eggs whether or not stored up till 10 weeks. The probably weak procedure to sanitize the shell surface (submersion in 95% ethanol) and the unclear information about the detection limits have to be taken into consideration. Very recently Musgrove *et al.* (2004) determined the variety of *Enterobacteriaceae* species associated with eggshells as they processed through the wash processing chain of three plants. The study was undertaken to characterize *Enterobacteriaceae* species not only with unwashed eggs and washed eggs, but also those micro-organisms that persisted during operations in three commercial shell egg washing facilities in the US. Three plants were sampled on three separated processing days;

from each collection site twelve eggs were sampled. Table 1.8 includes genera that were recovered at least once during one of the nine egg processing plant visits. In the second column of Table 1.7 ('before processing') the identified isolates recovered from the shell of unwashed eggs are listed. *Escherichia coli* and *Enterobacter* spp. were isolated from the eggshell of unwashed eggs of each of the nine plant visits. *Enterobacter sakazakii* as well as *Salmonella* spp. was also identified in each of the three plants but never isolated from fully processed (washed) eggs.

Table 1.8: Identification (genus) of isolates randomly selected from violet red bile glucose agar plates of shell egg rinses obtained from eggs collected before, during or after processing at three US egg processing facilities (three visits / plant) (Musgrove 2004)

Genus[a]	Before processing	During processing	After processing
Aeromonas	5/9[b]	4/9	2/9
Cedecea	2/9	0/9	0/9
Chryseomonas	1/9	0/9	0/9
Citrobacter	8/9	1/9	1/9
Enterobacter	9/9	3/9	3/9
Erwinia	1/9	0/9	0/9
Escherichia	9/9	5/9	3/9
Hafnia	5/9	1/9	0/9
Klebsiella	8/9	1/9	2/9
Kluyvera	2/9	1/9	0/9
Leclercia	3/9	0/9	0/9
Listonella	6/9	2/9	1/9
Morganella	2/9	1/9	0/9
Proteus	1/9	0/9	0/9
Providencia	5/9	2/9	1/9
Pseudomonas	5/9	0/9	0/9
Rahnella	1/9	0/9	0/9
Salmonella	7/9	3/9	0/9
Serratia	3/9	2/9	0/9
Sphingobacterium	1/9	0/9	0/9
Vibrio	2/9	0/9	1/9
Xanthomonas	2/9	0/9	0/9

[a] Isolates were identified using API biochemical test strip reactions and software.

[b] Number of visits the genus was recovered/number of sampling visits.

5.2 *Salmonella* contamination of eggs

Eggshell contamination with Salmonella

Eggshells can become contaminated with salmonellas either as a result of infection of the oviduct or by faecal contamination. With salmonellas other than *Salmonella* Enteritidis the latter route would seem to be more important (Humphrey 1994a). Eggshells can also be contaminated with *Salmonella* Enteritidis as a result of intestinal carriage; Gast and Beard (1990) reported a correlation between *Salmonella* positive faeces and shell contamination after artificial infection of hens with *Salmonella* Enteritidis PT13a. With *Salmonella* Enteritidis PT4, infection of reproductive tissue may be more important. Humphrey *et al.* (1991a), working with artificially infected specific pathogen-free hens, found that eggshells were *Salmonella*-positive in the absence of faecal carriage. Infected birds laid eggs with contaminated shells over 6 weeks after intestinal carriage had ceased. Eggs with contaminated shells were also laid by five birds that were faeces-negative throughout the course of the study. These results suggest the possibility that the shell gland or another part of the oviduct may be a site of infection.

The evidence for eggshell contamination by *Salmonella* Enteritidis is very variable. In Spain, Perales and Audicana (1989) examined 372 eggs from flocks implicated with human cases of salmonellosis; *Salmonella* Enteritidis PT4 was found on 0.8% of the shells. In a laying house in which *Salmonella* was isolated from 72% of the environmental samples, 7.8% of the eggshells were contaminated (Jones *et al.* 1995). A study of the UK Food Standards Agency in 2003 did not find significant differences in *Salmonella* spp. contamination on the shell due to the production system (Anon. 2004d). On a total of 4 753 retail samples of six eggs, the eggshell of 9 samples was contaminated; statistical analysis of the survey results showed an overall prevalence of *Salmonella* in a box of six eggs of 0.34%; *i.e.* 1 box in every 290 boxes. Seven of the 9 isolates were *Salmonella* Enteritidis, 3 were phage type 4. The prevalence was significantly lower in comparison with a previous survey in the UK in 1995 - 1996 with 1/100 boxes positive. Finally Musgrove et al. (2005) identified one out of 105 *Enterobacteriaceae* isolates, isolated from 84 shell surfaces, as *Salmonella*.

Almost no information is available on the numbers of salmonellas on eggshells. In one old study (Baker *et al.* 1985), dirty ‘duck’ eggs were found to be carrying 5×10^5 salmonellas per egg, compared to less than 1×10^2 per egg on ‘clean’ eggs.

Contamination of egg contents with Salmonella

The observed prevalence of eggs with *Salmonella*-positive contents can be variable. There are a number of factors, including the size of sample, timing of sampling, site(s) within the egg that are tested, used techniques, investigations with eggs of artificially and naturally infected hens, … which have an influence on the observed prevalence of eggs with *Salmonella* positive contents (Humphrey 1994b). Interpretation of the results of the various surveys outlined below should, therefore, take account of the factors described above.

In the earlier mentioned study of the UK Food Standards Agency in 2003, none of the 4 753 pooled egg contents of retail samples were *Salmonella* positive (Anon. 2004d). Poppe *et al.* (1998) found 0.07 - 0.4% table eggs (n = 1 512) (eggshell and egg content) positive for *Salmonella*; *Salmonella* Agona was isolated. In a study of de Boer and Wit (2000) 14 on 46 200 or 0.03% eggs sampled in The Netherlands in 1998 – 1999 were *Salmonella*-positive. Most other work has been done on eggs from flocks known or thought to be infected with *Salmonella* Enteritidis. Studies on naturally infected layer flocks show mostly a prevalence below 3% (Kinde *et al.* 1996; Schlosser *et al.* 1999). In a larger study of Humphrey *et al.* (1991b), over 5 700 eggs from 15 naturally infected flocks were examined, of which only 32 or 0.6% were contaminated. In the majority, levels of contamination were low (< 20 CFU/egg). The prevalence of egg content contamination of eggs from battery or free-range were comparable; 0.73 and 0.64% respectively. Storage at room temperature had no significant effect on the prevalence of *Salmonella* positive eggs but those held for more than 21 days at ambient temperature were more likely ($P < 0.01$) to be heavily contaminated (> 100 CFU/egg). When it was possible to identify the site of contamination in eggs, the albumen (80%) was more frequently positive than the yolk (13%). The populations present in the contents of freshly laid eggs from either naturally (Humphrey 1989; Humphrey *et al.* 1989; Mawer *et al.* 1989; Humphrey *et al.* 1991b) or artificially infected hens (Gast and Beard 1990) are usually low. One exception to the above findings is the isolation of > 10^7 *Salmonella* Enteritidis CFU/g during outbreak investigations from the contents of a clean, intact egg thought to be five days old (Salvat *et al.* 1991).

In artificially infected hens the percentage of infected eggs can range from 0 - 27.5% (Keller *et al.* 1995; Okamura *et al.* 2001). Gast and Beard (1992), using experimentally infected hens, showed that storage of eggs before testing influenced the rate of detection. Only 3% of freshly laid eggs from experimentally infected hens were identified as contaminated, whereas 16% were detected after storage for 7d at room temperature.

5.3 Other contaminating pathogens

Campylobacter jejuni is commonly associated with poultry and there is thus the possibility that eggshells and egg contents can become contaminated. Doyle (1984) infected laying hens at 20 weeks of age. Of 226 eggs from hens faecally excreting *C. jejuni*, the organism was isolated from two shell surfaces but no egg contents. Egg penetration studies revealed that the organism would not penetrate into the contents of egg but could be isolated occasionally from the inner shell membranes. Sahin *et al.* (2003) tested the presence of *Campylobacter* separately in the shell membranes and contents of a total of 1 000 eggs obtained from a commercial hatchery over a period of a year; the pathogen was not detected. Likewise, *Campylobacter* was not recovered from any of 500 fresh eggs obtained from commercial broiler breeder flocks that were actively shedding *Campylobacter* in faeces. When *C. jejuni* was directly inoculated into the egg yolk, and eggs were stored at 18°C, the organism was able to survive for up to 14 days. However, viability of *C. jejuni* was dramatically shortened when injected into the albumen or the air sac. When freshly laid eggs from Campylobacter-inoculated specific pathogen-free layers were tested, *C. jejuni*-contamination was detected in three of 65 pooled whole eggs (5 - 10 eggs in each pool). However, the organism was not detected from any of the 800 eggs (80 pools), collected from the same specific pathogen free flock, but kept at 18°C for 7 days before testing. These results suggest that survival of *C. jejuni* is probably a rare event (Sahin *et al.* 2003).

Nitcheva *et al.* (1990) isolated *Listeria monocytogenes* from the eggshell (1 of 71 samples). Until now no data are available on the prevalence of *L. monocytogenes* in whole eggs. Brackett and Beuchat (1992) studied the survival of the organism on shells of unbroken eggs over a 6-week period at 5 and 20°C. Low (10^2 CFU per egg) and high (10^4 CFU per egg) populations of *L. monocytogenes* on the surface of eggshells decreased to < 10 CFU per egg after 6 days of storage at 5 and 20°C. After 6 weeks of storage the pathogen was still detectable but unquantifiable at both temperatures. Sionkowski and Shelef (1990) studied the viability of *L. monocytogenes* in raw and heat-treated (121°C, 15 min) whole eggs, albumen and yolk during storage at 5 and 20°C. The studies with raw eggs showed that the organism grew only in egg yolks, where initial numbers (10^6 CFU/g) increased to 10^8 CFU/g (generation times of 1.7 days and 2.4 h at 5 and 20°C, respectively). Cell numbers in whole eggs initially declined and then levelled off. A sharp decline was observed in the raw albumen

(to 10^2 CFU/g after 22 days at 5°C and to < 10 after 55 h at 20°C). In contrast, the organism grew in all heat-treated egg samples. On the other hand, the organism has been isolated, with high frequency, from samples of eggs collected at processing plants. Leasor and Foegeding (1989) obtained 45 *Listeria* isolates from 15 of 42 (36%) commercially broken raw liquid whole egg samples from 11 processing establishments across the US. *L. monocytogenes* was obtained from 5% (2) of the egg samples. Moore and Madden (1993) sampled in-line filters removing solids from raw blended whole eggs in an egg pasteurizing plant for the presence of *Listeria* species. Overall, 173 samples were studied, with 125 (72%) being *Listeria* positive; the species isolated were 62.2% *Listeria innocua* and 37.8% *L. monocytogenes*. A total of 500 daily samples of pasteurized product were also studied, and all proved to be negative for *Listeria*, confirming the safety of the pasteurization process with regard to listeriae.

Schoeni and Doyle (1994) challenged 1-day-old laying hens orally with *Escherichia coli* O 157:H7. *E. coli* O 157:H7 colonization persisted at least 10 - 11 months when chicks were administered 10^8 *E. coli* O 157:H7 bacteria. Eggs from 5 hens that were faecal shedders of *E. coli* O157:H7 until the termination of the study (10 - 11 months) were assayed for *E. coli* O157:H7. The organism was isolated from the shell of 14 of 101 (13.9%) eggs but not from the albumen and yolks.

Favier *et al.* (2005) evaluated a total of 352 eggs for the presence of *Yersinia enterocolitica* strains on the eggshell. No isolates were obtained by direct culture; however eight *Y. enterocolitica* strains were recovered after enrichment, which represents a prevalence of 2.27% eggshell samples. *Y. enterocolitica* was not detected in 45 content samples.

6 HOUSING SYSTEMS FOR LAYING HENS

During recent decades the housing of layers for commercial egg production has become widely discussed, especially in Europe. The debate has focused on the barren environment and restricted area available in conventional cages and the welfare of hens housed in such cages has been questioned (Craig and Swanson 1994). In 2004 429 layer farms were registered (min. 200 layers) in Belgium; 55 with free range systems, 56 with barn productions, 307 with cages and 27 with organic production (free range or barn) (Anon. 2004a). Conventional cage housing for laying hens will be prohibited from 2012 in the

European Union, following EU-directive 1999/74 (Anon. 1999). From 2012 onwards, only furnished cages and alternative non-cage systems like barn or deep litter systems and aviary systems will be allowed.

6.1 Conventional cages

It is estimated that in 2001, 70 - 80% of world egg production is derived from conventional caged laying hens. According to Walker *et al.* (2001) these cages offer the advantages of low production costs and high standards of hygiene. Cage arrangements can vary from single-deck cages to multiple-decked cages. In case the cage rows are mounted directly above one another, dropping belts and frequent manure removal is required (Figure 1.12).

Figure 1.12: Commercial multiple-decked cage system housing brown layers and equipped with dropping boards, feed thoughts and egg conveyor belts.

The stocking density mentioned in the EU-directive 1988/166 (Anon. 1988) of 450 cm^2/bird was increased to 550 cm^2/bird from January 1st 2003 for existing cages and from then there is also a ban on the installation of new or replacement of old conventional cages (Anon. 1999).

6.2 Furnished cages

In furnished cages, hens have more space than in traditional cages (750 versus 550 cm^2/bird), access to a nest and a perch, and an area with litter for pecking and scratching. Birds are kept in relatively small groups, ranging from 5 to 50 birds depending on the system (Rodenburg *et al.* 2005).

Furnished or enriched cages should meet to the following standards (Anon. 1999):

1) A minimum area of 750 cm^2/hen; 600 cm^2 of which has to be usable. The usable area must have a minimum height of 45 cm; the other at least 20 cm.
2) A minimum total cage area of 2000 cm^2
3) A nest
4) A littered area for scratching and pecking
5) Appropriate perches allowing at least 15 cm/bird
6) A feed trough provision of at least 12 cm/bird
7) Access by each bird to at least 2 nipple or cup drinkers
8) A suitable claw shortening device
9) A minimum aisle width of 90 cm
10) A minimum space between the floor of the building and the bottom tier of the cages of 35 cm

Figure 1.13 shows a design of a furnished cage; another design is outlined in chapter 3.

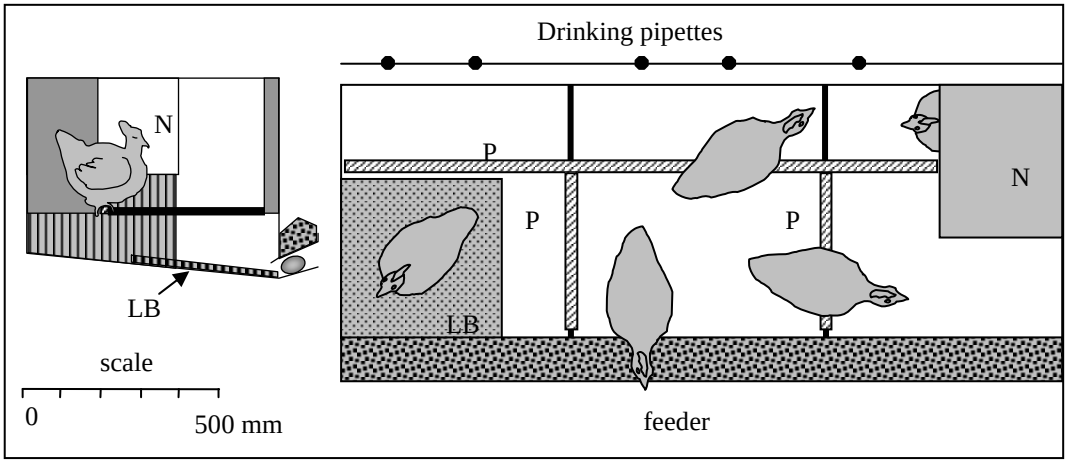

Figure 1.13: Commercial furnished cage of the Piers model for 15 birds with 1134 cm^2 cage floor area per bird (P= Perches, N = Nest, LB = Litter bath) (Mallet *et al.* 2004).

6.3 Alternative systems

From January 1st 2007 the new standards for the alternative systems are (Anon. 1999):

1) A maximum of 9 hens/m^2 and a headroom of at least 45 cm height.
2) At least 250 cm^2 of littered area per hen, the litter covering at least one third of the ground floor.
3) Elevated levels must be of such construction so that droppings do not fall on the levels below

4) No more than 4 tiers
5) At least one nest for every seven hens. If group nests are used, there must be at least 1 m^2 of nest space for a maximum of 120 hens
6) Drinking and feeding facilities must be distributed in such a way as to provide equal access to all hens (linear feeders 10 cm and circular feeders 4 cm per hen – continuous drinking trough providing 2.5 cm and circular drinking trough providing 1 cm per hen).
7) At least 15 cm perch per hen (horizontal distance between perches at least 30 cm).

If laying hens have access to open runs:

8) There must be several pop holes giving direct access to the outer area, at least 35 cm high and 40 cm wide, a total opening of 2 m per group of 1 000 hens must be available
9) Stocking density on free range must not exceed 1 000 hens/hectare.

Alternative housing systems (non-cage) can be either aviary or (single-tiered) floor housing systems. When hens also have access to open runs, the systems are called free range systems. In the aviary system there are several different designs, but in all part of the floor is covered with litter for scratching and pecking, there are wire platforms at several levels with food and water adjacent to the wire platforms. Some systems, particularly in the UK, make use of perches at different levels attached to an A-frame. Figure 1.14 shows a cross section of an aviary housing system. Another type of aviary system is outlined in chapter 3. Floor housing systems are also called barn or deep litter systems. The floor is usually partially covered with litter and an elevated perforated floor area (*e.g.* slats or wire mesh) is available. Birds are kept at floor level in these systems; but perches may also be available (Figure 1.15). (Tauson 2005)

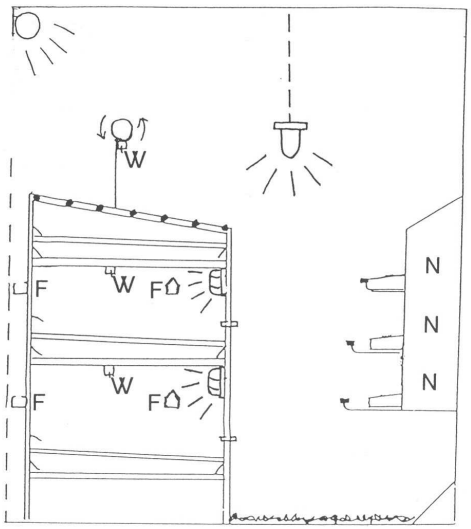

Figure 1.14 Cross section of the Marielund aviary system. F = Feed trough, N = Nests, W = Water (Abrahamsson and Tauson 1995).

Figure 1.15 Floor housing system for laying hens (barn system).

6.4 Productivity, welfare, health and hygiene in different housing systems

The ban of the conventional cages caused recent intensive evaluations of the alternatives in terms of costs, productivity and bird welfare. Abrahamsson and Tauson (1995) concluded that in a good aviary system, egg production, although being less predictable, may be similar to that in conventional cages, while hygiene and bird welfare are still in several respects better in cages than in new aviary tiered systems. Furnished cages can combine the advantages of small group size of the conventional cages and reduce the disadvantages of poor air conditions, outbreaks of cannibalism, parasitic disorders, and inferior hygiene in alternative systems (Tauson 2002). The major differences between furnished cages and alternative systems are related to group size, freedom of movement, and complexity of the environment (Table 1.9) (Rodenburg *et al.* 2005).

Table 1.9: Major differences between furnished cages and alternative systems (Rodenburg *et al.* 2005).

	Furnished cages	*Alternative systems*
Group size	*Small*	*Large*
Risk of feather pecking and cannibalism	Medium	Large
Freedom of movement	*Limited*	*Yes*
Space allowance per bird	750 cm^2	1111 cm^2
Space allowance per group	Small	Large
Complexity of the environment	*Medium*	*Large*
Litter	Limited amount	Large amount
Perches	Low	High
Access to different tiers	No	Yes / no
Air quality (dust, ammonia, bacteria)	Good	Poor

In alternative systems, birds have more possibilities to express various behaviours, resulting in stronger bones and higher levels of foraging, dust bathing and other comfort behaviours than in furnished cages. On the other hand, the large group size leads to an increased risk of feather pecking, although some studies also found a poorer plumage in furnished cages than in alternative systems (Rodenburg *et al.* 2005).

6.5 Traceability of eggs

The EU has introduced directive 2002/4/EC to make traceability of eggs possible, consumers can identify exactly where each egg they buy comes from and how it was produced. Every egg is individually stamped with a code, which makes it fully traceable to the hen-house where it was produced. The code consists of three parts: (1) a number (0, 1, 2 or 3) referring to the farming system or housing facility of the hens (Table 1.10), (2) two letters referring to the country of origin (*e.g.* BE for Belgium, NL for the Netherlands, IE for Ireland,…) and (3) the registration code of the producer. This is shown in Figure 1.16.

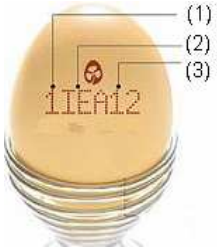

Figure 1.16: Traceability of the eggs; code on eggs.

Four farming types or housing facilities can be distinguished in accordance to the Council Directive 2002/4/EC (Table 1.10).

Table 1.10: Summary of the identification of the farming method or housing system for laying hen.

Code	Housing system
Code 3	**Eggs from caged hens**
Code 2	**Barn eggs** Eggs from alternative housing systems were hens are kept in a building
Code 1	**Free range eggs** Eggs from alternative housing systems were hens have access to open runs
Code 0	**Organic eggs** Eggs from alternative housing systems were hens have access to open runs Stocking density in building is lower; 6 hens/m^2, hens must be fed mainly with organic feed, no beak treatment is allowed (Anon. 1991)

7 THE EGG PRODUCTION CHAIN

Eggs are one of the few foods that are used throughout the world; thus the egg industry is an important segment of the world food industry. The egg industry of the world is primary based on hen (*Gallus domesticas*) eggs (Stadelman 1995a). Using FAO's statistics, between 1961 and 2002, annual world egg production rose almost 4 times to reach about 57.8 million tons, of which 53.5 million are hen eggs (about 6% are hatching eggs). Production is further predicted to increase another 36% by the year 2015 and further increasing 27% by 2030. The increases are due to the rapid expansion in egg production in Asian countries, mainly in China. (Gillin and Sakoff 2003)

The most common commercial egg production chain in Belgium and other European countries is outlined below.

Hatchery and type of animal breed

Egg-type hatcheries deliver chicks to the rearing farms within one to two days after hatching. The past decades genetic improvement in the performance of layers has been achieved by primary breeders using different breeding concepts. In Europe the brown shell egg strains (ISA Brown, Bovans Goldline and other strains) are mostly used (depending on the country). The strain selection of layers is based on rate of lay, early maturity, good feed efficiency, relative small body size, and adaptability to various climates. (Stadelman 1995a)

Rearing farms

At arrival the chicks are reared in a pullet house. At the hatchery and rearing farms the chicks are vaccinated according to a typical vaccination schedule (see also paragraph 3.7) (Anon. 2004f).

Layer farms

At the age of 17 - 19 weeks the hens arrive at the production farm. The layers quickly reach peak egg production (> 90 percent lay) around 26 - 28 weeks of age followed by a steady decline with advancing age of the laying flock. In general the laying cycle lasts approx 52 weeks (from 20 - 72 weeks of age) (Zoons and De Baere 2000). Then an economic decision has to be made by the egg producer. The producer has to decide whether he wants to end the production or moult the flock to increase egg production by introducing a second laying cycle, instead of sending the hens to slaughter.

In layer farms there are two primary methods of egg collection and packaging. In either case, hens lay eggs on an angled floor (wire or other) from where the egg rolls towards an egg collection belt (Figure 1.17a). The belt inside the hen house and a central egg collecting belt (Figure 1.17b) transports eggs out of different houses either directly to the egg processing facility or to a collection facility (Figure 1.17c). Since eggs are normally collected on a daily basis, eggs may reside on the belt of the hen house for as long as 24 h, but most are collected within a few hours after lay. In an in-line layer facility, eggs move directly from the layer house to the egg processing/packing facility. In an off-line layer facility, eggs are collected on open carton trays. The eggs remain at the farm for approximately 1 - 3 days at ambient temperature and then they are transported to an egg processing facility by truck. These eggs are there treated identically as those from the in-line operations.

a. Conventional cage house with angled floor and egg belt

b. Central conveyor belt collecting eggs from belts of each hen house

c. Conveyor belt in an in-line facility moving eggs to the egg packaging station

d. Visual inspection of eggs in a candling booth

e. Grading and packaging of grade A (right) and grade B (left) eggs

f. Display of eggs in shop rack

Figure 1.17: Different parts in the egg production chain.

Egg processing centre or packaging station

Once the eggs enter the egg processing centre or packaging station they are in most cases visually inspected (checked for eggshell problems, cracks, blood spots, presence of faeces …), graded and packaged. Visual inspection is done in a candling booth (Figure 1.17d). Originally, candling procedures were developed to separate fresh eggs from stored and partially incubated eggs. With the advances in production practices the role of candling changed, so that the primary function is now to detect and remove cracked or abnormal eggs, such as an egg with a internal blood spot (Stadelman 1995b). Modern egg processing centers are equipped with an in-line automatic crack detector; eggs are scanned by means of an acoustical system in a very accurate way (Coucke 1998). Grading involves the sorting of eggs into categories based on size or weight, quality factors (visual inspection) and cracks. Grade A eggs, “fresh eggs” or “table eggs”, should have a normal, clean and undamaged shell, a clear egg white, a yolk in the centre of the egg, no germ cell development, an air space not exceeding 6 mm and should be free from extraneous odours. In the EU, grade A eggs should not be washed or cleaned before or after grading, and will not be chilled or treated for preservation. Grade B eggs, *i.e.* egg “which do not meet requirements applicable to eggs in grade A”, may only be used by the food or non-food industries (Anon. 2003b). Grade A eggs

are graded by weight as outlined in Table 1.11. After candling and grading, grade A eggs are mostly packaged automatically in closed cartons while grade B eggs are packaged in open carton trays (Figure 1.17e). Grading by weight (grade A eggs) is done automatically; separating grade A from grade B eggs is done manually or automatically. Grade B eggs are used for egg products.

Table 1.11: Weights of the official weight grades (Anon. 2003b).

Weight grade	Weight
XL-very large	73g and more
L-large	From 63 up to 73g
M-medium	From 53 g up to 63g
S-small	under 53g

Retail and consumer

Egg producers and/or packers commonly deliver grade A eggs to the food store chain or directly to the retail outlets within one week of lay. At the warehouse of a food chain's distribution centre, eggs are mostly stored cooled (approx 8°C) and delivered within one week to the local food shops (Figure 1.17f).

In Europe, according to the commission regulation 2295/2003 (Anon. 2003b), grade A eggs may not be treated for preservation or chilled in premises or plants where the temperature is artificially maintained at less than 5°C. However, eggs which have been kept at a temperature below 5°C during transport of not more than 24h or in retail for max 3 days shall not be considered as 'chilled eggs'. Grade A eggs must be delived to the packaging centers every third working day or once a week for eggs kept on the farm at an ambient temperature artificially maintained at less than 18°C. For eggs marketed as 'extra' grade eggs must be delived each working day or every other working day for eggs kept at less than 18°C. The commission regulation 2073/2005 on microbiological criteria for foodstuffs does not mention criteria on shell eggs (Anon. 2005a).

Some examples of egg production chains are also outlined in detail in chapter 2 and 4.

8 WASHING OF SHELL EGGS

8.1 Introduction

The microbial quality of table eggs (grade A) became more important since egg-borne *Salmonella* Enteritidis emerged as a major cause of food poisoning (Humphrey 1994a). Egg washing therefore has drawn attention of the industry, although currently in the EU the washing of table eggs or grade A eggs is not allowed. Also with the move to alternative housing systems for laying hens there is currently a resurgence of interest in this topic (Bain 2005). Egg washing has shown to significantly reduce the number of micro-organisms on the shell surface however it can under certain circumstances also cause damage to the cuticle and encourage food poisoning and spoilage organisms to be forced from the surface to the egg contents (Bain 2005). In the US egg washing of consumption or table eggs is a common practice. Current commercial egg washing practice is done by egg packing companies. The modern in-line egg washing procedure can be divided into four stages: pre-washing or wetting (stage 1), washing (stage 2), rinsing (stage 3) and drying (stage 4) (Hutchison *et al.* 2003) (Figure 1.18).

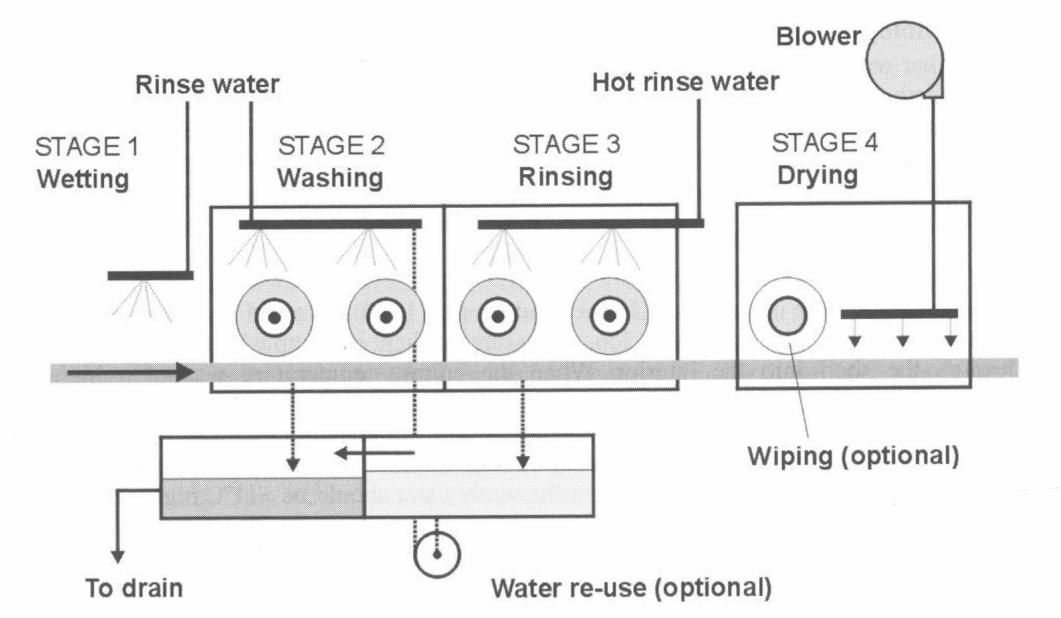

Figure 1.18: Diagram showing the key stages in commercial egg washing (Hutchison *et al.* 2003).

Pre-washing or wetting process

The pre-washing or wetting stage enables the softening of debris such as faecal material and egg varnish on the shell. This is usually little more than a light spray of warm (approx 40°C)

water, sufficient to moisture the shell surface and any debris. To achieve maximum benefit, there should be a period of several minutes to enable the penetration of the water into the soil material, before the main wash. In practice this interval is often minimal.

Washing

The main washing process typically involves rubbing the eggs with brushes while being sprayed with warm (40 - 50°C) water containing appropriate sanitising chemicals. High pressure water jets are also used in some equipement. The conveyor usually has rollers that turn the eggs. Within the washer two or three district zones or stages of washing may exist with increasing temperatures of water being applied. There may also be recycling zones with the drained water from the final stages being used for the pre-wash in the first stage.

Rinsing process

In the final stage of the wet part of the process eggs are rinsed with clear hot water to remove any loose debris that eggs picked up during the main washing and also to remove any chemicals or other dissolved matter.

Drying process

The drying process is carried out in two or more stages. It involves two distinct physical processes: 1) the mechanical removal of 70 - 80% of the surface water carried by the egg and 2) the removal of the remainder by evaporative mechanism. The first stage involves an element of drainage usually assisted by the use of air jets; evaporation is enhanced by the same air jets. An alternative for both types of drying is the use of very soft brushes to “wipe” the eggs dry.

8.2 Microbiological considerations of the egg washing process

A number of publications indicated that the historical practices of egg washing resulted in an increase of internal bacterial contamination (Haines and Moran 1940; Lorenz and Starr 1952; Brant and Starr 1962), whereas recent studies testing modern egg washing procedures indicate the opposite (Lucore *et al.* 1997; Hutchison *et al.* 2003; Hutchison *et al.* 2004).

The major parameters which influence egg washing are: water temperature, wash water quality and mineral content, wash chemicals, pH of wash water and the use of brushes and jets (Hutchison *et al.* 2003).

Water temperature and washing time

Water temperature is important. A fundamental requirement is that the temperature of the water should exceed the temperature of the eggs being washed to prevent the set up of a pressure gradient which draws the bacteria through the shell into the interior (see also paragraph 4.3) (Hutchison *et al.* 2003). Brant and Starr (1962) concluded that the temperature of the wash water should be > 10°C higher than the egg temperature.

Studies have shown that increasing the water temperature has beneficial effects upon the inactivation of microbes. Leclair *et al.* (1994) found the inactivation (> 4 log reduction) of *Salmonella* Typhimurium and *L. monocytogenes* significantly affected by increasing wash water temperature from 38 to 46°C. Bartlett *et al.* (1993) reported that in the presence of wash chemicals, there was an inverse correlation ($r^2 > 0.65$) between temperature of egg wash water and the total counts that the water contained. Hutchison *et al.* (2004) recently demonstrated that for a spray-jet washer the temperature of water was the most important parameter for inactivating micro-organisms on the eggshell and for preventing the ingress of *Salmonella* spp. into the egg. Work by Lucore *et al.* (1997) has questioned the traditionally held view that washing in cold water represents a high risk. They used a spray wash system (short treatment: 10 s washing and 3 s rinsing) to compare the effect of three wash water temperatures (15.5, 32.2 and 48.9°C) upon internal and external shell surface bacterial counts. They concluded that spray washing of eggs at lowest temperature did not increase internal shell bacterial counts. An additional consideration is that as wash water temperature rises, there is an increased risk of cuticle damage and thermal cracking. For this reason Wesley and Beane (1967) recommended that wash water temperatures above 45°C should be avoided.

Brant and Starr (1962) and Hutchison *et al.* (2004) concluded that treatment time was relatively unimportant in terms of bacterial contaminants on the eggshell or in the egg contents; treatment time should be determined by considerations of shell cleanliness.

Water quality, mineral content and pH

Egg wash water must be of a standard equivalent to potable water (Hutchison *et al.* 2003). Due to the role of iron in the unhindered growth of bacteria (see also paragraph 2.5) water

used to wash eggs should have an iron level of < 2 ppm (Garibaldi and Bayne 1960; Garibaldi and Bayne 1962). Egg washing water is normally high in pH (9 to 11), due to chemicals used for washing. However, successful trials have also been carried out using acid chemicals such as peracetic acid, with pH 5 (Anon. 2005b). Bartlett *et al.* (1993) found a strong relationship between high pH (≥ 10.5) and low counts of total aerobic bacteria in wash water sampled in commercial facilities. Jones *et al.* (1995) found *Salmonella* Heidelberg on the shells of eggs washed under commercial conditions when the pH of wash water fell below pH 10.2.

Wash chemicals

Although chemicals may reduce the bacterial load on eggshells, they may damage the cuticle or shell, rendering the egg more vulnerable to subsequent microbiological invasion. Moats (1978) concluded, in a review of egg washing, that eggs washed with sanitising chemical in wash water invariable spoiled less eggs than eggs washed in water alone. Favier *et al.* (2000a) compared how the survival of mesophilic aerobic bacteria and *Yersinia enterocolitica* was influenced by the use of hypochlorite, lactic or acetic acid in wash water. Highest reductions of mesophilic aerobic bacteria were 1.28 and 2.15 log with 100 and 200 mg/l of chlorine, 0.28 and 0.36 log with 1% and 3% acetic acid, and 0.70 and 0.71 log with 1% and 3% lactic acid, respectively. On *Y. enterocolitica* inoculated eggs, reductions ranged from 2.47 to 2.92 log for previously mentioned treatments. Jones *et al.* (2004) studied the effect of a commercial dual-tank washer (quaternary ammonium compound detergent and 200 ppm chlorine sanitizer) on the natural eggshell contamination. Aerobic counts of washed eggs decreased with 2 log CFU/ml; respectively from approx 4 log CFU/ml to approx 2 log CFU/ml. For yeast and moults a reduction ($P < 0.0001$) from 1.5 log CFU/ml to < 0.3 log CFU/ml was obtained; also a significant reduction ($P < 0.05$) of *Enterobacteriaceae* was found. Finally no increase in population levels of total aerobes or yeast and moulds in the egg contents of washed eggs was found throughout a storage period of 10 weeks. Soljour *et al.* (2004) evaluated the efficacy of three commercial cleaning and sanitizing compounds (sodium carbonate, sodium hypochlorite, and potassium hydroxide) for bactericidal activity at pH values of 10, 11, and 12 against various concentrations (10^2, 10^4, or 10^6 CFU/ml) of *Salmonella* Enteritidis inoculated onto the eggshell surface. None of the chemicals applied at the recommended manufacturer's concentrations (sodium carbonate, 36 ppm; other treatments, 200 ppm) could completely eliminate *Salmonella* Enteritidis from eggshells artificially contaminated with the highest concentrations (10^4 or 10^6 CFU/ml). Higher concentrations (at least 5 to 20 times

greater than recommended doses) were needed to destroy the bacteria on egg surfaces. However, at or slightly above manufacturer's recommended use concentrations, all three formulations were effective against *Salmonella* Enteritidis in aqueous suspension (10^8 CFU/ml). Inactivation occurred at lower concentrations at pH 12 than at pH 11 and pH 10. Recently Hutchison *et al.* (2004) described the effects of spray jet washing under various commercial processing conditions to shell surface counts of *Salmonella* and the presence of bacteria in egg contents. In the experiments used eggs were artificially contaminated with *Salmonella* Enteritidis and *Salmonella* Typhimurium before cuticle hardening. Washing of contaminated eggs under optimum conditions resulted in a more than 5-log reduction of *Salmonella* counts from the shell surface. *Salmonella* was not isolated from the yolk or albumen of any egg washed by the optimal protocol. However, contamination did arise if strict control was not maintained over the wash and rinse water temperature. Both pathogens entered the egg content when wash temperatures were lowered.

Wang and Slavik (1998) using scanning electron microscopy reported that washing with sodium carbonate severely damaged the cuticle while washing in 100 ppm sodium hypochlorite did not. Eggs washed with sodium hypochlorite and then inoculated with *Salmonella* Enteritidis were penetrated for 16,7% compared to 76,7% penetration of eggs washed with sodium carbonate. Washing with quaternary ammonium also appeared to preserve the cuticle but residues of the compound remained on the shell after washing and drying. Favier *et al.* (2000b) studied the efficacy of different surfactants and their effect on the shell microstructure. The Tergitol/100 ppm chlorine combination caused the most marked alterations of the eggshell microstructure in contrast with only 100 ppm chlorine which caused the least change.

Musgrove *et al.* (2004) studied the persistence of *Enterobacteriaceae* species during egg washing operations in three commercial shell washing facilities in the US. Genera that persisted on eggshells following washing operations included *Aeromonas, Citrobacter, Enterobacter, Escherichia, Klebsiella, Listonella, Providencia* and *Vibrio* (see also Table 1.8 in paragraph 5.1).

Use of brushes and jets

Apart from damage to eggs caused by chemicals, there is also the possibility that the cuticle and shell may be eroded or damaged by the physical action of brushes. High pressure jets of water and sanitizers remove the risk of cross contamination that is associated with brushes

and prove to clean eggs effectively. However, further work is required to confirm that at the high pressure there is no risk of shell damage or wash water being forced through the pores in the shell potentially causing contamination of the egg contents. (Hutchison *et al.* 2003)

Drying of washed eggs

After eggs have been washed, they must be promptly and thoroughly dried prior to packing. If eggs are still wet when they are packed, then there is an increased risk of muold growth. Bacteria may also be drawn into the egg through the shell as it cools (see also paragraph 4.3).

Oiling of washed eggs

In the US, it has been estimated that 50% of eggs are oiled after washing. The practice is adopted mainly in warmer regions of the country where there is a risk of inadequate refrigeration or if the eggs are destined for export. It is not considered necessary when eggs were distributed using refrigeration conditions (< 12°C) and likely to be consumed quickly (Hutchison *et al.* 2003). It has been reported (Ball *et al.* 1976) that shells of eggs oiled after washing are physically stronger than those of un-oiled eggs. However the main benefit is a reduction in the rate of decline of internal egg quality by reducing the rate of water and carbon dioxide loss from the egg and possibly also inhibiting entry of micro-organisms.

8.3 Balancing advantages and disadvantages of washing shell eggs

It has been demonstrated that egg washing can reduce the number of micro-organisms on the shell of the egg. However it can, under certain circumstances, cause food poisoning and spoilage organisms to be moved from the surface of the shell into the contents of the egg. The egg washing machines must be equipped with comprehensive control systems which ensure that key operating parameters (*e.g.* water temperature, pH, detergent levels) are constantly met. The full advantages of egg washing can also only be obtained if all eggs are visually or mechanically evaluated prior to washing and unsuitable eggs (e.g. cracked, corrugated eggs) are removed. Therefore ultraviolet irradiation could be a more favourable alternative for decontamination and is test out and discussed in chapter 5.

Chapter 2

The use of total aerobic and Gram-negative flora for quality assurance in the production chain of consumption eggs

Redrafted after:

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CHAPTER 2: The use of total aerobic and Gram-negative flora for quality assurance in the production chain of consumption eggs

Abstract

Washing eggs in sterile plastic bags with diluent is an efficient sample preparation method for the determination of the bacterial contamination on eggshells. Total count of aerobic and Gram-negative bacteria on the eggshell can be used to detect critical points for contamination in the egg production chain. The number of eggs to be sampled at a point of the production chain was determined on a statistical basis and fixed on 40 for non-graded eggs and on 20 for graded eggs. In two production chains, one cage production and one organic production system, critical points for contamination were identified. The most critical point for the cage production system was a short conveyor belt at the entrance of the candling, grading and packaging area, for the organic production system it was the initial contamination at the nest boxes. With the exception of heavily soiled shells, like shells from eggs collected from the ground (ground eggs), there is a poor correlation between the level of bacterial contamination and the visual eggshell contamination. A positive correlation was found between the initial bacterial eggshell contamination and the concentration of bacteria in the air of the poultry houses.

1 INTRODUCTION

In literature few data are published about the bacterial contamination on the shell of consumption eggs. Data available concern mostly research on hatching eggs because trans-shell contamination of hatching eggs may reduce hatchability (Quarles *et al.* 1970). The extent of contamination of hatching eggs was reported by Board and Tranter (1995) with a variation ranging from 10^2 up to 10^7 CFU for individual eggshells. In egg washing experiments Knape *et al.* (2002), Favier *et al*. (2000a), Knape *et al.* (1999) and Lucore *et al.* (1997) reported an average initial eggshell contamination of respectively 6.33, 4.55, 3.86 and 5.10 log CFU/eggshell.

The shell can already be infected when passing through the vent, but many researchers suggest that the main contamination occurs within a short period after laying due to contact with dirty surfaces (Harry 1963; Board *et al.* 1964; Quarles *et al.* 1970; Gentry and Quarles 1972).

External eggshell contamination could be important for the shelf life and the food safety of consumption eggs and egg products. It is hypothesized that bacterial contamination of the internal egg content could be the result of the penetration of the shell by bacteria deposited on the surface of the egg after it has been laid (Haines 1938; Harry 1963; Schoeni *et al.* 1995). Smith *et al*. (2000) also reported that increasing excreta moisture gave a linear increase ($P < 0.001$) in numbers of micro-organisms on the eggshell and consequently increase the risk of microbial contamination of the internal contents of ostensibly clean eggs.

In this chapter the development of a methodology to quantify the bacterial contamination on the eggshell and to detect critical points of contamination in the entire production chain is discussed. Different methods for the recuperation of the bacteria from the eggshell are published. Haines (1938) and Board *et al.* (1964) crushed the shell together with membranes in a sterile plastic bag with diluent after removal of the egg content. Gentry and Quarles. (1972) and Pienaar *et al.* (1995) washed the intact eggs in a sterile plastic bag by rubbing. Sacco *et al.* (1989) swabbed a part of the eggshell. Knape *et al.* (2002) placed an intact egg into a sterile plastic bag containing 50 ml Phosphate Buffered Saline (PBS) that was serial diluted immediately. Pienaar *et al*. (1995) used a method based on optical density to determine bacterial contamination on hatching eggs. The mentioned researchers used different counting media. In this chapter the comparison of the different recuperation methods and the optimisation of one method are discussed. The total count of aerobic bacteria and Gram-

negative bacteria were determined and used to determine the bacterial contamination on the shell of consumption eggs through the production chain. Based on the level and the variation of the bacterial contamination on the eggshell a sampling method for the detection of the critical points for contamination was developed.

2 MATERIALS AND METHODS

2.1 Determination of bacterial eggshell contamination

For the recuperation of bacteria from the eggshell different methods were compared. One method concerned removal of the egg content and crushing of the shell and shell membranes in a sterile plastic bag with 10 ml 1/4 Ringers solution (Ringers Solution, Oxoid, Basingstoke, UK) for 2 times 1 min with an interval of 5 min rest in between (Haines 1938). Another method considered the washing of intact eggs in a sterile plastic bag with 10 ml diluent. The bag was held at an angle with the egg and the diluent in the corner. The washing of the egg was done by rubbing the eggshell through the bag (Gentry and Quarles 1972) (Figure 2.1) or by placing the bag with the egg in an ultrasonic bath (Bransonic 2200, The Netherlands). For both methods this was done for different time intervals: (1) 2 times 1 min washing with in between an interval of 5 min resting, (2) 2 times 30 s washing with in between an interval of 2.5 min resting and (3) 1 min washing. Each washing method was followed by plating out of the diluent. Buffered Peptone Water (BPW, Oxoid) and Phosphate Buffered Saline (PBS, Oxoid) were used as diluents. In a third method the half egg was swabbed with a swab moistened with 1/4 Ringers solution (Oxoid) and soaked off in 10 ml 1/4 Ringers solution (Oxoid).

Figure 2.1: Recuperation of the bacteria from the eggshell by washing the egg by means of rubbing the eggshell through the bag.

The total count of aerobic bacteria determined on Nutrient Agar (NA, Oxoid), Tryptone Soya Agar supplemented with 0.6% Yeast Extract (TSA and YE, Oxoid), Brain Heart Infusion Agar (BHI, Oxoid) and Plate Count Agar (PCA, Oxoid). The incubation temperature/time combinations of 3 days at 37°C, 3 days at 30°C, 5 days at 25°C and 10 days at 10°C were studied on 4 times 20 eggs. NA (Oxoid) with 0,0001% crystal violet (VWR, Darmstadt, Germany) was used for counting Gram-negative aerobic bacteria (Mossel and Jacobs-Reitsma 1990). The spiral inoculation method (Eddy Jet, IUL Instruments, Barcelona) was used. The eggs used for the method evaluation were cage production eggs sampled at sales-outlets.

2.2 Statistical analysis of data

The bacterial counts were log 10 transformed prior to statistical analysis (Jarvis 1989). Significant differences were assessed using an analysis of variance (ANOVA), done in Statistica 7.0 (Statsoft Inc., Tulsa, USA). The underlying assumptions for an ANOVA were verified: the homogeneity of variances using the Bartletts χ^2 test and the absence of a correlation between means and variances was checked on a plot. Post-hoc inter factor differences were calculated using Duncan's test (Kendall and Stewart 1968).

2.3 Sampling, collection and transport of eggs

In the points of the production chain before packaging, eggs were picked up with the fingertips and placed in new carton trays. Between each sampling point the fingertips were disinfected. In the points after packaging closed cartons (first category eggs) or carton trays (second category eggs) filled with eggs were sampled. First category eggs had a normal, clean and undamaged shell; second category eggs did not meet these requirements (see also chapter 1, paragraph 7). The eggs were brought by car, in ambient conditions, to the laboratory were they were kept for maximum 56 h in ambient conditions before analysing. Our sampling method was compared with the method used by Gentry and Quarles (1972) who collected the eggs with sterilized metal tongs and also filled in egg cartons. Therefore a batch of 40 eggs was sampled at a sales-outlet; 20 eggs were sampled with sterilized metal tongs while the other 20 eggs were picked up by hand (fingertips). For both sampling methods the total count of aerobic and Gram-negative bacteria on the eggshell was determined. As the eggs were analysed during a period of 56 h after sampling, the influence of 56 h storage at ambient conditions was evaluated. From a batch of 40 caged eggs sampled at a sales-outlet, 20 eggs were analysed within 2 h after sampling while the other 20 eggs were analysed after 56 h storage at ambient conditions in the laboratory. In both cases the total count of aerobic and Gram-negative bacteria on the eggshell was determined.

2.4 Influence of time, temperature and atmospheric humidity on the bacterial shell contamination

A batch of 80 eggs from the same caged production was sampled in a sales-outlet. Twenty eggs were analysed immediately, 2 times 20 eggs were analysed after being kept at room temperature with an average atmospheric humidity (RH) of 50% for 7 and 14 days, respectively, and 20 eggs were analysed after 14 days storage in a refrigerator at 5°C with an average RH of 85%. The total count of aerobic and Gram-negative bacteria on the eggshell were determined.

2.5 Number of samples

To produce statistically reliable results, a minimum number of eggs need to be sampled at a certain point in the production chain. The minimum number of samples can then be found as the number of samples from which the standard error on the average total count of aerobic bacteria of a batch of eggs starts converging to an asymptotic value (Grijspeerdt and

Verstraete 1997). To obtain an even larger variation in the bacteriological contamination present at one point of the production chain, a batch of non-graded eggs from three successive points from the hen house up to the candling was sampled.

2.6 Sampling through the production systems

Cage production

The caged layer house contained the brown-shell breed ISA Brown. The farm housed a total of 153 600 hens in 4 adjoining hen houses connected by a large corridor. The cage arrangement consisted of four-storey cages, holding 6 layers per cage. The cage rows were mounted directly above one another with a dropping board in between. The eggs of one hen house (38 400 hens) were followed through the production chain. Eighteen-week-old layers were transferred to the hen house and sampling of the eggs was done when hens were 30 weeks old. The eggs were gathered from each row of roll-out cages to a cross conveyor which took them to a lift cage which transported the eggs from the hen house to the corridor. In the corridor a second conveyor belt assembled the eggs and brought them to the entrance of the candling, grading and packaging area. A short conveyor with metal grid brought the eggs from the conveyor of the corridor to the conveyor of the candling booth. The eggs were graded and packaged automatically after visual evaluation in a candling booth ECM 1200 Staalkat (Staalkat International B.V., The Netherlands). First category eggs were packed in closed cartons; second category eggs in carton trays. The same evening the first category eggs, after being stored at ambient conditions, were loaded into a refrigerated lorry (6-8°C), which brought them the next morning to the cooled warehouse (6-8°C) of a food chain's distribution centre. The same day a refrigerated lorry (6-8°C) from the food chain brought the packaged eggs to the refrigerated storage area (6-8°C) of the local food shop. The eggs were kept there for four days and then replenished in the shop racks in ambient conditions.

At 10 points in the production chain samples were taken:

1) in the hen house at the conveyor belts;
2) on the large conveyor belt of the corridor which connects the conveyors of each hen house;
3) at the entrance of the candling, grading and packing area where a short conveyor with metal grid brought the eggs from the conveyor of the corridor to the conveyor of the candling booth;
4) in the candling booth;

5) first category consumption eggs immediately after packaging in closed cartons;
6) second category eggs packaged in open carton trays;
7) first category consumption eggs in closed cartons at the refrigerated lorry at the hen house;
8) first category consumption eggs in closed cartons at the cooled warehouse of the food chain's distribution centre;
9) first category consumption eggs in closed cartons at the refrigerated storage of the local shop;
10) first category consumption eggs in closed cartons at the shop rack of the local shop.

Samples 8-10 were taken respectively 1, 1 and 5 days after egg laying. In chapter 1 paragraph 7, especially in Figure 1.17, an egg production chain is also visualized.

Organic production

The organic production unit housed 5 000 brown-shell breed Bovans Goldline hens. It was an aviary hen house of 700 m^2 with 240 roll-out nest boxes on the side wall, 18 cm roosts per bird, 600 m^2 open space in the hen house, with 450 m^2 open-air free range with concrete floor next to the hen house and free range in grasslands. Twenty-two-week-old layers were transferred to the hen house and the sampling of the eggs was done at the hen age of 39 weeks. The eggs from the roll-out nest boxes (45 x 45cm, with Astroturf® mat) were gathered in front of the boxes on a cross conveyor with cover. The conveyor belt transported the eggs from the hen house directly to a small collecting area where the eggs were visually evaluated and collected by hand in open carton trays. From the collecting area the eggs were brought by van to the candling and packaging area, located in a building 100 m from the hen house. The eggs were visually evaluated in a candling booth (MOBA, The Netherlands) and first category eggs were automatically packaged in closed cartons. The next day the packaged eggs, after being stored at ambient conditions, were loaded into a refrigerated lorry (6-8°C), which brought them to the cooled warehouse (6-8°C) of a food chain's distribution centre. The same day a refrigerated lorry (6-8°C) from the food chain brought the packaged eggs to the refrigerated storage area (6-8°C) of the local food shop. Eggs were kept for four days in the local food shop and then replenished in the shop racks at ambient conditions.

Samples were taken at 7 points in the production chain:

1) in the hen house at the covered conveyor belt;
2) at the end of the conveyor belt at the collecting area;

3) at the candling and packing area just before handling;
4) first category consumption eggs immediately after packaging in closed cartons;
5) first category consumption eggs in closed cartons sampled at the refrigerated lorry at the hen house;
6) first category consumption eggs in closed cartons taken at the shop rack;
7) eggs collected from the ground (ground eggs from the hen house).

Samples 5 and 6 were taken respectively 1 and 5 days after egg laying.

2.7 Environmental conditions in the production chain

An Air Sampler RCS (Biotest AG, Dreieich, Germany) was used to determine total count of aerobic bacteria per m^3 air in each part of the production chain. Strips in the air sampler contained Nutrient Agar (Oxoid). Strips were incubated for 3 days at 30°C. Also temperature and RH (hear-hygrometer) were measured.

2.8 Visual examination of the shell eggs

Each egg was thoroughly evaluated visually and placed into one of the following five categories: clean, faeces and/or blood, egg white and/or egg yolk, dust and/or feathers, straw and/or earth and/or dirt (Anon. 1996). Cracked eggs, open or closed, were removed. The visual examination of the eggshell was performed using a candling light.

3 RESULTS AND DISCUSSION

3.1 Determination of bacterial eggshell contamination

For the determination of the total count of aerobic bacteria on eggshells, no statistical significant differences were found between the different counting media used (Figure 2.2).

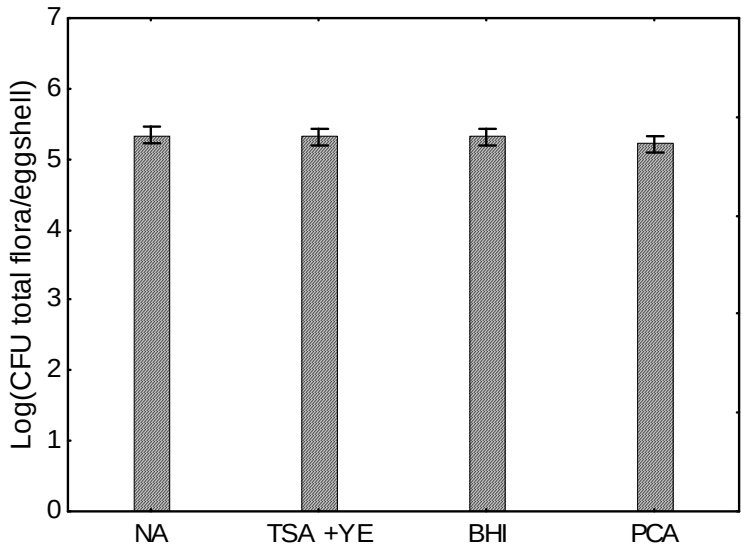

Figure 2.2: Methodology: Influence of the counting medium on the determination of the total count of aerobic bacteria. Vertical bars denote 95% confidence intervals (n = 160). (NA: Nutrient Agar; TSA+YE: Tryptone Soya Agar supplemented with Yeast Extract, BHI: Brain Heart Infusion Agar; PCA: Plate Count Agar)

Also no statistically significant differences were obtained between the studied temperature/time combinations of 3 days at 37°C, 3 days at 30°C and 5 days at 25°C while the combination 10 days at 10°C was slightly less sensitive. On the contrary, the sample preparation methods showed large statistically significant differences (Figure 2.3). The washing of intact eggs in PBS or BPW by rubbing 2 times 1 min with an interval of 5 min rest in between gave statistically significant higher ($P < 0.001$) counts than the two other sample recuperation methods. This washing method was also the most practical method. Crushing the shell and shell membranes ("Method 1" in Figure 2.3) gave similar results compared to swabbing half of the surface of the eggshell ("Method 4" in Figure 2.3). The swabbing method is comparable with the method used by Sacco *et al*. (1989) who swabbed a circular area with a diameter of 3 cm on the side of the egg. The lower recovery found by crushing the shell is probably because a thorough rubbing of the shell is not possible to avoid rupture of the plastic bag. Swabbing has on the other hand the disadvantage that not all bacteria are swabbed up and absorbed and/or recovered from the swab for counting.

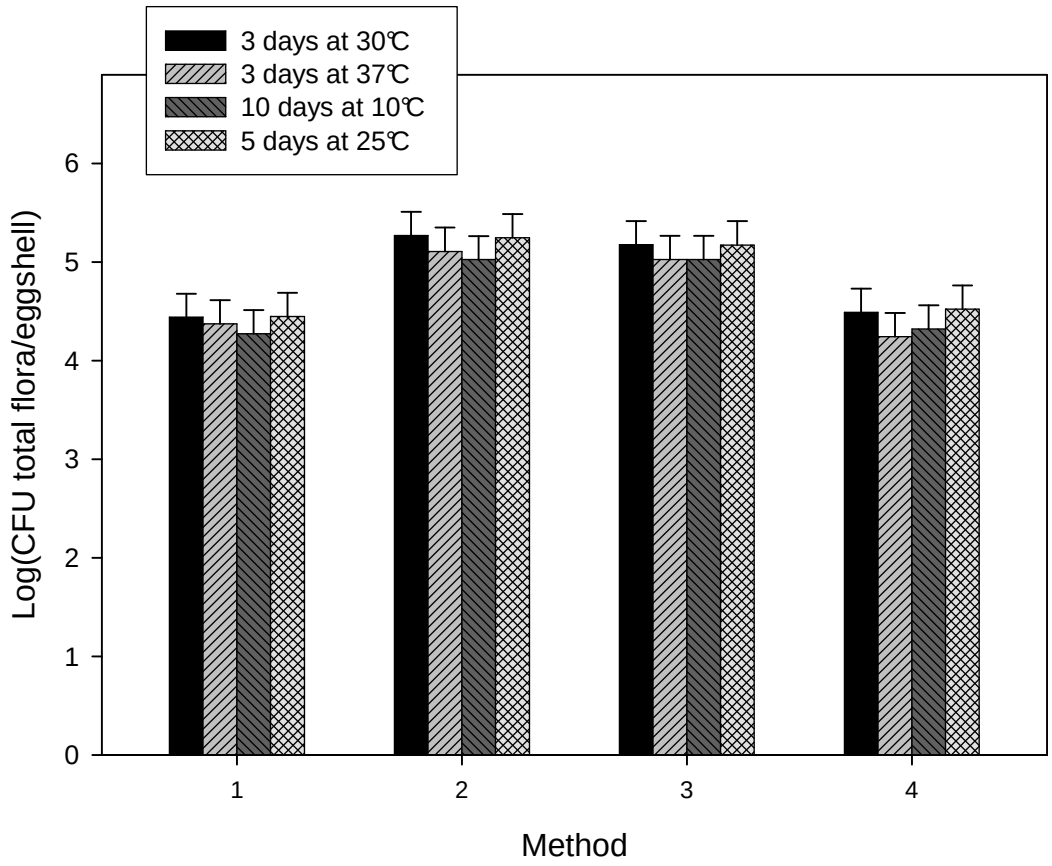

Figure 2.3: Methodology: Influence of the incubation temperature/time combinations and the sample preparations on the determination of the total count of aerobic bacteria using Nutrient Agar. Vertical bars denote 95% confidence intervals (n = 20). (Method 1: Removal of egg content and crushing of shell and shell membranes; Method 2: Washing – by rubbing - intact eggs with PBS; Method 3: Washing – by rubbing - intact egg with BPW; Method 4: Swabbing half egg)

As the washing procedure of intact eggs was very time-consuming, it was shortened to 2 times 30 seconds rubbing with an interval of 2.5 min rest in between and also to 1 min rubbing immediately followed by plating out, without significant loss of sensitivity compared to the original method. Although no statistical significant difference was observed, washing through 1 min rubbing was shown to give higher counts than treatment by 1 min in the ultrasonic bath (results not shown). To estimate the efficiency of the washing method by 1 min rubbing, a second washing of the same eggs was performed by the same method. The average counts on the second washing of 10 eggs never exceeded 2% of the original counts. To test the presence of bacteria in the pores of the eggshell, the eggshell was isolated and crushed to very small particles and analysed for total bacterial flora. The average count of 5 tests (eggs) did not exceed 3% of the original counts of the bacteria found on the eggshell. These results indicate that the plastic bag washing procedure is an efficient sample recuperation method for measuring the bacterial contamination on the eggshell.

Based on these results the following final method was used for application in further experiments (also in next chapters): the egg was placed in a plastic bag with 10 ml PBS (Oxoid) and the egg was rubbed through the bag for 1 min. The diluent was plated by a spiral-enter on NA (Oxoid) for the determination of the total count of aerobic bacteria (detection limit 100 CFU/eggshell) and on Nutrient Agar with 0,0001% crystal violet (VWR) for the total count of Gram-negative bacteria (detection limit 33 CFU/eggshell). Plates were incubated for 3 days at 30°C.

3.2 Sampling, collection and transport of eggs

Collecting the eggs by hand did not influence the results significantly compared to the method of Gentry and Quarles (1972) using sterilized metal tongs. The 20 eggs picked up by hand (fingertips) had an average total count of aerobic and Gram-negative bacteria on the eggshell of respectively 5.24 and 3.60 log CFU/eggshell while the other 20 eggs sampled with sterilized metal tongs had respectively average counts of 5.33 and 3.70 log CFU/eggshell.
As the eggs sampled in the production chains were analysed during a period of 56 h after sampling, the influence of 56 h storage at ambient conditions was evaluated. The average total count of aerobic and Gram-negative bacteria of the 20 eggs analysed within 2 h after sampling deviated respectively less than 0.01 and 0.04 log CFU/eggshell compared to the other 20 eggs of the same batch analysed after 56 h storage at ambient conditions in the laboratory. Also Haines (1938) reported no significant difference in the total bacterial flora on the egg between eggs examined immediately and after keeping for 4 days at room temperature.

3.3 Influence of storage time, temperature and atmospheric humidity on the bacterial shell contamination

The study on the influence of time, temperature and atmospheric humidity on the bacterial shell contamination showed that the total count of aerobic bacteria decreased (not statistically significant) during the storage time of 14 days; neither at room temperature and a RH of approx 50% (from 5.44 to 5.22 log CFU/eggshell) nor at refrigerator temperature (5°C) and a RH of approx 85% (from 5.44 to 5.33 log CFU/eggshell). Gentry and Quarles (1972) reported no marked differences in viable counts after 1 day storage of the freshly laid eggs at 4°C. Contrary to the total count of aerobic bacteria, the total count of Gram-negative bacteria decreased statistically significantly ($P < 0.001$) at room environment (from 4.04 to 3.23 log

CFU/eggshell) but not at refrigeration environment (from 4.04 to 3.66 log CFU/eggshell; P = 0.59). This was probably due to the lower humidity at room temperature.

3.4 Number of samples

The standard error on the average total count of aerobic bacteria of a batch of non-graded eggs from the layer house up to the candling booth converged to its final value after about 35 eggs (Figure 2.4). Consequently, the minimum number of samples to be taken was set at a safe value of 40 for non-graded eggs. Following the same procedure, the required number of samples was set at 20 for a batch graded eggs from the shop rack.

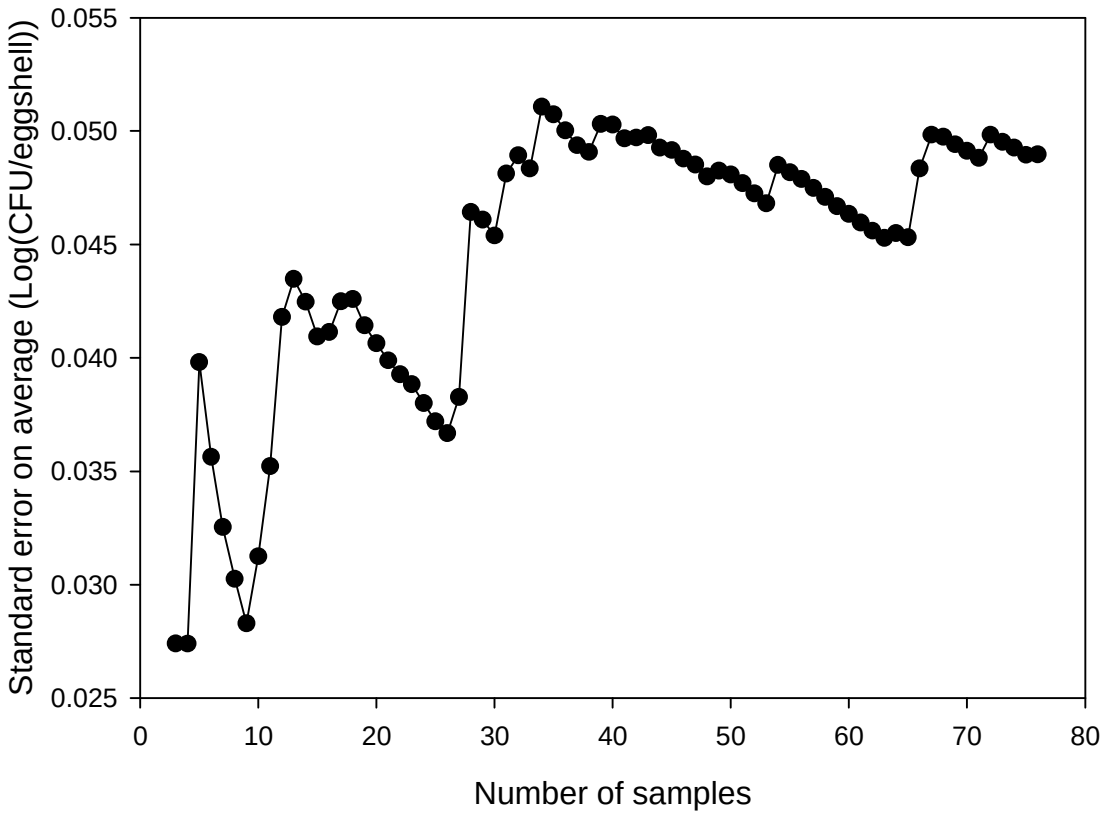

Figure 2.4: Standard error on the average total count of aerobic bacteria of a batch eggs sampled from the hen house up to the candling.

3.5 Sampling through the production chain

Cage production

Figure 2.5 shows an increase in total count of aerobic and Gram-negative bacteria, at the moment the eggs enter the candling, grading and packing area ('3. Entrance packaging area' in Figure 2.5). For both parameters the increase was statistically significant ($P < 0.001$). This point in the production chain was indicated as a critical point for increase of bacterial eggshell contamination. Here all eggs passed the same small surface, a short conveyor with metal grid. Visual examination of the shell could not be used to detect this critical point for contamination as 60% of the eggs sampled at the corridor showed visual contamination, while for the eggs sampled at the critical point it concerned only 45%. The rolling of all eggs on the same short surface caused cross-contamination due to eggshell dirt and broken egg content.

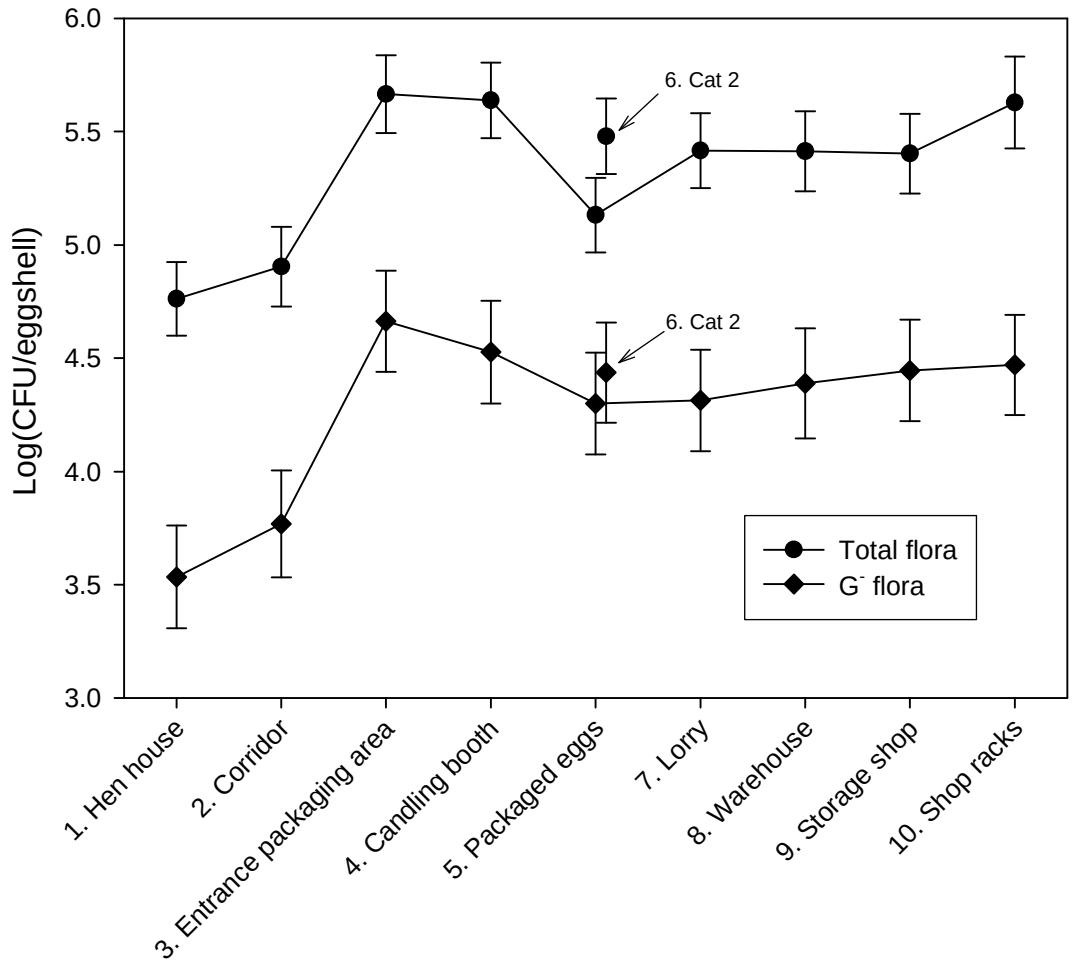

Figure 2.5: Total count of aerobic (Total flora) and Gram-negative (G^- flora) flora at the different points of the caged production chain ($n = 40$). Vertical bars denote 95% confidence intervals. (Cat 2: Second category eggs)

The air contamination was lower in the candling, grading and packaging area (3.8 log CFU/m^3 air) compared to the corridor (4.1 log CFU/m^3 air) (Table 2.1), which confirmed that the significant increase of shell contamination was due to contact with the metal grid.

Also a difference in bacterial contamination of first (point 5) and second category (point 6) eggs was shown immediately after packaging. This difference was only limited statistically significant ($P < 0.05$) despite the visual contamination in a limited degree of 97.5% of the second category eggs compared to 23.8% of the first category eggs. The three visual contamination categories faeces and/or blood, egg white and/or egg yolk and dust and/or feathers were respectively present on 77.5, 50.0 and 20.0% of the second category eggs compared to 7.5, 20.0 and 2.5% on first category eggs.

From the critical point, the short conveyor, to the end of the production chain, at the shop rack, the total aerobic and Gram-negative flora on first category consumption eggs remained at a constant level. The moment of sampling at the shop rack, the eggs were already 5 days laid. First category eggs just after packaging (‘5. Packaged eggs’ in Figure 2.5) showed a lower contamination compared to the two previous and the four following points in the chain; yet the decrease was only limited significant ($P < 0.05$). Moreover, at this stage the total count of Gram-negative bacteria was not significantly lower compared to the two previous and the four following points.

The total count of Gram-negative bacteria was approx 1 log CFU/eggshell lower on average compared to the total aerobic flora, indicating that Gram-positive bacteria dominated the flora on eggshells, probably because of their higher tolerance to dry conditions. Mayes and Takeballi (1983) and Board and Tranter (1995) also found Grampositive bacteria dominating the eggshell.

The average bacterial contamination of minimum 40 eggs sampled at the hen house and placed in the 4 categories; clean, faeces and/or blood, egg white and/or egg yolk and dust and/or feathers, was respectively 5.04 (stdev 0.57), 5.11 (stdev 0.54), 5.20 (stdev 0.69) and 5.15 (stdev 0.52) log CFU/eggshell for total aerobic count and 3.71 (stdev 0.89), 3.58 (stdev 0.74), 3.80 (stdev 1.02) and 3.65 (stdev 0.81) log CFU/eggshell for Gram-negative count. These differences were not statistically significant. So, in the sampled cage production, no correlation between the level of contamination and the appearance of the shell was shown which means that the bacterial contamination of the shell could not be judged by evaluation of the visual shell contamination. This is in agreement with Board and Tranter (1995), who reported, that with the exception of heavily soiled shells, there is a poor correlation between the level of contamination and the appearance of the shell.

Table 2.1: Total count of airborne flora (aerobic bacteria) per production system (log CFU/m^3 air)

	Hen house	Corridor	Collecting area	Packaging area	Ref. lorry	Ref. warehouse	Ref. storage shop	Shop racks
Cage	4.4	4.1	n.p.	3.8	3.7	2.9	2.9	3.2
Organic	5.6	n.p.	5.5	4.3	4.3	2.8	2.4	2.9

Ref. = refrigerated; n.p. = not present

Organic production

Compared to the caged layer house, the bacterial eggshell contamination through the organic chain showed fewer fluctuations (Figure 2.6).

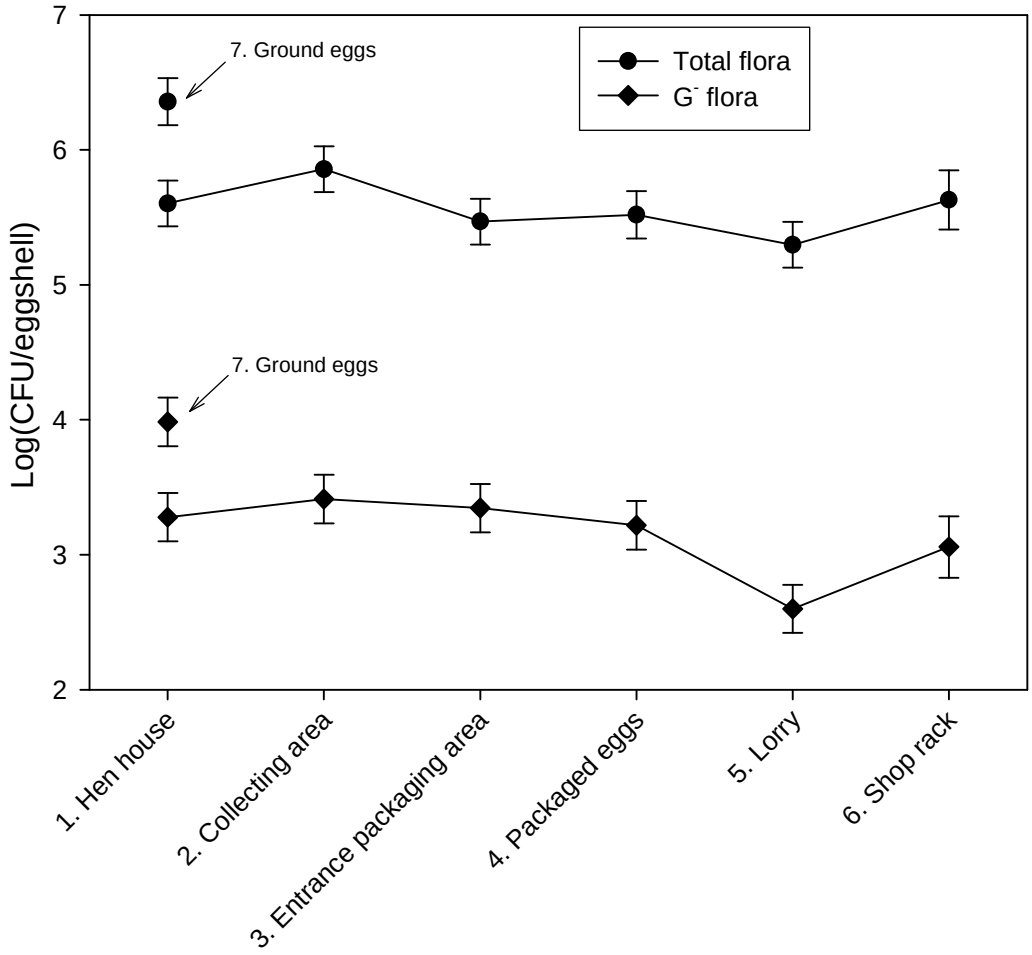

Figure 2.6: Total count of aerobic (Total flora) and Gram-negative (G^- flora) flora at the different points of the organic production chain (n = 40). Vertical bars denote 95% confidence intervals. (7. Ground eggs: Eggs collected from the ground)

The initial contamination with aerobic bacteria (5.8 log CFU/eggshell) of the eggs from the covered conveyor belt of the nest boxes was 1 log higher compared to the eggs on the conveyor belt next to the cages of the caged production. This raise of initial contamination makes the nest boxes in the organic housing system a critical point for bacterial eggshell contamination. The higher initial contamination was also reflected in the air where a much higher contamination (5.6 log CFU/m^3 air) was measured compared to the caged stable (4.4 log CFU/m^3 air) (see Table 2.1). Harry (1963) and Quarles *et al.* (1970) also reported correlations between initial eggshell contamination and the concentration of bacteria in the air of the hen house. Table 2.1 shows that the air contamination in the hen house, collecting area and packaging area of the organic production was higher compared to the comparable points; hen house, corridor and packaging area, of the caged production. On the other hand the average total count of Gram-negative bacteria through the entire organic chain was more than 1 log and at the end of the chain even more than 1.4 log CFU/eggshell lower compared to the eggs from the caged layer house. Possibly the higher initial contamination of the organic eggs with Gram-positive bacteria oppressed the adhesion of Gram-negative bacteria. ANOVA testing revealed a statistically significant ($P < 0.001$) lower contamination in Gram-negative flora for the eggs available at the lorry compared to those sampled directly after packaging and in the shop. This decrease was not found for the total count of aerobic bacteria.

Contrary to the second category eggs of the caged layer house, the contamination of eggs collected from the ground (ground eggs) was significantly higher for both parameters compared to the contamination of eggs at other points in the chain. A comparable amount of eggs were visually contaminated, but ground eggs in a much higher degree. Only 5% clean eggs collected from the ground were present and besides the three visual contamination categories faeces and/or blood (82.5%), egg white and/or egg yolk (12.5%) and dust and/or feathers (32.5%) also a fourth category straw and/or earth and/or dirt, not present in the cage production, was found on 37.5% of the organic eggs. Gentry *et al.* (1972) also found significant differences in bacterial counts from eggs classified as clean, soiled and dirty; approximately 3.5, 4.5 and 5.5 log CFU/eggshell, respectively. As the eggs collected from the ground were heavily soiled, this confirmed the report of Board and Tranter (1995) that mentioned that only for heavily soiled shells a correlation exists between the level of bacterial contamination and the appearance of the shell.

Despite the clear difference in critical points for bacterial contamination, the total bacterial count on the eggshell for the 2 production systems was comparable at the end of the chain. It

can therefore be hypothesised that eggshells of consumption eggs reach a maximum of bacterial contamination of about 6 log CFU/eggshell.

4 CONCLUSIONS

In this chapter a concept for sampling of eggs in the production chain was evaluated and a methodology to recover and count the bacterial eggshell contamination was optimized. Washing eggs in plastic bags with diluent and by rubbing is an efficient sample preparation method. The minimum number of eggs to be sampled at a certain point in the production chain was determined and set on 40 for non-graded eggs and on 20 for graded eggs. The concept was used in a preliminary study on the evolution of the bacterial eggshell contamination and the detection of critical points for introducing eggshell contamination in two production chains. The study in chapter 2 also showed that bacterial contamination of the eggshell can not be judged by evaluation of the visual eggshell contamination.

In the next two chapters the above described methodology will be used to study in detail the initial bacterial shell contamination from eggs from different experimental housing systems (chapter 3), and to study thorough the progress of the bacterial eggshell contamination and identification of critical points for introducing bacterial contamination in more production chains of different commercial housing systems (chapter 4).

Chapter 3

Influence of the housing system for laying hens on the initial bacterial eggshell contamination

Redrafted after:

Bacterial eggshell contamination in conventional cages, furnished cages and aviary housing systems for laying hens

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British Poultry Science, 46, 149 - 155

CHAPTER 3: Influence of the housing system for laying hens on the initial bacterial eggshell contamination

Abstract

The influence of the housing system on the initial bacterial contamination of the eggshell was studied. Two long-term experiments were performed. The bacterial eggshell contamination, as expressed by total count of aerobic and Gram-negative bacteria, was periodically analyzed for eggs from a conventional cage, a furnished cage with nest boxes containing artificial turf or grids as nest-floor material and an aviary housing system. For these experiments no systematic differences were found between the conventional cage and furnished cage. The type of nest-floor material in the nest boxes of the furnished cages also did not systematically influence the bacterial shell contamination. A possible seasonal influence on the eggshell contamination with a decrease in the winter period (up to > 0.5 log CFU/eggshell) of total count of aerobic and Gram-negative bacteria was observed in the first experiment. The contamination with total aerobic flora was higher (> 1.0 log) on eggs derived from the aviary housing system compared to the conventional and the furnished cage systems. For Gram-negative bacteria this was not the case. During the entire period of both experiments, independent of the housing system, shell contamination was not influenced by age of hens or period since placing the birds in the houses. For the total count of aerobic bacteria a positive correlation ($r^2 = 0.66$, $P < 0.001$) was found between the concentration of total bacteria in the air of the poultry houses and the initial bacterial eggshell contamination.

1 INTRODUCTION

It is estimated that 70 to 80% of the world egg production is derived from conventional caged laying hens. These cages offer the advantages of low production costs and high standards of hygiene, but due to bird welfare considerations there are calls for cages to be banned (Walker *et al.* 2001). In 1999 the European Commission passed a directive 99/74/EC (Anon. 1999) requiring that conventional cages should not be used as a new investment from 2003 and must be banned from 2012 in the European Union. Alternatives such as furnished cages, aviary systems and perchery systems have been proposed (see also chapter 1, paragraph 6). While the conventional cage provides approx 450 cm^2 cage area and 10 cm trough length for each hen, furnished cages provide at least 750 cm^2 per hen, a nest box, a dust bath and 15 cm perch per bird. Aviary systems provide platforms of slats at different heights, litter area on the ground and nest boxes. The perchery system also uses the vertical space of houses like the aviary system but rather by perches than by platforms. During a transitional period from 2003 to 2012 the usable area in conventional cages has to be increased from 450 cm^2 to 550 cm^2. The alternatives for the conventional cages have been evaluated both commercially and by researchers in terms of productivity and bird welfare (Abrahamsson and Tauson 1995; Tauson *et al.* 1999; Tauson 2002; Wall *et al.* 2002).

Little attention was given to the differences in bacterial eggshell contamination, although this may be important for the shelf life and the safety of eggs and egg products. Bacterial contamination of the internal egg content could be the result of the penetration of the shell by bacteria deposited on the surface of the egg after it has been laid (Harry 1963; Quarles *et al.* 1970; Schoeni *et al.* 1995). In early studies bacterial shell contamination has been compared in litter and wire floor houses. Quarles *et al.* (1970) reported litter floor houses had on average approximately 9 times more bacteria in the air, and 20 to 30 times more aerobic bacteria on the shell than wire floor houses. Harry (1963) reported that the shells of deep litter eggs had on average 15 times more bacteria and a higher proportion of potential spoilage organisms than did battery eggs. More recently, Ellen *et al.* (2000) reported that dust concentrations in the air were lowest in cage systems and up to 4 or 5 times higher in other systems, such as percheries and aviaries. Micro-organisms, like bacteria, may represent only a minor percentage (< 1%) of the number of airborne particles (Pedersen *et al.* 2000), but have a marked negative effect on the health of the livestock and probably lead to higher bacterial contamination on the shell of aviary and perchery systems. In our study of chapter 2, higher

bacterial contamination in the air was indeed correlated with higher bacterial counts on the eggshell.

The objective of the study in this chapter was to compare the initial bacterial eggshell contamination in conventional cages, furnished cages and aviary housing systems build in experimental hen houses. The methods developed in chapter 2 to quantify the bacterial contamination on the eggshell were used.

2 MATERIALS AND METHODS

2.1 Housing

The different types of experimental housing systems were arranged in two separated identical buildings (1 and 2) with the same climate (temperature and atmospheric humidity), located side by side. Each building contained two hen houses (A and B) each 6.10 m wide and 34.00 m long, separated by a wall (see also paragraph 2.5).

2.2 Conventional cages

The conventional cage measured 50 × 51 × 43 cm (width × depth × height) with a floor slope of 7°. The 4-hen cages provided approx 640 cm^2 cage area per hen. The arrangement of conventional cages consisted of two rows of three-storey cages; housing laying hens at both sides (Figure 3.1 and 3.5). Each row contained 56 cages per floor at each side. In total 2 688 commercial Brown layers were housed per hen house. Food and water was available *ad libitum* by a feed trough and by nipple drinkers, manure was dried on a manure belt and removed at least once a week.

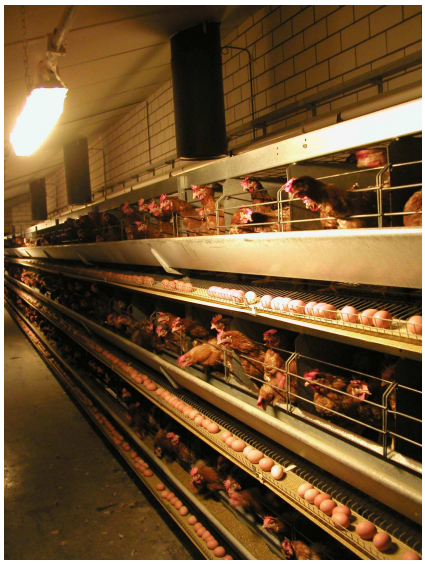

Figure 3.1: Arrangement of the studied conventional cages.

2.3 Furnished cages

Cages were of wire mesh with a floor slope of 7°, with galvanised metal partitions between cages and fully opening fronts consisting of widely spaced horizontal bars. The living area, containing 15 cm perch per hen, was 240 cm long and 110 cm deep while the nest section was 60 cm long and 55 cm deep; both sections were 53.5 cm high (Figure 3.2 and 3.3). The nest box was positioned at one end of the cage. The bottom of the nest boxes consisted either of wire floor or was lined up with artificial turf (XPNP long Astroturf®). The opening to the nesting area was 22 cm wide and 33 cm high. The litter baths, positioned at a height of 20 cm at the other end of the cage, contained sawdust and opened for 4.5 h in the afternoon. The cages were stocked with 39 hens; feed and water was available *ad libitum*, by a feed trough and by nipple drinkers. The furnished cages provided approx 750 m^2 area per hen. The commercial Brown layers were housed in two rows of three-storey cages (Figure 3.5) with 10 cages per row; with approx 2 400 birds per hen house. Manure was dried on a manure belt and removed at least once a week.

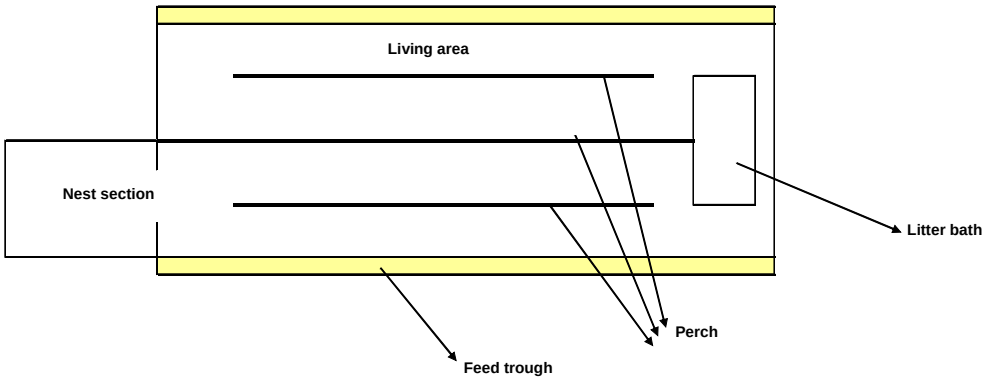

Figure 3.2: Design of the studied furnished cages.

Figure 3.3: Studied furnished cages with eggs on the conveyor belt next to the nest boxes (left); laying hen entering a nest box (right).

2.4 Aviary housing

The aviary system was divided in 4 pens, each 7.2 m long and 6.10 m wide. Each pen contained 500 commercial Brown layers. Each pen incorporated a central 2 m wide slatted platform with two levels (85 cm height between platforms), a 1 m wide littered floor area at each side of the platform and 3 rollaway nest boxes, 240 cm long and 42 cm wide, at each side wall (Figure 3.4 and 3.5). The nest boxes and the first floor slatted platform were mounted at 85 cm from the ground. The littered floor area under the nest boxes and the slatted platform was also accessible for the birds. A manure belt mounted under the slatted platforms removed the dried manure weekly. The nest boxes were lined up with artificial turf (XPNP long Astroturf®) and the entrance was covered by a curtain made of plastic with two openings of 20 cm. Beside the slatted platform and the nest boxes alighting rails were fixed. The littered floor area contained a thin layer of white sand. Water and food were supplied *ad*

libitum from nipple drinkers and feed pans at the platform, with nipple drinkers also at the entrance of the nest boxes.

Figure 3.4: Studied aviary housing system with nest boxes, alighting rails and nipple drinkers at the side wall (left); a central slatted platform with nipple drinkers and feed pans (middle); the system housed with laying hens (right).

2.5 Experiments

Two experiments were performed; from August 2001 to May 2002, and from January 2003 to August 2003. Three and four designs were compared, respectively. Table 3.1 summarises the two experiments with their different designs.

Table 3.1: Description of the experimental arrangements.

Experiment 1 (August 2001 to May 2002)				
Design	Housing system	Nest material	Hen house	Sampled cages
1	Conventional cages	Wire floor	2B	10
2	Furnished cages	Wire floor	1A	3
3	Furnished cages	Artificial turf	1A	3
Experiment 2 (Januari 2003 to August 2003)				
1	Conventional cages	Wire floor	2B	10
2	Furnished cages	Wire floor	1A/2A	4
3	Furnished cages	Artificial turf	1A/2A	4
4	Aviary	Artificial turf	1B	n.a.

1A, 1B, 2A and 2B = building 1 hen house A, building 1 hen house B, building 2 hen house A and building 2 hen house B; n.a. = not applicable.

Figure 3.5 shows cross sections of the houses of experiment 2. In both experiments 17 weeks old commercial Brown layers were transferred to the experimental buildings where they received 12 h of light per day increasing to 16 h from week 21 onwards.

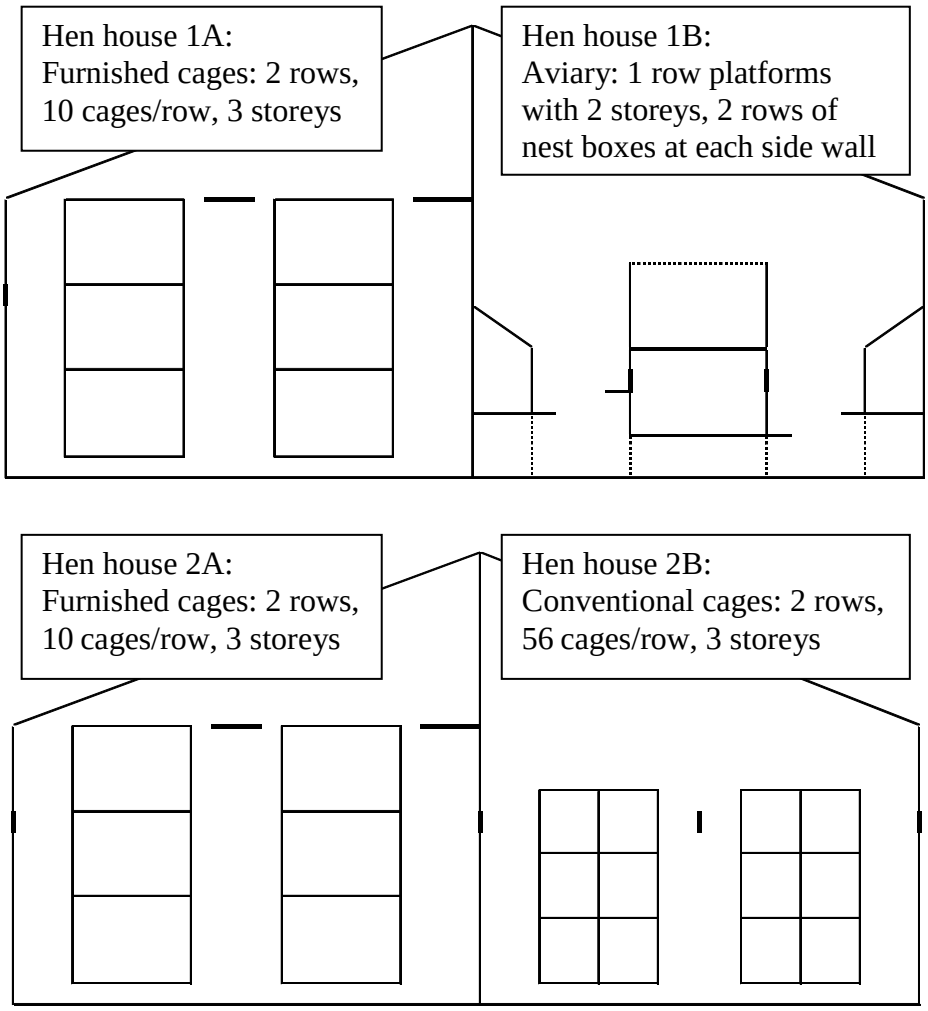

Figure 3.5: Cross section of the hen houses of experiment 2 showing the arrangements.

2.6 Sampling

In the first experiment (August 2001 to May 2002) samples were taken at about 8-week intervals: namely at the hen age of 24, 32, 41, 50, 57 and 65 weeks; in the second experiment (January 2003 to August 2003) at the hen age of 33, 38, 48, 57 and 61 weeks. To produce statistically reliable results a minimum of 40 eggs from each housing system (design) were sampled (see chapter 2; paragraph 3.4). Sampling, collection and transport of the eggs occurred as described in chapter 2; paragraph 2.3.

In the second sampling period bacterial air contamination, temperature and atmospheric humidity were measured as described in chapter 2; paragraph 2.7.

2.7 Determination of bacterial eggshell contamination

To recover bacteria from the eggshell, the intact egg was placed in a plastic bag with 10 ml Phosphate Buffered Saline (PBS, Oxoid, Basingstoke, UK) and the egg was rubbed through the bag for 1 min. The diluent was plated by a spiral-enter on Nutrient Agar (NA, Oxoid) to count the total of aerobic bacteria and on NA with 0.0001% crystal violet (VWR, Darmstadt, Germany) to count the Gram-negative bacteria. For full details on the used methodology; reference is made to chapter 2; paragraph 3.1.

2.8 Statistical analysis of data

Statistical analysis of the data was performed as outlined in chapter 2; paragraph 2.2. In addition a simple linear regression was carried out to determine the influence of the air contamination on the initial bacterial eggshell contamination.

3 RESULTS AND DISCUSSION

Figure 3.6 shows shell contamination with total aerobic flora on the different sampling dates during experiment 1 (August 2001 to May 2002) for the three designs and two housing systems: conventional cages, furnished cages with wire floor nest boxes and furnished cages with nest boxes with artificial turf (Table 3.1). Figure 3.7 shows the same data for the Gram-negative flora on the shells of the same eggs.

The results for experiment 2 (January 2003 to August 2003) are shown in Figure 3.8 and 3.9. Figure 3.8 shows shell contamination with total aerobic flora on the different sampling dates for the four designs and three housing systems; conventional cages, furnished cages with wire floor nest boxes, furnished cages with artificial turf lined nest and an aviary housing system (Table 3.1 and Figure 3.5). Figure 3.9 shows the same data for the Gram-negative flora on the shell of the same eggs. Table 3.2 summarises the significant differences per sampling date for both experiments. More data are available upon request.

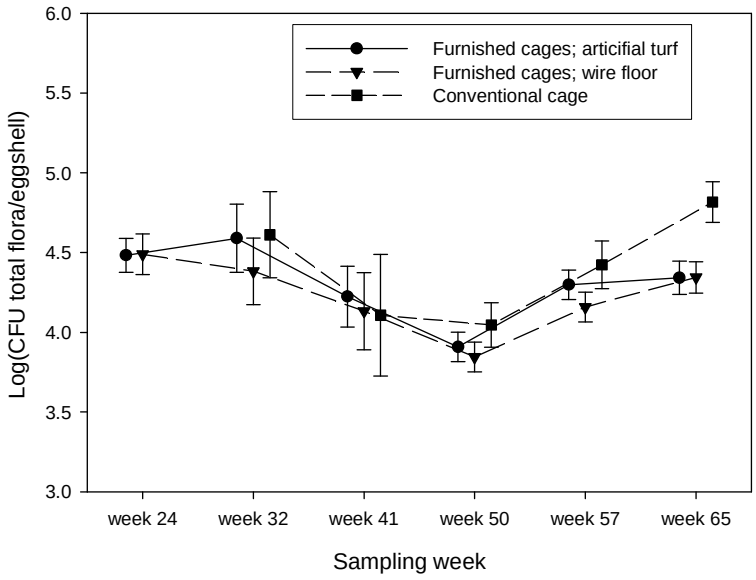

Figure 3.6: Experiment 1: Eggshell contamination with total aerobic flora on different dates for the three compared designs including two housing systems (period: August 2001 to May 2002).

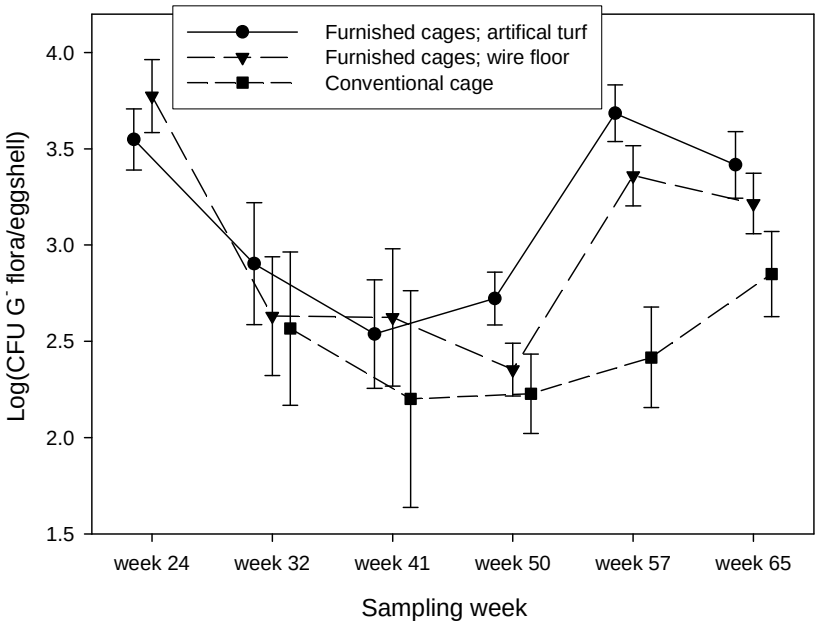

Figure 3.7: Experiment 1: Eggshell contamination with Gram-negative flora on different dates for the three compared designs including two housing systems (period: August 2001 to May 2002).

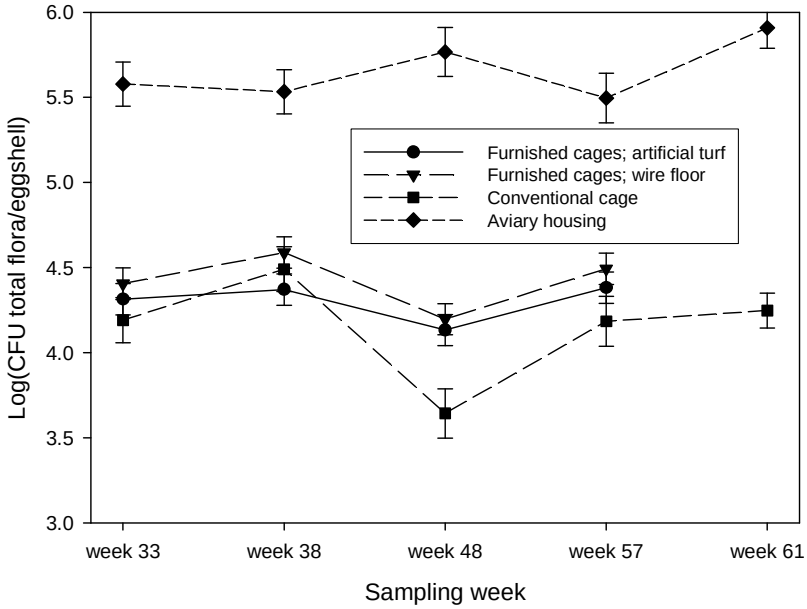

Figure 3.8: Experiment 2: Eggshell contamination with total count of aerobic flora on different dates for four compared designs including three housing systems (period: January 2003 to August 2003).

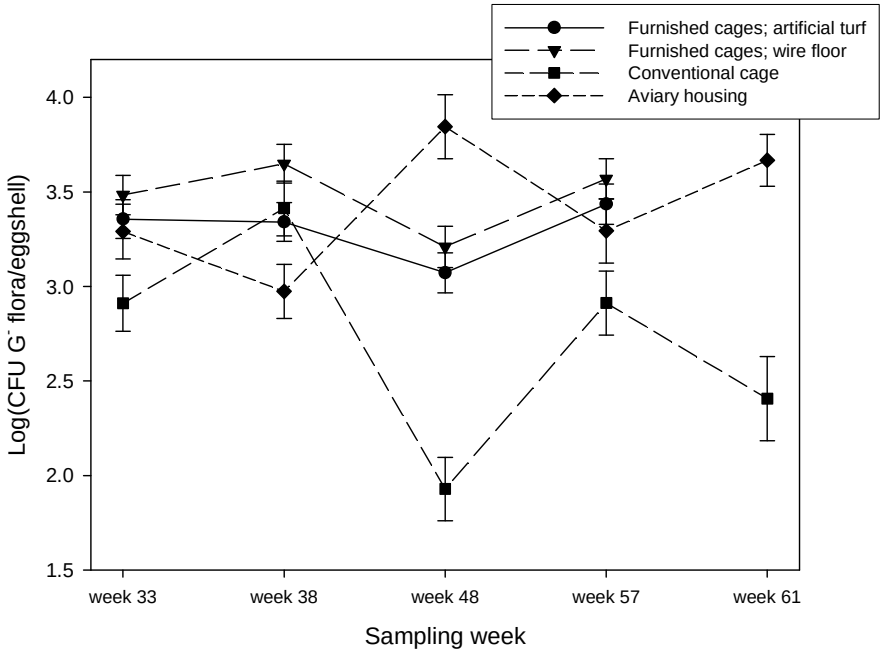

Figure 3.9: Experiment 2: Eggshell contamination with Gram-negative flora on different dates for four compared designs including three housing systems (period: January 2003 to August 2003).

Table 3.2: Summary of the statistical significant differences ($P < 0.05$, 0.01 or 0.001) per sampling date for both experiments (ANOVA).

Experiment 1 (August 2001 to May 2002)

Total aerobic flora							Gram-negative flora						
Week →	24	32	41	50	57	65	Week →	24	32	41	50	57	65
System ↓							System ↓						
Conventional cages (2B)*	-	A	A	A	A	A	Conventional cages (2B)	-	A	A	A	A	A
Furnished cages; wire floor (1A)	A	A	A	A	B	B	Furnished cages; wire floor (1A)	A	A	A	B	B	B
Furnished cages; artificial turf (1A)	A	A	A	A	B	B	Furnished cages; artificial turf (1A)	B	A	A	C	C	B

Experiment 2 (January 2003 to August 2003)

Total aerobic flora						Gram-negative flora					
Week →	33	38	48	57	61	Week →	33	38	48	57	61
System ↓						System ↓					
Conventional cages (2B)*	A	A/C	A	A	A	Conventional cages (2B)	A	A	A	A	A
Furnished cages; wire floor (1A/2A)	C	A	C	C	-	Furnished cages; wire floor (1A/2A)	B	C	C	C	-
Furnished cages; artificial turf (1A/2A)	C	C	C	A/C	-	Furnished cages; artificial turf (1A/2A)	B	A	C	C	-
Aviary housing (1B)	B	B	B	B	B	Aviary housing (1B)	B	B	B	B	B

Systems in the same column with common letter are not significant different. * = identification hen house, - = no data available.

For both experiments an ANOVA showed no systematic statistically significant differences between conventional cages and furnished cages, for either total aerobic flora or Gram-negative flora (Table 3.2, Figures 3.6 to 3.9). On the final sampling dates (week 57 and 65) of experiment 1, shell contamination with total aerobic flora was significantly higher at the 95%-confidence level on the eggs from the conventional cages (Table 3.2 and Figure 3.6). Figure 3.6 and the ANOVA data ($P < 0.05$ week 57 and $P < 0.001$ week 65) show this difference was only very significantly different in week 65. This high value in week 65 can probably be attributed more to coincidence (a manure heap next to the conventional cage housing division) than to the type of housing system itself. At the date of sampling (week 65), manure from a period of 6 weeks before was stocked outside, next to house B of building 2, whereas on the other sampling dates manure was more regularly removed. This increase of total count of aerobic bacteria in the conventional cages was not observed during experiment 2, confirming this assumption (Figure 3.8). In experiment 2 the differences in total aerobic flora on the eggshell for cage and furnished cage production were again not systematic (Table 3.2 and Figure 3.8). Only in week 48 was there a very significant difference ($P < 0.001$). In both sampling periods contamination with Gram-negative flora on shells of eggs from conventional cages was much lower for one sampling point (week 57) in experiment 1 and two sampling points (week 48 and 61) in experiment 2. This lower contamination level was not observed on the previous and/or following sampling dates (Figure 3.7 and 3.9). In both experiments there were no systematic differences in contamination with Gram-negative flora between conventional and furnished cages (Table 3.2, Figure 3.7 and 3.9).

Both experiments also showed that accumulation of eggs in the furnished cages in an area of about 60 cm width did not necessary increase shell contamination. Tauson (2002) reported that furnished cages increased contact between eggs and in some cases the proportion of dirty and cracked egg. This was caused by the accumulation of the eggs on a short part of the conveyor belt next to the nest section. In our experiments only eggs laid at the nest boxes were sampled.

Both experiments showed that the shell contamination was not systematically influenced by whether the nest-floor material was wire or artificial turf. (Table 3.2, Figure 3.6 to 3.9). The results for total aerobic flora did not differ significantly for 9 of the 10 sampling dates and for Gram-negative flora did not differ significantly for 6 of the 10 sampling dates (Table 3.2). For the other dates no systematic difference was observed. Wall *et al.* (2002) also found no significant effect of the nest-floor material on the egg production or proportions of cracked or

dirty eggs in furnished cages; on the other hand the use of the nests was significantly increased where cages had nests with 100% Astroturf®, compared with 50 or 30% lining.

In both experiments there was no influence of the age of hens or the interval since placing the hens in the houses on shell contamination (data not shown). Comparing Figure 3.6 with Figure 3.7 and Figure 3.8 with Figure 3.9 shows that, regardless of housing design, a comparable graphical trend was found for both total aerobic and Gram-negative flora. This suggests that the sampling date influenced the bacterial contamination; more specifically in experiment 1 the season appeared to affect shell contamination, with both total aerobic and Gram-negative flora. During the winter period, week 41 (beginning of December) and week 50 (end of January), shell contamination was lower (at least $P < 0.05$) compared to the warmer periods; week 24 (August), week 32 (September) and week 65 (May) (Figure 3.6 and 3.7). Takai *et al.* (1998) also reported a seasonal influence on the dust concentration in poultry houses. Some results of Quarles *et al*. (1970) also suspected that high temperatures might influence shell contamination. However, this possible seasonal influence was not confirmed in the second experiment (Figure 3.8 and 3.9). During experiment 2, in the conventional cages and the aviary system, an additional sampling was performed during the heat wave period in week 61 (August 2003; outside-temperature up to 40°C – inside temperature 30°C, see Table 3.3). Shell contamination was not higher than in the winter period; week 33 and 38. Similarly, Quarles *et al.* (1970) could not always confirm their supposition of the influence of the season on the shell contamination.

Experiment 2 showed that shell contamination with total count of aerobic flora was more than 1 log unit higher, during the entire experiment, for eggs from the aviary system (Table 3.2 and Figure 3.8). For Gram-negative flora (Table 3.2 and Figure 3.9) no systematically differences were found between the four designs including the aviary system. In chapter 2 was found that contamination with aerobic bacteria (5.8 log CFU/eggshell) of organic eggs from the conveyor belt next to the nest boxes, was also 1 log unit higher compared to eggs from the conveyor belt of the caged hen house. The housing system for organic eggs resembles the aviary system of our experiment. Higher contamination with total count of aerobic flora was also measured in the air of the organic house (5.6 log CFU/m^3 air) compared to the cage house (4.4 log CFU/m^3 air) (chapter 2). In experiment 2 the influence of bacterial air contamination on the shell contamination was examined and, for total aerobic count, a positive correlation of $r^2 = 0.66$ was found ($P < 0.001$). Figure 3.10 shows the bacterial air contamination with total count of aerobic flora for each system; the air contamination in the aviary system was higher compared to the other two systems.

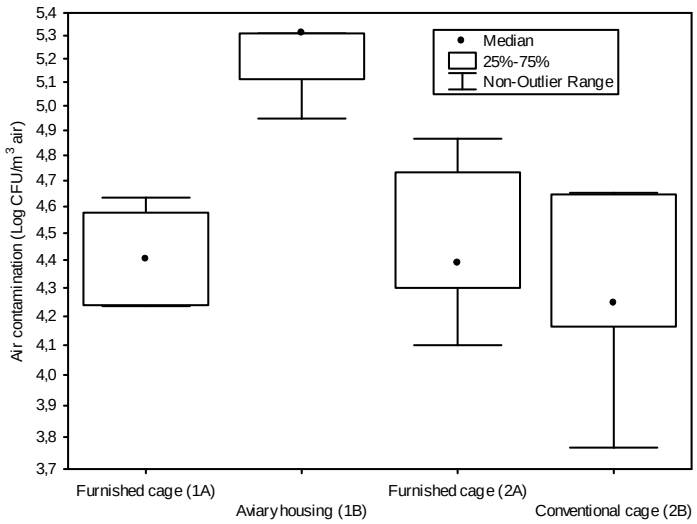

Figure 3.10: Bacterial air contamination in each housing system of the second experiment.

Harry (1963) and Quarles *et al*. (1970) also reported correlations between initial eggshell contamination and the concentration of bacteria in the house. Quarles *et al*. (1970) reported a significant difference for air contamination between litter floor houses (sawdust on the floor and wood shavings in the nests) and wire-floor houses (sloping wire floors and plastic roll away nests); 3.97 log CFU/m^3 and 3.03 log CFU/ m^3 respectively. We obtained averages of 4.3 log CFU/m^3 for the conventional cage housing, 4.4 log CFU/m^3 for the furnished cages and > 5.3 log CFU/m^3 for the aviary housing system. The concentration of airborne bacteria in animal houses was also studied by Hartung and Seedorf (1999). According to their study the incidence of total aerobic bacteria was highest in poultry houses (6.4 log CFU/m^3) compared to 5.1 log CFU/m^3 and 4.3 log CFU/m^3 in pig and cattle sheds, respectively. Lyngtveit (1992) described the behaviour of animals affecting the dust concentrations. In aviary systems the hens can move both horizontally and vertically and perform dust bathing. Their study showed significantly higher concentrations of dust in the afternoon than in the morning, owing to dust bathing behaviour. Because all our sampling was performed in the morning this factor could not have influenced our data. Ellen *et al.* (2000) reported a variation of the dust concentration in poultry houses from 0.02 - 81.33 mg/m^3 for inhalable dust and from 0.01 - 6.5 mg/m^3 for respirable dust. Houses with caged laying hens showed the lowest dust concentrations, less than 2 mg/m^3, while the dust concentrations in perchery and aviary systems were often four to five times higher. Other factors affecting the dust concentrations were animal category, animal activity, bedding materials and the season. Important sources of dust are the bird, excreta, food, bedding materials, floor materials and soil (Lyngtveit 1992). As dust contains micro-organisms like bacteria (Lyngtveit and Eduard 1997; Pedersen *et al.*

2000) this also explains the higher air contamination with total aerobic flora that we found in the aviary system.

Table 3.3 summarises the measured temperature and atmospheric humidity in the hen houses during the second sampling period.

Table 3.3: Temperature and atmospheric humidity in the different hen houses during the second experiment.

Temperature (°C)					
Week →	33	38	48	57	61
System ↓					
Conventional cages (2B)*	16.8	17.5	21.8	n.d.	30.1
Furnished cages (1A/2A)	17.7/17.5	19.7/17.5	22.1/22.3	n.d.	30.1/30.6
Aviary housing (1B)	17.5	19.1	21.1	n.d.	29.1
Atmospheric humidity (%)					
Week →	33	38	48	57	61
System ↓					
Conventional cages (2B)	48	51	59	n.d.	64
Furnished cages (1A/2A)	50/47	55/45	56/59	n.d.	66/69
Aviary housing (1B)	53	51	55	n.d.	64

* = identification hen house, n.d. = not determined.

In contrast to the bacterial air contamination in experiment 2, no correlation was found between shell contamination and the temperature or atmospheric humidity measured in the houses.

In the current studies on the improvement of the alternative laying hen production facilities it is desirable to include their effects on shell contamination and air contamination, to improve the bacterial shell quality. It will also document the possible influence on food safety, health of the laying hens and the development of a healthier working environment in the alternative housing systems.

4 CONCLUSIONS

The long-term experiments with pilot housing systems made it possible to evaluate the alternatives for the conventional cage in terms of initial bacterial eggshell contamination and air contamination. No systematic differences in eggshell contamination with total aerobic and Gram-negative flora were found between conventional and furnished cages. In the selection of

the most suitable nest bottom material for productivity and animal welfare, the studied lining did not influence the eggshell contamination. Obvious was the 1.0 log higher contamination with total aerobic flora on eggs derived from the aviary system. Finally a positive correlation was found between the concentration of total bacteria in the air of the experimental poultry houses and the initial bacterial eggshell contamination.

The study in the next chapter must check that differences in initial eggshell contamination, found in the pilot housing systems; are also applicable on conventional and alternative commercial housing systems. The second aim of the study was to analyze the evolution of the bacterial eggshell contamination progress and to identify critical points for introducing bacterial eggshell contamination in more production chains and at different stages in the laying period (compared to the preliminary study in chapter 2).

Chapter 4

Bacterial eggshell contamination in the production chain of different commercial housing systems

Redrafted after:

Bacterial eggshell contamination in the egg collection chains of different housing systems for laying hens

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CHAPTER 4: Bacterial eggshell contamination in the production chain of different commercial housing systems

Abstract

The bacterial eggshell contamination of consumption eggs in different commercial housing systems; two conventional cages, one organic aviary system and one barn production, were compared. The total count of aerobic bacteria and the total count of Gram-negative bacteria on the eggshell were used to detect critical points for introducing bacterial eggshell contamination and to study the progress of the eggshell contamination in the egg production chains.

The critical points for the bacterial eggshell contamination were the accumulation of eggs on a short conveyor belt, the initial eggshell contamination in the alternative housing systems and the extra nest boxes placed on the ground. A high bacterial load of ground eggs (> 6.3 log CFU total aerobic flora/eggshell) was observed.

On average a significant higher ($P < 0.001$*) initial eggshell contamination with total count of aerobic bacteria was found for eggs from the alternative housing systems compared to the conventional systems; respectively 5.46 compared to 5.08 log CFU/ eggshell. However, initial contamination with total count of Gram-negative bacteria on the eggshells was significantly lower (*$P < 0.001$*) in the alternative housings; 3.31 compared to 3.85 log CFU/eggshell.*

A moderated and not significant ($r^2 = 0.77$*;* $P = 0.099$*) positive correlation was found between the initial bacterial eggshell contamination and the concentration of bacteria in the air of the poultry houses.*

Storing shell eggs, whether temporary refrigerated or not, for 9 days or more, resulted in a significant decrease in bacterial eggshell contamination for both bacterial variables.

1 INTRODUCTION

Due to the EU-directive 99/74, implying a ban on conventional cages from 2012 onwards and the introduction of furnished cages and alternative systems, more recent research was focused on the comparison of the initial bacterial eggshell contamination of eggs laid in conventional cages, furnished cages and aviary or perchery housing systems; see chapter 3, Protais *et al.* (2003b) and Mallet *et al.* (2004). All studies were performed in experimental hen houses. At the moment it remains unknown whether the differences in bacterial numbers among eggs produced in different housing systems have an impact on the quality of eggs and egg products. Only Petrak *et al.* (1999) reported a direct relationship between initial eggshell contamination and the final contamination of the egg products. Harry (1963), Smeltzer *et al.* (1979b) and we (chapter 6) found a correlation between bacterial eggshell contamination and egg infection.

To our knowledge, in literature only limited data are published about the bacterial eggshell contamination of consumption eggs through the production chain. The aim of our study in chapter 4 was to compare the initial eggshell contamination in different commercial production chains from different housing systems, to study the contamination progress and to detect critical points introducing bacterial eggshell contamination in the chain.

2 MATERIALS AND METHODS

2.1 Determination of bacterial eggshell contamination

The method used for the recuperation of bacteria from the eggshell and the determination of the total count of aerobic and Gram-negative bacteria is described in chapter 3; paragraph 2.7.

2.2 Sampling, collection and transport of eggs

Sampling, collection, transport and storage (prior to analysis) of the eggs were done as described in chapter 2; paragraph 2.3.

2.3 Sampling through the production chain

Cage production 1

The detailed description of the cage production 1 (C1 in Table 4.1) is explained in chapter 2, paragraph 2.6. Sampling of the eggs was done when hens were 30 weeks old. Due to technical problems with the ventilation, all hens were removed before the end of lay, making sampling at end of lay impossible. At 10 points in the production chain samples were taken; samples 8 - 10 were taken respectively 1, 1 and 5 days after egg laying.

Cage production 2

The second sampled caged layer house, housed the brown-shell breed ISA Brown. The farm housed a total of 75 000 hens in 3 adjoining houses connected by a large corridor. The cage arrangements were comparable with cage production 1. The eggs of one hen house (35 000 hens) were followed through the production chain. Eggs were sampled when hens were 26 and 71 weeks old and were gathered from each row of roll-out cages to a cross conveyor which took them to the corridor. In the corridor a second up and down moveable horizontal conveyor belt assembled the eggs from each deck of cages and took them to the collecting area where the eggs were visually evaluated and automatically collected in carton trays. From the collecting area the eggs were taken to the candling, grading and packaging area, located in a building 20 m from the collecting area. The eggs were graded and packaged automatically after a second visual evaluation in an ECM 1200 Staalkat (Staalkat International B.V., The Netherlands) candling booth. First category eggs were packed in closed cartons. By the second sampling date (week 71), the hen house had been partly rebuild; the collecting area was eliminated and the candling, grading and packaging area was now connected directly with the corridor. Packaged first category eggs were stored at ambient conditions at the shell egg processing plant and loaded two days later into a non-refrigerated lorry, which took them the next day to the cooled warehouse (6-8°C) of a food chain's distribution centre. Five days later the manager of the local shop took the eggs, in a non-refrigerated lorry, to the storage area of his local food shop. The eggs were kept there for 5 days and then replenished at the shop racks, both at ambient conditions. In week 26 (w26) and 71 (w71), respectively 7 and 4 points in the production chain were sampled (C2B and C2E in Table 4.1). Samples 6 and 7 of week 26 were taken respectively 3 and 13 days after egg laying.

Organic production

The detailed description of the organic production is explained in chapter 2, paragraph 2.6. Sampling of the eggs was done when hens were 39 and 71 weeks old; respectively 7 and 8 points in the production chain were sampled (OB and OE in Table 4.1). In week 39 sample 7 was taken 5 days after egg laying; in week 71 samples 6 and 7 were taken respectively 1 and 9 days after egg laying. Because there were too many ground eggs, extra roll-out nest boxes were build during the laying period. These nest boxes were not connected to a cross conveyor and were placed on the ground at different places in the hen house. Eggs laid in those nest boxes were sampled in week 71.

Barn production

The barn production housed 6 200 brown-shell breed Bovans Goldline hens in each of two hen houses. The eggs of one hen house were sampled. The 794-m^2 hen house with 420 m^2 roosts contained 84 roll-out nest boxes located at each side of a central conveyor belt. Sampling was done at the age of 56 weeks. Eggs from the roll-out nest boxes (120 x 42 cm, Astroturf® mat) were gathered to the covered cross conveyor at the middle of the hen house. The conveyor belt transported the eggs from the hen house directly to a small collecting area where the eggs were visually evaluated and collected by hand in carton trays. Eggs were stored at ambient conditions. Next day, eggs were taken by a refrigerated lorry (6-8 °C) to a shell egg processing plant 50 km away. There, the same day, eggs were candled, graded and packaged using an ECM 1200 Staalkat candling booth. First category eggs (see also chapter 1 paragraph 7 and chapter 2) were automatically packaged in closed cartons and stored at the shell egg processing plant at ambient conditions. Four days later the packaged eggs were loaded into a refrigerated lorry (6-8°C), which took them to the refrigerated warehouse (6-8°C) of a food chain's distribution centre. The next day a refrigerated lorry (6-8°C) took the packaged eggs to the refrigerated storage area (6-8°C) of the local food shop, where the eggs were kept for 3 days and then replenished at the shop racks at ambient conditions. Samples were taken at 5 points in the production chain (B in Table 4.1). Samples 3, 4 and 5 were taken respectively, 1, 1 and 10 days after egg laying.

Table 4.1: Summary of the sampled points during the samplings of the production chains.

Production system	Cage production 1	Cage production 2		Organic production		Barn production
	C1	C2B	C2E	OB	OE	B
Sampling moment	w30*	w26	w71	w39	w71	w56
Sampling point						
Hen house at the conveyor belt	1**	1	1	1	1	1
Large conveyor belt of the corridor which connects the conveyors of each hen house	2	2	2	n.p.	n.p.	n.p.
At the entrance of the collecting area	n.p.	3	n.p.	2	2	2
At the entrance of the candling, grading and packaging area (shell egg processing plant)	3	4	4	3	3	3 (d1)
In candling booth	4	-	-	-	-	-
First category consumption eggs just packaged in closed cartons	5	5	5	4	4	4 (d1)
Second category eggs packaged in open carton trays	6	-	-	-	-	-
First category consumption eggs in closed cartons at the refrigerated lorry at the hen house	7	-	-	5	-	-
First category consumption eggs in closed cartons at the refrigerated warehouse at the food chain's distribution centre	8 (d1)	6 (d3)	-	-	6 (d1)	-
First category consumption eggs in closed cartons at the refrigerated storage of the local shop	9 (d1)	-	-	-	-	-
First category consumption eggs in closed cartons at the shop rack of the local shop	10 (d5)	7 (d13)	-	7 (d5)	7(d9)	5 (d10)
Eggs collected from the ground (ground eggs) of the hen house	n.p.	n.p.	n.p.	8	8	6
Eggs collected from the extra build nests	n.p.	n.p.	n.p.	n.p.	9	n.p.

C1 = cage production 1; C2B = cage production 2 begin lay; C2E = cage production 2 end lay; OB = organic production begin lay; OE = organic production end lay; B = barn production

* age of layers in weeks; ** number sampling point; (dx) = sampled x days after egg laying; n.p. = sampling point not present; - = not sampled

2.4 Statistical analysis of data

The statistical analysis of the data was performed conform to chapter 3; paragraph 2.8.

2.5 Environmental conditions in the production chain

Bacterial air contamination, temperature and atmospheric humidity were measured in the different parts of the production chain. For full details on the used methods; reference is made to chapter 2; paragraph 2.7.

2.6 Visual examination of the shell eggs

The visual examination of the eggshell was performed as specified in chapter 2; paragraph 2.8.

3 RESULTS

3.1 Cage production 1

Figure 4.1 shows an increase in total count of aerobic and Gram-negative bacteria on the eggshells, at the moment the eggs enter the candling, grading and packaging area (‘3. Entrance packaging area’ in Figure 4.1). For both parameters this increase was statistically significant ($P < 0.001$). Also a difference in eggshell contamination with total count of aerobic bacteria of first (point 5) and second category (point 6) eggs was shown immediately after packaging, this difference was limited statistically significant ($P < 0.05$). Finally from point 3 onwards, the bacterial eggshell contamination with total count of aerobic and Gram-negative bacteria remained at a constant level and was, respectively, in 7 of the 8 and in all 8 points significantly higher compared to the first two points (Figure 4.1).

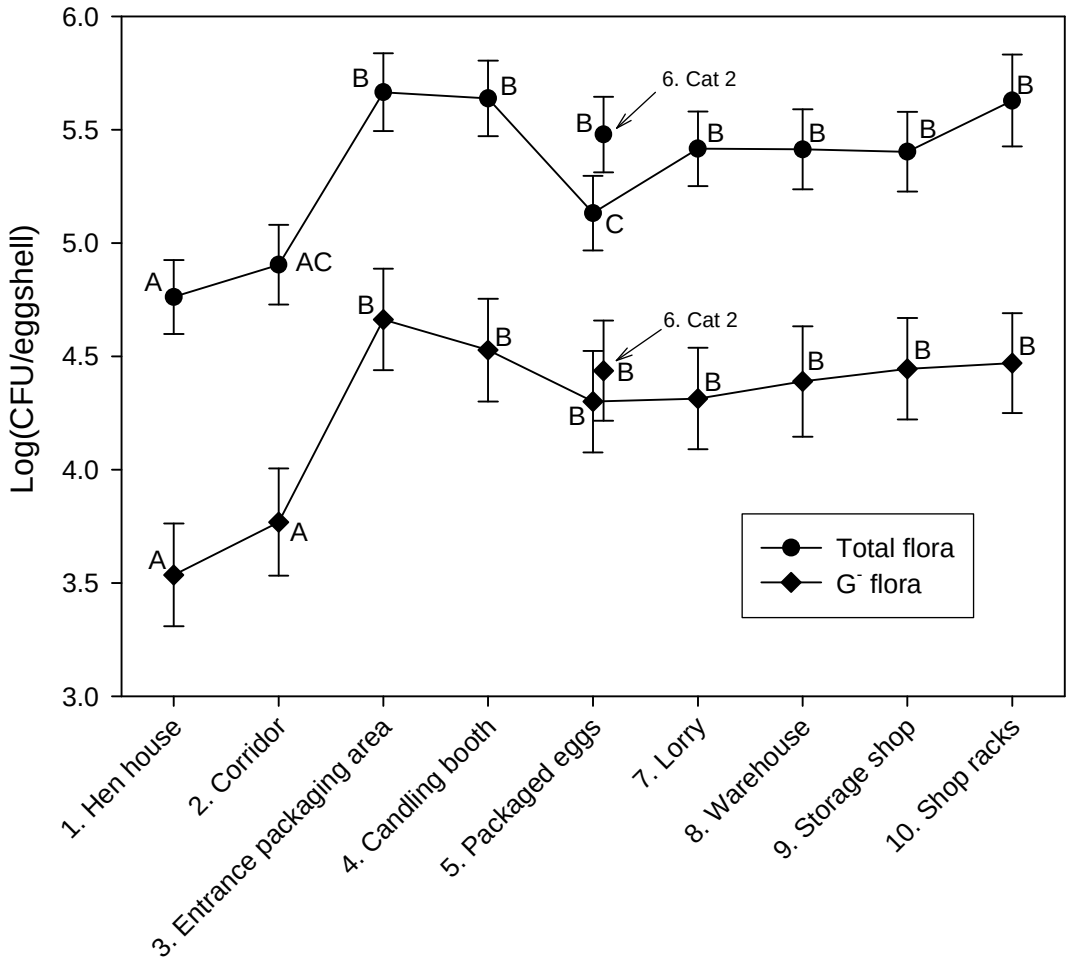

Figure 4.1: Total count of aerobic (Total flora) and Gram-negative (G^- flora) flora in the different points of the caged production chain 1. Vertical bars denote 95% confidence intervals. Points of the same curve without common letters are significant different.

3.2 Cage production 2

There was no significant increase in eggshell contamination (total count of aerobic and Gram-negative flora) through the production chain at the beginning of lay (Figure 4.2). At the end of the chain, in the warehouse and the shop racks (point 6 and 7), a significantly lower ($P < 0.001$) eggshell contamination with both hygiene indicators was found.

At the end of lay (week 71), fewer points (4) were sampled (Table 4.1). Comparable to the beginning of lay, there was no increase or fluctuation for total counts of aerobic bacteria through the chain (points 1, 2, 4 and 5 in Figure 4.2). No systematic increase or decrease, but more fluctuations for Gram-negative bacteria were found; most fluctuations or differences were minor but significant ($P < 0.05$ and $P < 0.01$).

Comparing the beginning and end of lay, we observed minor but significantly higher contamination with total aerobic flora at the end of lay in the points 1, 4 and 5 (Figure 4.2).

For Gram-negative bacteria, in 3 of the 4 points no significant difference was found, while in point 2 a significant lower eggshell contamination was found at the end of lay (Figure 4.2).

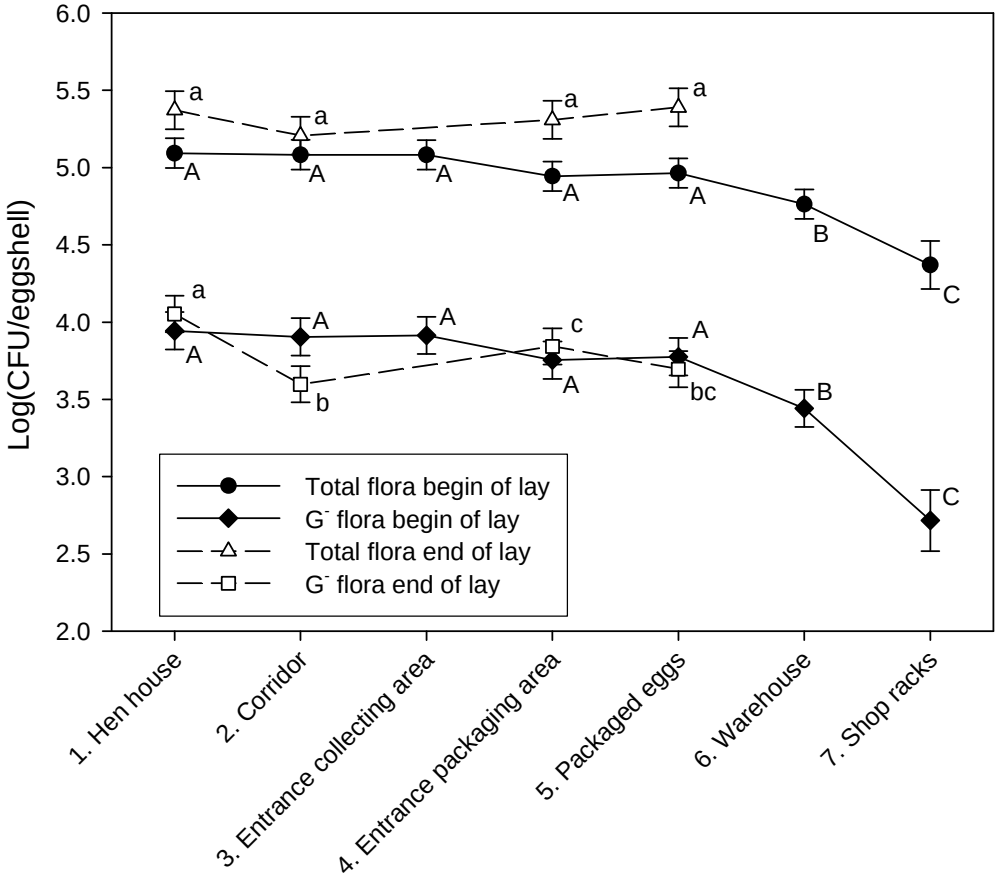

Figure 4.2: Total count of aerobic (Total flora) and Gram-negative (G^- flora) flora in the different points of the caged production chain 2. Vertical bars denote 95% confidence intervals. Points of the same curve without common letters are significant different (beginning lay = capital letters – end lay = small letters).

The initial and the average (points 1, 2, 4 and 5) eggshell contamination with total count of aerobic bacteria was, respectively, 0.28 and 0.30 log CFU/eggshell higher at the end of lay. For Gram-negative bacteria the corresponding figures were 0.09 and 0.04 log CFU/eggshell lower at the end of lay.

3.3 Organic production

The sampling of eggs at the beginning of lay (week 39) showed no systematic increase or decrease of total count of aerobic and Gram-negative bacteria through the chain (point 1 - 7). The observed fluctuations for both parameters (Figure 4.3) ranged between 5.30 and 5.86 log CFU/eggshell for aerobic flora and between 2.60 and 3.41 log CFU/eggshell for Gram-

negative bacteria. The observed statistical differences or fluctuations for total count of aerobic bacteria were of minor importance ($P < 0.05$ or $P < 0.01$); only a major difference was found ($P < 0.001$) between the eggs sampled in the collecting area (point 2) and the eggs sampled on the lorry (point 5). For Gram-negative flora ANOVA revealed a statistically significant ($P < 0.001$) lower eggshell contamination of the eggs available at the lorry (point 5) compared to all other points in the chain. The differences found between the other 5 points had all a *P*-value < 0.05. The eggshell contamination of the eggs collected from the ground (ground eggs, point 8, not shown in Figure 4.3) was higher for both variables (>0.5 log CFU/eggshell; $P < 0.001$) compared to the contamination of the eggs sampled at other points in the chain; respectively 6.36 log CFU total aerobic flora/eggshell and 3.98 log CFU Gram-negative flora/eggshell.

At the end of lay, a very similar course of eggshell contamination through the chain (points 1 to 7) was found (Figure 4.3). However, the significantly lower contamination with both parameters at the end of the chain in the shop racks (point 7) was striking, compared to the contamination found in the previous 5 sampling points. Eggshell contamination of the ground eggs (point 8) was again major significantly higher for both variables (> 1.5 log CFU/eggshell; $P < 0.001$) compared to eggs sampled at other points in the chain; 7.94 log CFU total aerobic flora/eggshell and 5.80 log CFU Gram-negative flora/eggshell. The eggs sampled in the extra nest boxes (point 9) were also major significantly higher contaminated (> 1.0 log CFU/eggshell; $P < 0.001$); respectively with 6.88 log CFU total aerobic flora/eggshell and 4.67 log CFU Gram-negative flora/eggshell.

Comparing beginning and end of lay, contamination of the eggshell with total count of aerobic bacteria was lower at the end of lay in 5 of the 6 sampling points (Figure 4.3). However, the contamination of the ground eggs was >1.50 log CFU/eggshell higher at the end of lay. For Gram-negative bacteria an opposite trend was found; eggshell contamination at the end of lay was in 5 of the 6 points higher (Figure 4.3); this was also the case for the ground eggs.

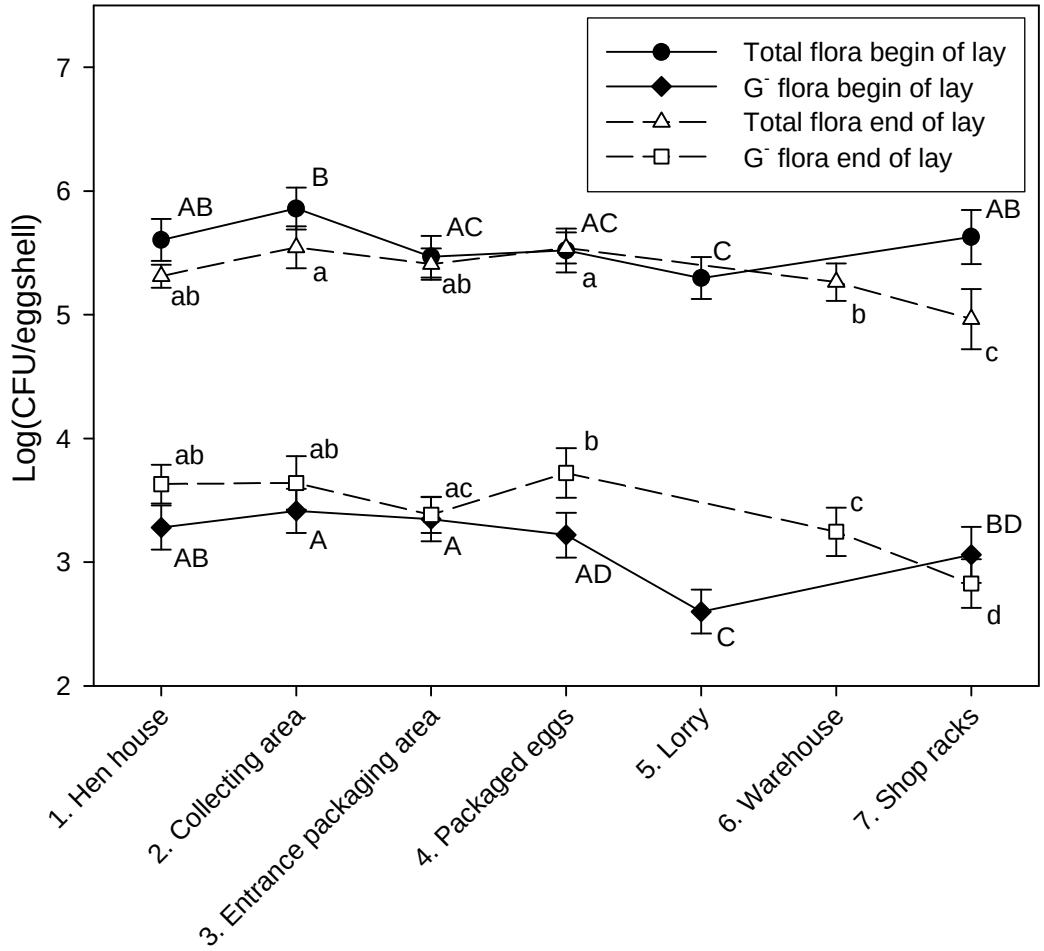

Figure 4.3: Total count of aerobic (Total flora) and Gram-negative (G^- flora) flora in the different points of the organic production chain. Vertical bars denote 95% confidence intervals. Points of the same curve without common letters are significant different (beginning of lay = capital letters – end lay = small letters).

Initial and average (points 1, 2, 3, 4, 5 or 6 and 7) eggshell contamination with total count of aerobic bacteria was, respectively 0.29 and 0.23 log CFU/eggshell lower at the end of lay; while for Gram-negative bacteria the initial and average contamination was 0.35 and 0.26 log CFU/eggshell higher.

3.4 Barn production

Figure 4.4 shows no significant increase of bacterial eggshell contamination through the chain; only minor fluctuations were found. Contamination was significantly lower in the last point of the chain, the shop racks (point 5), both for total count of aerobic bacteria and for Gram-negative bacteria. The contamination of eggs collected from the ground (ground eggs, point 6) was again major significantly higher ($P < 0.001$) for both parameters compared to the contamination of eggs sampled at the other points in the chain.

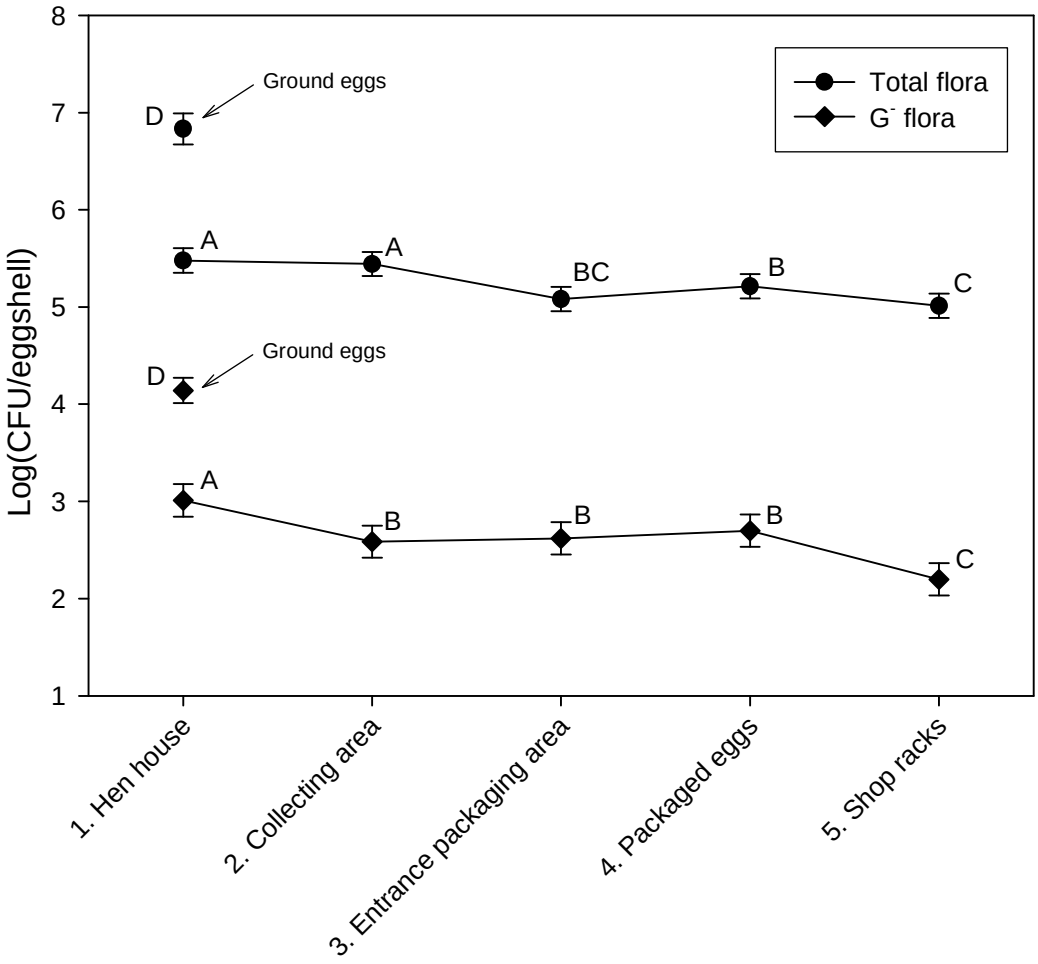

Figure 4.4: Total count of aerobic (Total flora) and Gram-negative (G^- flora) flora in the different points of the barn chain. Vertical bars denote 95% confidence intervals. Points of the same curve without common letters are significant different.

3.5 Bacterial air contamination in the production chains

Table 4.2 summarizes the total count of airborne flora in different parts of the production chains. The total count of aerobic bacteria in the air of the alternative housing systems was higher compared to the conventional cages; 5.6 (OB), 5.6 (OE) and 5.4 (B) compared to 4.4 (C1), 4.6 (C2B) and 4.5 (C2E) log CFU/m^3, respectively. For each production chain; air contamination is lower at the warehouse, the storage of the shop and in the shop compared to previous sampled points in the chain.

Table 4.2: Total count of airborne flora in the different parts of the production systems (log CFU/m^3 air).

	Code	Hen house	Corridor	Collecting area	Packaging area	Ref. lorry	Ref. warehouse	Ref. storage shop	Shop racks
Cage production 1; week 30	C1B	4.4	4.1	n.p.	3.8	3.7	2.9	2.9	3.2
Cage production 2; week 26	C2B	4.6	3.9	3.7	3.1	3.7*	2.8	2.6	3.1
Cage production 2; week 71	C2E	4.5	4.1	n.p.	3.4	n.d.	n.d.	n.d.	n.d.
Organic production; week 39	OB	5.6	n.p.	5.5	4.3	4.3	2.8	2.4	2.9
Organic production; week 71	OE	5.6	n.p.	5.0	3.9	n.d.	n.d.	n.d.	n.d.
Barn production; week 56	B	5.4	n.p.	3.8	3.7	3.7	2.9	2.7	3.0

Ref. = refrigerated; n.d. = not determined; n.p. = not present; * not refrigerated

3.6 Initial eggshell contamination at the hen house

Comparing the initial bacterial eggshell contamination of the eggs sampled in the hen house (points 1 in Figures 4.1 - 4.4), we found on average a statistically significant higher contamination ($P < 0.001$) with total count of aerobic bacteria for the alternative systems compared to the conventional cages; 5.46 (average of point 1 at OB, OE and B) compared to 5.08 (average of point 1 at C1, C2B and C2E) log CFU/ eggshell, respectively. On the other hand the initial contamination with total count of Gram-negative bacteria on the eggshells was significantly lower ($P < 0.001$) in the alternative housings; 3.31 (average of point 1 at OB, OE and B) compared to 3.85 (average of point 1 at C1, C2B and C2E) log CFU/eggshell.

4 DISCUSSION

Only in one of the four sampled production chains, cage production 1, a major statistically and microbiologically significant (> 1 log) increase in one of the sampled points was found (ignoring ground and extra nest eggs present at some of the production chains). At the moment the eggs enter the candling, grading and packaging area ('3. Entrance packaging area' in Figure 4.1) the eggshell contamination with both total count of aerobic flora as well as Gram-negative flora increased significantly. Here, all eggs from the 4 hen houses passed the same small surface, and the rolling of all eggs on the same surface caused bacterial cross-contamination due to eggshell dirt and broken egg content. This critical point for contamination could not be detected by visual inspection of the eggshell (see also chapter 2, paragraph 3.5). The type of conveyor, a metal grid, can also contain more dirt and egg content compared to (double) roller conveyor belts. The air contamination (Table 4.2) was lower in the candling, grading and packaging area (3.8 log CFU/m^3 air) compared to the corridor (4.1 log CFU/m^3 air), which also confirmed that the significant increase of bacterial shell contamination was due to contact with the metal grid.

Comparing the initial bacterial eggshell contamination of eggs laid in different pilot housing systems, Protais *et al.* (2003a) and we (chapter 3) also found a higher eggshell contamination with mesophilic aerobic bacteria in aviaries or percheries compared to conventional and furnished cages. The aviary and perchery housing system resembled, respectively, the organic and barn system of this study. The increase found in the alternative housings of these published experimental studies was more than 1 log CFU unit (up to a total of 6.0 log

CFU/eggshell) (ignoring outside nest eggs and ground eggs), compared to only 0.4 log CFU units increase of the alternative versus conventional cage systems in the study of this chapter. It should also be noted that, in agreement with Protais *et al.* (2003a), also in our present study a weak association between visual soiling of eggs and shell bacterial load was observed (ignoring ground eggs) (data shown in chapter 2). For Gram-negative bacteria, in our former study (chapter 3) we found no systematic significant differences in initial eggshell contamination between the three pilot housing systems (aviary, conventional and furnished cages), in comparison to an average 0.5 log unit lower initial contamination found in the alternative commercial housings (OB, OE and B) of this present study.

Our study showed a higher contamination of the air with total counts of aerobic bacteria for the alternative housing systems compared to the conventional cages. A positive but not significant correlation (r^2= 0.77; P = 0.099) between air contamination and initial shell contamination was found. Protais *et al.* (2003a) and we in our former study (chapter 3) also found a correlation between the air contamination of the hen house and the initial bacterial eggshell contamination of the eggs sampled at the henhouse (total count of aerobic bacteria). In these studies, on average, 4 log CFU/m^3 air for the conventional and furnished cages was found compared to a 100 times higher average (> 6 log CFU/m^3 air) in the aviary or perchery housing. Similarly, Zoons *et al.* (2005) also reported a 5-fold higher contamination of dust in an aviary system compared to furnished cages (10.1 versus 2.1 mg/m^3).

As in the study of this chapter, in the study of chapter 3 comparing pilot housing systems, also no systematic significant difference in bacterial eggshell contamination with total count of aerobic and Gram-negative bacteria was found comparing beginning and end of lay.

In comparison to the second category eggs of caged layer house 1, the eggshell contamination of eggs collected from the ground in the alternative housings (OB, OE and B) was major significantly higher for both eggshell contamination parameters, compared to eggs sampled at the other points of those chains. The high contamination of the extra nest eggs (OE) was also striking; indicating that the extra nest boxes placed on the ground were also critical points for the bacterial eggshell contamination. Protais *et al.* (2003a) and we in our previous study (chapter 2) found counts up to 7 log CFU/eggshell on those eggs laid on the floor.

For all four production chains the total count of Gram-negative bacteria was on average >1 log CFU/eggshell lower compared to the total aerobic flora, indicating that Gram-positive bacteria dominate the flora on eggshells; probably because of their greater tolerance to dry conditions (Board and Tranter 1995).

Finally, for all samplings of the production chains where eggs were available at the shop racks within 5 days after lay (C1 and OB), no significant decrease in eggshell contamination with both parameters was found compared to the previous sampled points (Table 4.1, Figure 4.1 and 4.3). For the other three samplings (C2B, OE and B), eggs were available at the shops racks after 13, 9 and 10 days respectively, and showed significant less eggshell contamination with both parameters compared to the previous points (Table 4.1, Figure 4.2, 4.3 and 4.4). These findings show that storing of shell eggs, whether temporary refrigerated or not, for 9 days or more, causes a significant decrease in bacterial eggshell contamination. In a previous study (chapter 2) we also reported a decrease in bacterial eggshell contamination after 14 days storage at room temperature and approx 50% relative humidity; for Gram-negative bacteria the decrease was statistically significant. Despite the significant higher initial eggshell contamination with total count of aerobic bacteria for eggshell of alternative systems (5.46 versus 5.08 log), the average contamination was more comparable at the end of the chain (5.20 versus 5.00 log). For Gram-negative bacteria still a >0.5 log lower contamination was found for eggshell from alternative housing systems.

5 CONCLUSIONS

In the four sampled production chains the only critical points that are responsible for introducing bacterial eggshell contamination were; accumulating eggs on a short conveyor belt (metal grid), the initial eggshell contamination in the alternative housing systems and the extra nest boxes placed on the ground. The high bacterial load of ground eggs explain why they cannot be used as consumption eggs. The major differences in eggshell contamination with total count of aerobic bacteria, found between conventional and alternative housing systems in pilot studies (see chapter 3) are less pronounced in the sampled commercial housing systems.

Beside the identification of critical points and further studies to develop a less bacteriological contaminated alternative non-cage housing system, also disinfection of the eggshell surface is an important tool to reduce bacterial eggshell contamination. In the next chapter the effect of the use of an UV irradiation system as integral part of a conveyor belt to decontaminate eggshell and belt is discussed.

Chapter 5

The effect of a commercial UV disinfection system on the bacterial load of shell eggs

Redrafted after:

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CHAPTER 5: The effect of a commercial UV disinfection system on the bacterial load of shell eggs

Abstract

The effect of UV irradiation on the bacterial load of shell eggs and of a roller conveyor belt was studied. The natural bacterial load on the eggshell of clean eggs was significantly reduced by a standard UV treatment of 4.7 s; from 4.47 to 3.57 log CFU/eggshell. For very dirty eggs no significant reduction was observed. Eggs inoculated with Escherichia coli *and* Staphylococcus aureus *(4.74 and 4.64 log CFU/eggshell, respectively) passed the conveyor belt and were exposed to UV for 4.7 and 18.8 s. The reduction of both inoculated bacteria on the eggshell was comparable and significant for both exposure times (3 and 4 log CFU/eggshell, respectively).* E. coli *was reduced but still detectable on the conveyor rollers. The internal bacterial contamination of eggs filled up with diluent containing* E. coli *or* S. aureus *was not influenced by UV irradiation.*

The penetration of UV into organic material appears to be poor and UV disinfection can be used as an alternative for egg washing of clean eggs.

1 INTRODUCTION

In chapters 2, 3 and 4 is shown that alternative housing systems for laying hens can cause an increase in bacterial eggshell contamination. In chapter 1 and 6 a correlation between bacterial eggshell contamination and internal egg infection is reported. Disinfection of the eggshell surface is therefore an important tool to reduce the number of micro-organisms on the shell surface and through this the prevention of egg spoilage and egg-related illnesses. The cuticle is an important physical barrier for egg invading organisms (chapter 1 paragraph 2.1 and chapter 6 and 7). It obstructs bacterial invasion by closing the pores resulting in a reduced permeability of the shell (Fromm and Margolf 1958). Egg-washing chemicals can damage the cuticle layer (Kim and Slavik 1996), change the microstructure of eggshells or leave chemical residues on shell surfaces (Kim and Slavik 1996; Wang and Slavik 1998; Favier *et al.* 2001) (see also paragraph 8 in chapter 1). Ultraviolet irradiation could be a more favourable alternative for decontamination of the eggshell (Kuo *et al.* 1997a). Studies using pilot UV irradiation systems have shown UV irradiation to be effective in reducing the bacterial load on the surface of visibly clean eggs (Kuo *et al.* 1997b; Chavez *et al.* 2002; Coufal *et al.* 2003). Gao *et al.* (1997) studied, also with a pilot system, the effectiveness of UV irradiation on different types of egg belt conveyor materials. The effect of UV irradiation on dirty (faeces) eggs and internal egg decontamination has not been published to our knowledge.

The work in this chapter aims to compare the effect of a commercial irradiation system, linked to a commercial roller system, on the elimination of aerobic bacteria on clean eggs and dirty eggs, to study the effect on recent surface contamination (eggshell and rollers) and to check the influence of UV irradiation on the contamination of the egg content.

2 MATERIALS AND METHODS

2.1 Egg samples

Clean eggs were collected from a commercial conventional housing system, with ISA Brown laying hens, on the day of lay. Very dirty eggs (eggs with visible faecal contamination) were collected from a commercial aviary housing system, with Bovans Goldline laying hens, on the day of lay.

2.2 Ultraviolet irradiation

A commercial UV-C disinfection system having a wavelength of 253.7 nm and an intensity of 10 mW/cm^2 was used (UV-disinfection unit MOBA; MOBA, Barneveld, The Netherlands) (Figure 5.1). The UV-disinfection system was linked to a MOBA plastic double roller conveyor belt. Two different speeds of the conveyor belt were used; one with a maximum speed of 10 000 eggs/h per row and another with a moderate speed of 2 500 eggs/h per row. This resulted in a speed of the belt of 0.2167 m/s and 0.0542 m/s, respectively. As the UV-C disinfection system had a length of 102 cm, the exposure time for one egg was 4.7 and 18.8 s, respectively.

Figure 5.1: MOBA UV-disinfection unit linked to a MOBA double roller conveyer belt.

2.3 Inoculation of eggs

Escherichia coli (ATCC 25922) and *Staphylococcus aureus* (ATCC 6535) were used to inoculate the eggshell of clean eggs. Inoculation was performed by immersing the whole egg for 1 min in Phosphate Buffered Saline (PBS, Oxoid, Basingstoke, UK) containing 10^5 - 10^6 CFU/ml of the selected bacterium and was allowed to dry at ambient temperature during 2 h. This resulted in an average eggshell contamination with 5.5×10^4 CFU *E. coli*/eggshell or 4.6×10^4 CFU *S. aureus*/eggshell.

E. coli (ATCC 11775) and *S. aureus* (ATCC 6535) were also used to inoculate the egg content. The egg contents (albumen and egg yolk) were drained after cutting a hole of approx 1 cm^2 with a rotary tool (Dremel, S-B Power Tool Company, Chicago USA) and a pair of

tweezers. The inner part of the shell was rinsed with sterile ¼ Ringers solution (Oxoid) to remove the albumen adhering to the membranes and after that the egg was filled up with ¼ Ringers solution containing 1.0×10^3 CFU *E. coli*/ml or 6.1×10^2 CFU *S. aureus*/ml. After filling up the eggs, the hole was closed with silicone.

2.4 Determination of the bacterial contamination of eggshell, conveyor rollers and internal egg fluid

The total aerobic mesophilic bacteria of uninoculated clean and uninoculated dirty eggs was determined by the washing procedure outlined in chapter 2, paragraph 3.1.

The *E. coli* or *S. aureus* count on eggshells was also determined by washing the egg with diluent as described before. The diluent was subsequently plated on Mc. Conkey No3 Agar (Oxoid) for *E. coli* and Baird-Parker medium with Rabbit Plasma Fibrinogen (Oxoid) for *S. aureus*. Plates were incubated at 37°C for 24 h and 48 h respectively.

Individual rollers of the conveyor belt were swabbed with plain cotton swabs, soaked in Buffered Peptone Water (BPW, Oxoid). Swabs were respectively immediately streaked on Mc. Conkey No3 Agar and enriched for 24 h at 30°C in BPW, followed by streaking the enrichment on Mc. Conkey No3 Agar. The selective plates were incubated at 37°C for 24 h.

After aseptic removal of the silicone, the internal egg *E. coli* or *S. aureus* count was determined by sampling 1 ml from the internal fluid with a sterile pipette through the hole and plating on Violet Red Bile Lactose Agar (Oxoid) for *E. coli* and Baird-Parker medium with Rabbit Plasma Fibrinogen (Oxoid) for *S. aureus*. Plates were incubated at 37°C for 24 h and 48 h respectively.

2.5 Decontamination experiments

In the first test cycle 80 clean and 80 dirty eggs were sampled, where both were not inoculated. The next day 40 eggs from both categories were irradiated at an exposure time of 4.7 s; the remaining 40 eggs from each category were used as control group. The total aerobic bacterial count was determined the day after the irradiation.

In a second test cycle 15 clean eggs were inoculated with a culture of *E. coli* bacteria and 15 clean eggs with *S. aureus* bacteria. After drying at ambient conditions, 10 inoculated eggs of both groups were passed on the conveyor belt, of them 5 eggs were UV irradiated for 4.7 s and the other 5 eggs for 18.8 s. After the test with *E. coli* the individual rollers of the

conveyor belt were swabbed. The remaining 5 eggs of both groups were used as control group. The *E. coli* and *S. aureus* shell contamination was determined the same day.

To study the influence of UV irradiation on internal bacterial egg contamination, the egg content of 40 clean eggs was removed; 20 eggs were filled up with ¼ Ringers solution containing *E. coli* and the other 20 eggs with ¼ Ringers solution containing *S. aureus*. From each set of filled up eggs, 10 eggs were irradiated with UV for 4.7 s and the remaining 10 eggs were used as control group. Microbiological analyses were performed the same day.

2.6 Identification

Identification was performed on 2 colonies picked up from the Nutrient Agar plates used for the determination of the total count of aerobic mesophilic bacteria of 2 non-UV treated clean eggs by partial 16S rDNA sequencing (Scheldeman et al. 2004).

2.7 Statistical analysis

Analysis of variance was done on the log 10 transformed counts according to paragraph 2.2 in chapter 2.

3 RESULTS

The natural bacterial load (total aerobic bacteria) on the eggshell of uninoculated clean eggs was significantly reduced ($P < 0.001$) by UV treatment; from 4.47 to 3.57 log CFU/eggshell (Figure 5.2). For the uninoculated dirty eggs a non-significant ($P > 0.05$) reduction from 6.17 to 5.99 log CFU/eggshell was observed (Figure 5.2). Identification showed that the 4 picked up colonies from the non-UV treated clean eggs were all member of the *Staphylococcus equorum* group (>97% similarity).

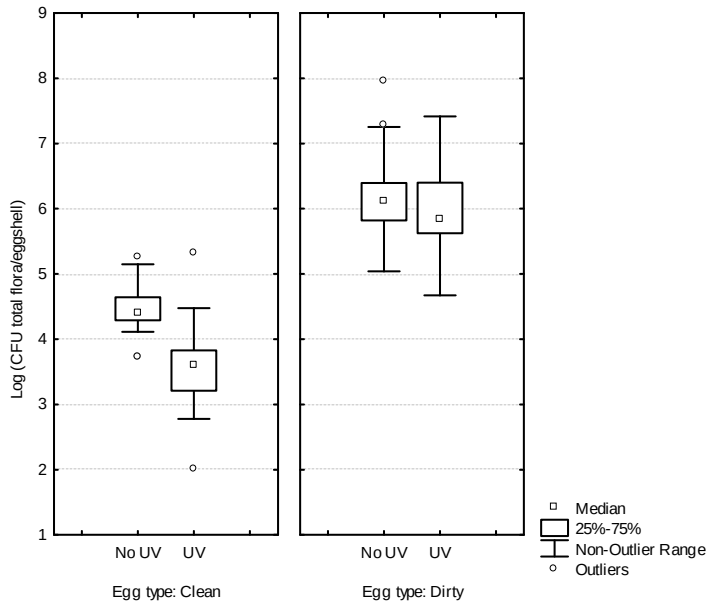

Figure 5.2: Influence of UV disinfection (253.7 nm, 10 mW/cm^2, 4.7 s) on the natural bacterial load (total aerobic bacteria) of uninoculated clean and dirty eggshells.

The reduction of *E. coli* surface contamination after inoculation was significantly ($P < 0.001$) for both exposure times respectively. A reduction of 3 log (4.7s UV) and 4 log (18.8 s UV) occurred, compared with the control group having an average contamination of 5.5×10^4 CFU *E. coli*/eggshell. For *S. aureus* comparable results were obtained; significant ($P < 0.001$) reductions of 3 log (4.7 s UV) and 4 log (18.8 s UV) occurred, compared with an initial eggshell contamination of 4.6×10^4 CFU *S. aureus*/eggshell.

After passing the UV device 3 times at both conveyor speeds; no *E. coli* could be isolated from the plastic rollers surface by direct plating of the swabs from the surface. However, after enrichment of the swabs taken after 3 and even 8 times passing the device, *E. coli* was still detectable.

UV treatment did not significantly influence the internal egg contamination. For *E. coli*, UV treated eggs contained on average 4.07 log CFU/ml compared with 4.37 log CFU/ml for non-treated eggs ($P < 0.05$), for *S. aureus* the count in UV treated eggs was even higher compared with non-treated eggs, 2.75 versus 2.64 log CFU/ml ($P = 0.14$) (Figure 5.3). To determine the effect of repeated UV-treatments, two eggs filled up with *E. coli* (4.37 log CFU/ml) were irradiated three times subsequently for 4.7 s and afterwards still contained 4.08 and 4.36 log CFU *E. coli*/ml respectively.

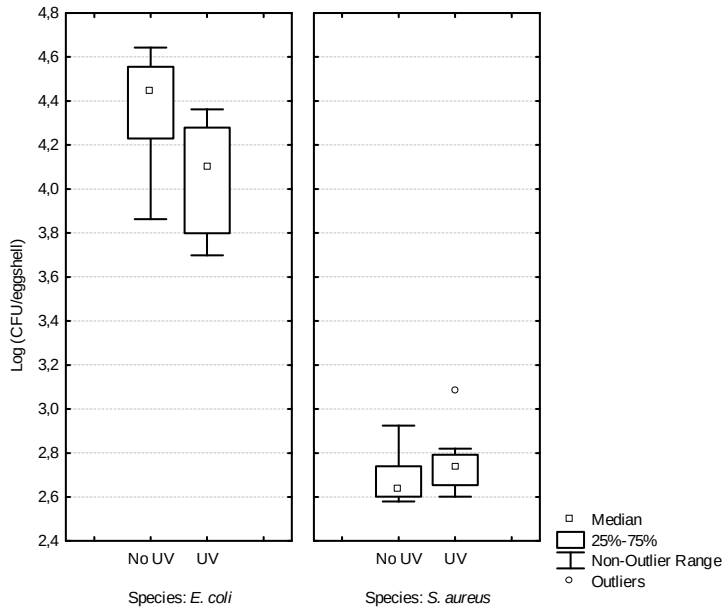

Figure 5.3: Influence of UV disinfection (253.7 nm, 10 mW/cm^2, 4.7 s) on the internal contamination of eggs.

4 DISCUSSION

Our data showed no significant reduction of the natural bacterial load on very dirty uninoculated eggs compared with a significant reduction on visible clean uninoculated eggs. Possibly the on top faeces particles on the shell of the dirty eggs formed a protective layer for the bacteria against the UV treatment. The penetration of UV into the organic material appears to be poor, only the outer surface layer was apparently exposed. Stermer *et al.* (1987) also found that the bactericidal effect of UV light was less effective on rough meat surfaces because bacteria were partly shielded from the radiation.

Kuo *et al.* (1997b) evaluated different UV (254 nm) treatment times (0, 15 and 30 min) at an intensity of 620 µW/cm^2 and different intensities (620, 1 350 and 1 720 µW/cm^2) at a treatment time of 15 min. For all UV treatments a 2 log reduction of CFU of aerobic bacteria per eggshell was observed. The visibly clean eggshell surfaces initially contained 5.0 log CFU aerobic bacteria per eggshell. Favier *et al.* (2001) found a reduction of 1.6 log on uninoculated clean eggs after an UV exposure for > 25 min (254 nm; 4 573 µW/cm^2). In one of the experiments of Chavez *et al.* (2002), visibly clean eggs were exposed to UV treatment (254 nm; 7.35 mW/cm^2) for 0, 15, 30 and 60 s. Exposure of eggshells to UV for 30 and 60 s resulted in a 0.8 - 2 and a 2 - 3 log reduction of the aerobic plate count per eggshell, respectively. Coufal *et al.* (2003), using an UV cabinet (254 nm, 4 min and 4 - 14 mW/cm^2), found a 1.3 log reduction. All previous mentioned studies used pilot UV irradiation systems.

In our experiment, using a 253.7 nm - 10 mW/cm^2 commercial UV treatment, a reduction of 0.9 log was found after the short period of 4.7 s UV treatment. Gao *et al*. (1997) came to the conclusion that the exposure time was more important than the UV intensity.

The significant reduction of the surface contamination after eggshell inoculation was also found by other researchers. Kuo *et al.*, (1997b) found a significant reduction of *Salmonella* Typhimurium inoculated on eggshell surfaces (2.5×10^6 CFU/eggshell); one minute of irradiation (254 nm; 620 µW/cm^2) decreased the population with approximately 3 log. Coufal *et al.* (2003) found a 4 log reduction for *Salmonella* Typhimurium and a 4 - 5 log reduction for *E. coli* (254 nm, 4 min and 4 – 14 mW/cm^2). The latter is comparable to our 4 log reduction for the inoculated *E. coli* bacteria (18.8 s UV). Favier et al. (2000b) found UV irradiation was more effective on groups of eggs with low *Yersinia enterocolitica* inoculum (2.4×10^4 CFU/eggshell) than on those groups with high inoculum (2.2×10^7 CFU/eggshell). A decrease of 4.39 and 1.43 log cycles was observed after 40 min of 4 573 µW/cm^2 UV exposure respectively.

Gao *et al.* (1997) demonstrated that *Salmonella* was easier to eliminate from plastic belt than from other materials tested; fibre belt was most difficult, eggshell and metal were within median range. In our study the contamination of the rollers with *E. coli*, a less dangerous substitute for *Salmonella*, was not completely eradicated.

Although *E. coli*, *S. aureus* (inoculated eggs) and *Staphylococcus equorum* (example of natural flora on clean eggs) have a comparable amount of energy needed to be deactivated by UV (6 600 µJ for *E. coli* and 5 720 – 6 600 µJ for *Staphylococcus* sp. respectively) (Srikanth 1995), our study showed that the UV decontamination was clearly more effective on *E. coli* and *S. aureus* inoculated eggs compared to naturally contaminated clean eggs. The freshness of the inoculum (which might lead to a higher susceptibility of the bacteria), the more protected position (shielded) of the natural flora on the eggshell or the presence of organisms that are only partly or effectively not deactivated by the UV system on clean eggs might explain this difference. As already mentioned in chapter 1 different other researchers also reported *Staphylococcus* spp. as natural flora present on the eggshell. In this study, no determination of the initial composition of all the microflora of the eggshells was performed.

Although the effect of UV treatment on internal *E. coli* contamination was for one test statistically less significant, in microbiology the decrease is limited relevant. Both organisms used for the internal egg contamination (*E. coli* and *S. aureus*) need the same UV deactivation energy; 6 600 µJ (Srikanth 1995). Our results show that UV cannot penetrate the eggshell.

Gao *et al.* (1997), using a UV sensor placed beneath a piece of eggshell, confirmed that UV penetration could not be detected on the other side of an eggshell.

5 CONCLUSIONS

We can conclude that there is a significant lethal effect of the commercial UV disinfection system on bacterial contamination of visibly clean eggshells and recent shell contamination, that contamination of rollers can be controlled but not completely eradicated, and that the internal contamination of eggs was not reduced by the UV irradiation used.

In the next chapter the correlation between bacterial eggshell contamination and eggshell penetration and whole egg contamination is discussed. For that purpose eggshells of agar-filled eggs and whole eggs were inoculated with phylogenetically diverse bacterial species. The influence of physical barriers of the egg (eggshell factors) and the hen age on the penetration and contamination was determined.

Chapter 6

Eggshell factors influencing eggshell penetration and whole egg contamination by different bacteria, including *Salmonella* Enteritidis

Redrafted after:

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CHAPTER 6: Eggshell factors influencing eggshell penetration and whole egg contamination by different bacteria, including *Salmonella* Enteritidis

Abstract

In a first study trans-shell infection routes and whole egg contamination of 7 selected bacterial strains; Staphylococcus warneri, Acinetobacter baumannii, Alcaligenes *sp.,* Serratia marcescens, Carnobacterium *sp.,* Pseudomonas *sp. and* Salmonella *Enteritidis, recovered from egg contents, were studied. A first objective was to correlate bacterial eggshell penetration with various eggshell characteristics and the identity of phylogenetically diverse bacterial strains. An agar approach was used to assess the eggshell penetration. A second objective was to assess the contamination of whole eggs with the bacterial strains; whole intact eggs were used in this case. The intact shells of agar-filled and whole eggs were inoculated with* 10^3 *-* 10^4 *CFU of the selected strains. Inoculated eggs were stored for 3 weeks at 20°C and 60% relative humidity. Bacterial eggshell penetration was regularly monitored and whole egg contamination was analyzed after 3 weeks. Contrary to the cuticle deposition, the eggshell characteristics shell surface area, shell thickness and number of pores did not influence the bacterial eggshell penetration. The whole egg contamination was not influenced by neither the area of the eggshell or the porosity of the eggshell. The results of the agar approach indicate that the Gram-negative, motile and non-clustering bacteria penetrated the eggshell most frequently; Pseudomonas sp. (60%) and* Alcaligenes *sp. (58%) were primary invaders followed by* Salmonella *Enteritidis (43%). All selected strains were able to penetrate; penetration was observed most frequently after approx 4 - 5 days. In comparison with the non-*Salmonella *strains,* Salmonella *Enteritidis was a primary invader of whole eggs in the first study: the membranes and/or the content of 32% of the whole eggs were contaminated. Penetrated eggshells and contaminated whole eggs showed a significantly higher bacterial contamination on the eggshell compared to respectively not penetrated eggshells and not contaminated whole eggs (general results of all strains). The influence of hen age on bacterial eggshell penetration and egg content contamination was not significant.*

In a second short study the whole egg contamination with four different Salmonella *Enteritidis strains and one* Salmonella *Typhimurium strain was studied. Contamination percentages ranged from 6% - 26%, with no special capacity of egg related* Salmonella

Enteritidis strains compared to other Salmonella *Enteritidis strains and the* Salmonella *Typhimurium strain.*

1 INTRODUCTION

The increasing consumer awareness of food safety issues has changed the public perception of a “good egg” from shell cleanliness and physical properties to that of microbial integrity. Micro-organisms can contaminate egg contents at different stages, from laying, handling to preparation and consumption. Transovarian or “vertical” transmission of micro-organisms occurs when eggs are infected during their formation in the hen’s ovaries. Horizontal transmission occurs when eggs are subsequently exposed to a contaminated environment and micro-organisms penetrate the eggshell (see also chapter 1, paragraph 4). Studies conducted by Barrow and Lovell (1991) suggest that most of the contamination is due to horizontal transmission, although others do not agree (Humphrey 1994a). Contents contamination of whole intact eggs with *Salmonella* Enteritidis should be mainly the result of infection of the reproductive tissue (Humphrey 1994a). Different researchers reported on the penetration of bacteria through the eggshell with associated membranes and on the following whole egg contamination. Some published reports suggest a relationship between eggshell quality and bacterial eggshell penetration and/or whole egg contamination (Sauter and Petersen 1974; Nascimento and Solomon 1991). Most research was focused on the penetration and/or contamination of *Pseudomonas* and various salmonellae. Bacteria of the genus *Pseudomonas* have been shown to more readily penetrate into whole eggs of poor shell quality (Sauter and Petersen 1969). Sauter and Petersen (1974) also found that whole eggs with low specific gravity or low shell quality were more likely to be penetrated by *Salmonella*. Berrang *et al.* (1998) reported on the influence of egg weight, specific gravity, conductance and flock age on the ability of *Salmonella* to penetrate the shell and the membranes. Because shell quality measures did not change greatly in relation to flock age and the *Salmonella* Typhimurium penetration patterns did vary, they concluded that it is likely that factors other than just shell quality are involved in bacterial penetration in eggshells. Nascimento *et al.* (1992) also reported an increasing eggshell penetration from 12.9% (beginning of lay) till 25.0% (end of lay) for *Salmonella* Enteritidis. Messens *et al.* (2005a) did not found a correlation between eggshell characteristics and eggshell penetration with *Salmonella* Enteritidis. Bruce and Johnson (1978) reported for hatching eggs an increasing contamination of eggs as flocks became older.

Until now no attention was given to the connection between bacterial eggshell penetration and whole egg contamination. In this study the influence of hen age and eggshell characteristics on the eggshell penetration on the one hand and the egg content contamination on the other

hand was investigated, using 7 selected bacterial strains isolated from the egg content of consumption eggs. To study more in detail the potential of *Salmonella* to contaminate whole eggs by the horizontal infection route, the whole egg contamination with different *Salmonella* strains was determined.

2 MATERIALS AND METHODS

2.1 Eggs

For the first study, eggs from a fixed stable of a commercial conventional housing system, housing ISA Brown laying hens, were collected at the day of lay. Upon storage overnight at 20°C the eggs were filled with agar and/or inoculated (as described in paragraphs 2.3 and 2.4 of this chapter). The laying hens were placed in production at the hen age of 24 weeks and eggs were sampled at the ages of 32, 34, 46, 60, 69 and 74 weeks. Eggs were visually inspected by candling and only intact eggs (no cracks, pin-holes…) were included in further analyses. In the second study, used eggs came from the higher mentioned system, housing a new flock of ISA Brown hens with an age of 45 weeks (middle of lay).

2.2 Bacterial strains and cultures

Seven phylogenetically diverse bacterial strains; *Staphylococcus warneri* (MB 2792), *Acinetobacter baumannii* (MB 2793), *Alcaligenes* sp. (MB 2794), *Serratia marcescens* (MB 2795), *Carnobacterium* sp. (MB 2796), *Pseudomonas* sp. (MB 2797) and *Salmonella* Enteritidis (MB 1409), all own isolates from egg contents, albumen or yolk, were used in the first study. The content isolations were obtained from commercial brown eggs from various production units that were analyzed at expiry date after storage at room conditions. The determination of the egg contents contamination was based on the aseptically removal of the egg contents (for details see paragraph 2.6 in this chapter) and separation of yolk from albumen followed by plating out of both on Nutrient Agar (NA, Oxoid, Basingstoke, UK) and incubation at 30°C for 72h. Species identification was done by 16S rDNA sequencing (Scheldeman et al. 2004). In the second study four different *Salmonella* Enteritidis strains and one *Salmonella* Typhimurium strain were used. The four *Salmonella* Enteritidis strains were originally respectively isolated from two different egg contents (MB 1409 and MB 1419), from a deer (MB 1535) and a lizard (MB 2499); the *Salmonella* Typhimurium (MB 2115) strain was isolated from overshoes taken at the outside environment of a pig farm.

Strains were selected for resistance to streptomycin. The streptomycin resistant bacteria, stored on Protect Beats at - 80°C, were resuscitated by incubation overnight at 30°C in Buffered Peptone Water (BPW, Oxoid) with 25 ppm streptomycin (Sigma-Aldrich, S 6501, St.Louis, USA). This culture was plated on NA with 25 ppm streptomycin and again incubated overnight at 30°C. One colony was grown overnight in 9 ml BPW with 25 ppm streptomycin and 2 ml of the culture was diluted in 200 ml ¼ Ringers solution (Oxoid) to obtain an immersion solution of 10^5 - 10^6 CFU/ml. Enumeration was done by plating 100 µl by spiral-enter (Eddy Jet, IUL instruments, Barcelona) on NA with streptomycin (25 ppm).

2.3 Agar method for the assessment of the eggshell penetration

An agar method described by Berrang *et al.* (1998) was adapted to study and visualize the bacterial eggshell penetration. The egg contents were drained after cutting a hole of approx 1 cm^2 with a rotary tool (Dremel, S-B Power Tool Company, Chicago, USA) and tweezing. After rinsing the inside of the shell with sterile ¼ Ringers solution (Oxoid), in order to remove the albumen adhering to the membranes, the egg was filled with molten (50°C) NA with 25 ppm streptomycin (Sigma-Aldrich), 50 ppm cycloheximide (Sigma-Aldrich, C 7698) and 0.1% 2,3,5 triphenyl tetrazolium chloride (Difco™-TTC, Becton Dickinson and Company, Sparks, USA) (Figure 6.1). After hardening of the agar, the hole was closed with commercial silicone. The addition of streptomycin to the agar assured that only the inoculated streptomycin resistant bacteria were able to grow on the agar, thus holding down all other natural flora competitors present on the fresh eggshell and able to penetrate. Cycloheximide was added to prevent yeast and mould growth. Where bacterial penetration occurred organisms grew on the agar and reduced the TTC to formazan which is red in color (Figure 6.1). Penetration was recorded when red colonies on the agar were visible by candling. Candling was performed daily during the first days of the experiments and three times a week later. Red colonies seen nearby the hole were assumed to result from contamination and not recorded as penetration.

Figure 6.1: Filling up drained eggs with supplemented Nutrient Agar (left); inoculation of eggshell by immersion in a bacterial suspension (middle); visualisation of penetration by candling (right).

2.4 Inoculation and storage

Agar-filled (agar approach) and whole eggs (intact egg approach) were inoculated by immersion for 1 min in Phosphate Buffered Saline (PBS, Oxoid) containing 10^5 - 10^6 CFU/ml of a streptomycin resistant strain of one of the selected species. This resulted in 10^3 - 10^4 CFU of the selected bacterium on the eggshell. After drying at ambient conditions (during 2 h) the eggs were stored in a climate chamber (Termaks KBP 6395 F, Solheimsvinken, Norway) at 20°C and 60% relative humidity (RH) for up to 21 days, *i.e.* the average sell by date in Belgium. This temperature/RH combination resembles the environmental conditions the eggs are exposed to most of the year at the packaging station and the store (see chapters 1, 2 and 4).

2.5 Determination of the eggshell contamination

At day 0 and day 21 the eggshell contamination (detection limit 10 CFU/eggshell) with the selected strains was determined by washing the egg in a plastic bag with diluent and rubbing the eggshell through the bag to detach the bacteria (see chapter 2, paragraph 3.1). The diluent was next plated by a spiral-enter on NA with 25 ppm streptomycin. Plates were incubated at 30°C for 72 h.

2.6 Determination of the egg content contamination

To remove the egg contents of whole eggs aseptically (intact egg approach), a modification of the method described by Himathongkham *et al.* (1999) was used. Each egg was placed in a petri-dish and sprinkled with 75% ethanol. Rolling the egg in the dish with tweezers, the alcohol was burned off during approx 5 s. After a second successive short flaming the

disinfected egg was broken by hand using a sterile blade and sanitized plastic gloves. The whole egg was separated in two fractions; the albumen with yolk and the burned off eggshell with the membranes. Both fractions were enriched in BPW at 30°C for 24 h and plated out on NA with 25 ppm streptomycin. Plates were incubated at 30°C for 72 h.

2.7 Eggshell characteristics

During the eggshell penetration and egg content contamination experiment of the first study, different eggshell characteristics were determined. The shell surface area, the shell thickness, the number of pores, and the cuticle score were studied in the penetration experiment. As the whole egg contamination experiment is a destructive method, only shell surface area and loss of weight at the pores were measured. The egg weight of the fresh eggs was measured and the formula $S = 4.67 \times W^{2/3}$ was used to calculate the shell surface area (Tyler 1953). *S* represents the surface area of the egg in cm^2 and *W* the fresh weight of the egg in g. The shell thickness was determined at three places with a micrometer and the mean value was used for calculations. The number of pores was determined by microscopic counting (ocular x 8, objective x 4) (Olympus BH2-RFCA, Tokyo, Japan) after immersion of pieces of the eggshell for 25 sec in 65% nitric acid solution (Tyler 1953), rinsing with distilled water and removal of the membranes. Fourteen places of approx 11 mm^2 were counted, 7 places at the apex and 7 places at the blunt end. The number of pores was summed and expressed as total number of pores of the entire eggshell. The cuticle score was analyzed by dying with an aqueous mixture of 7.2 g Tartrazine and 28 g Green S per litre (Barentz N.V., Zaventem, Belgium) (also referred to as Edicol Pea Green) (Board and Halls 1973). The cuticle was stained by immersion of the egg for a period of 1 min. The shell was then rinsed with distilled water to remove excess dye, followed by drying. The remaining red colour, *i.e.* the colour at places were the green dye did not bound to, was analysed with Paint Shop Pro version 8 (Jasc Software, Eden Prairie, MN 55344, USA) using the histogram function. Using this method, the red value score or cuticle score is oppositely correlated with the cuticle deposition. Using the intact egg method, the loss of weight was determined for the fresh eggs after exactly 24 h of storage at 20°C and 60%RH. This weight loss is an indicator for the shell porosity.

2.8 Statistical analysis

The bacterial counts were log 10 transformed prior to statistical analysis (Jarvis 1989). Differences in eggshell characteristics and eggshell contamination as function of the presence

of penetration or contamination were assessed with an analysis of variance. A simple linear regression was carried out to determine the influence of hen age on eggshell penetration, whole egg contamination and eggshell characteristics. All analyses were done in Statistica 7 (Statsoft, Tulsa, USA).

There were left and right censored data for bacterial counts simultaneously, as a part of the data consisted of values '<10 CFU/eggshell' and '>3 000 CFU/eggshell'. However, there were exact bacterial counts larger than 3 000 available as well. Hence, we took a different approach for the left and right censored part. Basically, we assumed that the data that were present is the best guess for the data that have to be reconstructed. We constructed distributions, derived from the available data, from which we sampled in a bootstrap procedure. As there are actual data available above 3 000, we constructed an empirical cumulative distribution based on these data. This is equivalent to supposing that the censored data had the same distribution as the available data. This was done separately for each strain inoculated on the agar-filled eggs and on the whole eggs. The values '>3 000' were then each replaced by a random sample from the corresponding distribution. Because there were no exact data available for counts '<10', we fitted a distribution to the data (excluding the censored values) of each strain (agar-filled and whole eggs separately) and extrapolated to the '< 10 zone'. A normal distribution was fitted to the log-transformed data and then truncated between 0 and 1. The values smaller then 10 were then replaced by random samples from this distribution. Finally, a 10 000 iteration bootstrap was done on the averages of each strain of the agar-filled eggs and the whole eggs where the censored data were sampled from the constructed distributions as outlined above. (Manly 1994)

3 RESULTS

3.1 Effects of egg(shell) characteristics on eggshell penetration and whole egg contamination

Table 6.1 shows the mean values with standard deviations (stdev) for each analyzed eggshell characteristic for all eggshells (T), penetrated eggshells (Y) and non-penetrated eggshells (N) (agar approach). Those data are available for the individually selected bacterial species as well as for all bacterial strains combined.

Table 6.1: Eggshell characteristics, shell contamination and eggshell penetration on day 21.

Strain		Nr[c]	Area eggshell (cm^2)[a]	Shell thickness (mm)[a]	Number of pores[a]	Cuticle score[a]	Shell contamination on day 21 (log CFU/shell)[b]
S. warneri	T[d]	61	74.3 ± 3.8	0.417 ± 0.036	6300 ± 2300	93 ± 34	2.5 ± 0.9
(MB 2792)	Y[e]	9	74.8 ± 5.4	0.407 ± 0.016	5500 ± 1600	120 ± 40	3.5 ± 1.1^{A}
	N[f]	52	74.2 ± 3.5	0.419 ± 0.038	6400± 2400	89 ± 32	2.3 ± 0.8^{A}
Carnobacterium sp	T	60	74.1 ± 4.6	0.410 ± 0.035	5900 ± 2200	85 ± 40	1.6 ± 1.4
(MB 2796)	Y	13	74.8 ± 3.0	0.412 ± 0.035	6000 ± 2800	122 ± 36^{AAA}	2.5 ± 2.1
	N	47	73.9 ± 4.9	0.409 ± 0.036	5800 ± 2100	75 ± 35^{AAA}	1.4 ± 1.1
Alcaligenes sp.	T	57	75.9 ± 4.2	0.424 ± 0.037	5800 ± 2100	93 ± 34	3.7 ± 2.4
(MB 2794)	Y	33	76.8 ± 3.8	0.419 ± 0.037	5900 ± 2000	100 ± 38	5.0 ± 1.9^{CCC}
	N	24	74.7 ± 4.5	0.432 ± 0.036	5800 ± 2400	81 ± 24	1.8 ± 1.7^{CCC}
A. baumannii	T	62	74.1 ± 5.4	0.418 ± 0.036	5700 ± 2600	84 ± 33	2.0 ± 1.3
(MB 2793)	Y	15	74.8 ± 3.3	0.418 ± 0.033	5500 ± 2200	98 ± 41	3.3 ± 1.5^{AA}
	N	47	73.9 ± 5.9	0.418 ± 0.037	5800 ± 2700	79 ± 28	1.7 ± 0.9^{AA}
Pseudomonas sp.	T	52	75.5 ± 3.8	0.417 ± 0.032	6700 ± 6700	98 ± 36	3.6 ± 2.2
(MB 2797)	Y	31	76.1 ± 4.1	0.417 ± 0.035	7600 ± 8400	103 ± 42	4.7 ± 1.7^{DDD}
	N	21	74.7 ± 3.3	0.417 ± 0.028	5400 ± 2800	91 ± 25	2.1 ± 1.8^{DDD}
Salmonella	T	51	75.0 ± 4.5	0.417 ± 0.029	5800 ± 2400	98 ± 38	2.5 ± 1.8
Enteritidis	Y	22	75.3 ± 4.5	0.426 ± 0.027	5800 ± 2300	107 ± 41	3.4 ± 1.7^{BB}
(MB 1409)	N	29	74.9 ± 4.6	0.411 ± 0.029	5700 ± 2500	92 ± 36	1.8 ± 1.6^{BB}
S. marcescens	T	60	74.7 ± 3.9	0.420 ± 0.033	5800 ± 2400	87 ± 28	1.0 ± 0.7
(MB 2795)	Y	8	76.0 ± 3.5	0.425 ± 0.039	6300 ± 2700	96 ± 25	1.9 ± 1.0^{B}
	N	52	74.5 ± 3.9	0.419 ± 0.032	5700 ± 2400	85 ± 28	0.9 ± 0.6^{B}
All bacterial strains	T	403	74.8 ± 4.4	0.418 ± 0.034	6000 ± 3300	91 ± 35	2.3 ± 1.8
	Y	131	75.8 ± 4.0	0.419 ± 0.033	6200 ± 4500	105 ± 39^{BBB}	3.8 ± 1.6^{EEE}
	N	272	74.3 ± 4.5	0.417 ± 0.035	5900 ± 2400	84 ± 31^{BBB}	1.6 ± 1.2^{EEE}

[a] Values are means ± stdev; [b] Values are means ± stdev after log 10 transformation; [c] Number of eggs; [d] Total eggshells; [e] Penetrated eggshells; [f] Non-penetrated eggshells

[A, B, ...] Means with the same letter are significantly different ($P < 0.05$); [AA, BB, ...] Means with 2 same letters are highly significantly different ($P < 0.01$); [AAA, BBB, ...] Means with 3 same letters are extremely significantly different ($P < 0.001$)

Table 6.2 shows the data for the whole egg contamination experiment; all whole eggs (T), contaminated whole eggs (Y) and non-contaminated whole eggs (N) (intact egg approach).

Table 6.2: Eggshell characteristics, shell contamination and whole egg contamination on day 21.

Strain		Nr[c]	Area eggshell (cm^2) [a]	Nr[c]	Loss of weight after 24 h (g)[a]	Nr[c]	Shell contamination on day 21 (log CFU/shell)[b]
S. warneri	T[d]	55	75.0 ± 3.7	45	0.308 ± 0.151	51	3.0 ± 0.7
(MB 2792)	Y[e]	8	76.0 ± 4.1	7	0.324 ± 0.234	7	3.3 ± 0.4
	N[f]	47	74.8 ± 3.7	38	0.304 ± 0.135	44	3.0 ± 0.7
Carnobacterium sp.	T	53	74.3 ± 4.0	44	0.335 ± 0.166	45	1.1 ± 0.7
(MB 2796)	Y	10	73.4 ± 3.3	10	0.263 ± 0.119	8	1.1 ± 0.7
	N	43	74.6 ± 4.1	34	0.356 ± 0.174	37	1.1 ± 0.6
Alcaligenes sp.	T	55	74.0 ± 3.8	45	0.322 ± 0.119	53	1.3 ± 0.8
(MB 2794)	Y	7	75.2 ± 4.8	7	0.311 ± 0.145	7	1.2 ± 0.6
	N	48	73.8 ± 3.7	38	0.324 ± 0.116	46	1.3 ± 0.8
A. baumannii	T	56	74.8 ± 3.7	46	0.305 ± 0.154	54	1.9 ± 0.7
(MB 2793)	Y	8	75.4 ± 3.8	8	0.290 ± 0.175	8	2.0 ± 0.9
	N	48	74.6 ± 3.7	38	0.308 ± 0.152	46	1.9 ± 0.6
Pseudomonas sp.	T	44	74.2 ± 4.4	43	0.358 ± 0.206	44	1.5 ± 1.0
(MB 2797)	Y	5	73.0 ± 3.2	5	0.256 ± 0.080	5	2.6 ± 1.2
	N	39	74.3 ± 4.6	38	0.371 ± 0.214	39	1.4 ± 0.9
Salmonella	T	45	75.5 ± 4.1	36	0.356 ± 0.220	45	1.3 ± 0.8
Enteritidis	Y	15	75.6 ± 4.7	10	0.402 ± 0.255	15	1.6 ± 0.9
(MB 1409)	N	30	75.5 ± 3.8	26	0.338 ± 0.207	30	1.2 ± 0.7
S. marcescens	T	56	74.8 ± 4.0	46	0.505 ± 1.147	47	1.4 ± 1.0
(MB 2795)	Y	5	73.8 ± 3.0	5	0.346 ± 0.137	4	2.4 ± 2.4
	N	51	74.9 ± 4.1	41	0.524 ± 1.215	43	1.3 ± 0.9
All bacterial strains	T	364	74.7 ± 3.9	305	0.356 ± 0.473	339	1.7 ± 1.0
	Y	58	74.8 ± 4.0	52	0.316 ± 0.178	54	1.9 ± 1.1[AA]
	N	306	74.6 ± 3.9	253	0.364 ± 0.513	285	1.7 ± 1.0[AA]

[a] Values are means ± stdev; [b] Values are means ± stdev after log 10 transformation; [c] Number of eggs; [d] Total whole eggs; [e] Contaminated whole eggs; [f] Non-contaminated whole eggs
[AA] Means with 2 same letters are highly significantly different ($P < 0.01$ and > 0.001)

Evaluation of the data (Table 6.1) showed no significant difference between area eggshell, shell thickness and number of pores and the presence or absence of bacterial eggshell penetration. For each individual strain and for the general results of all strains the mean eggshell area of the penetrated eggshells was higher, but not significant, compared to the non-penetrated eggshells. The mean cuticle score was higher for penetrated compared to non-penetrated eggshells (individual strain and all strains). For the individual strain *Carnobacterium* sp. and for the general result of all strains this difference was significant ($P < 0.001$). Using our method, the cuticle score is oppositely correlated with the cuticle deposition; the higher cuticle score corresponds with a lower cuticle deposition.

Table 6.2 shows that the whole egg contamination was not influenced by either the area of the eggshell or by the porosity of the eggshell (loss of weight after 24h).

3.2 Effect of bacterial survival on the eggshell penetration and whole egg contamination

The individual data per selected strain and the general data (all bacterial strains) obtained with the agar approach, showed a higher count of the inoculated strain on the eggshell at day 21

(shell contamination on day 21) for penetrated eggshells (Y) compared to non-penetrated eggshells (N) (Table 6.1). This higher count was even significant for the general data (P < 0.001) and for six of the seven selected strains; respectively for *S. warneri*, *Alcaligenes* sp., *A. baumannii*, *Pseudomonas* sp., *Salmonella* Enteritidis and *S. marcescens* (respectively P = 0.011, < 0.001, 0.0018, < 0.001, 0.0016 and 0.0038). Figure 6.2 shows the box plot of the bacterial count on the eggshell at day 21 for penetrated compared to non-penetrated eggshells, considering all selected strains.

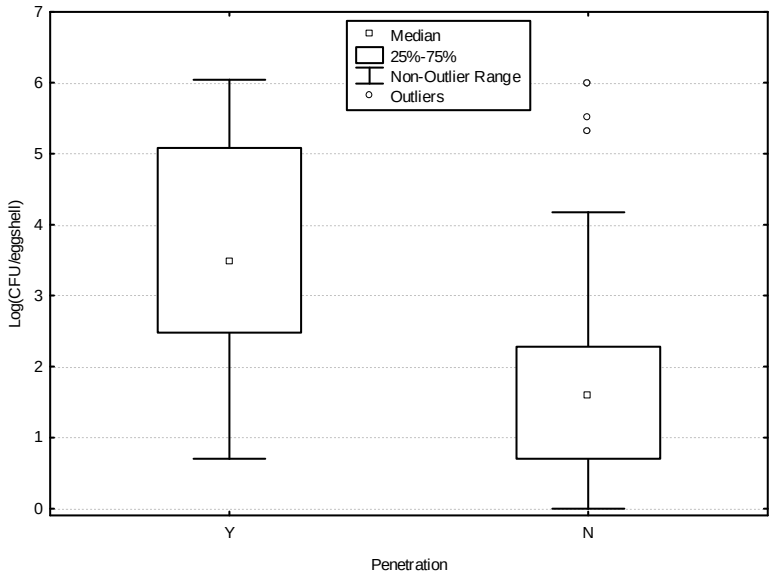

Figure 6.2: Total count of inoculated species on the eggshell of penetrated (Y) and non-penetrated eggshells (N) considering all strains.

The count of bacteria on the shell of whole eggs was on average 0.6 log CFU/shell lower compared to agar-filled shells; respectively 1.7 versus 2.3 log CFU/shell (Table 6.1 and 6.2). For 5 of the 7 selected strains the contaminated whole eggs had a (slightly) higher count of the inoculated strain on the eggshell at day 21; for none of the strains this was significant. The overall data of all strains showed that the count on the eggshell of the contaminated whole eggs was significantly higher (P = 0.0029); 1.89 log CFU/shell versus 1.66 log CFU/shell for the non-contaminated whole eggs (Figure 6.3).

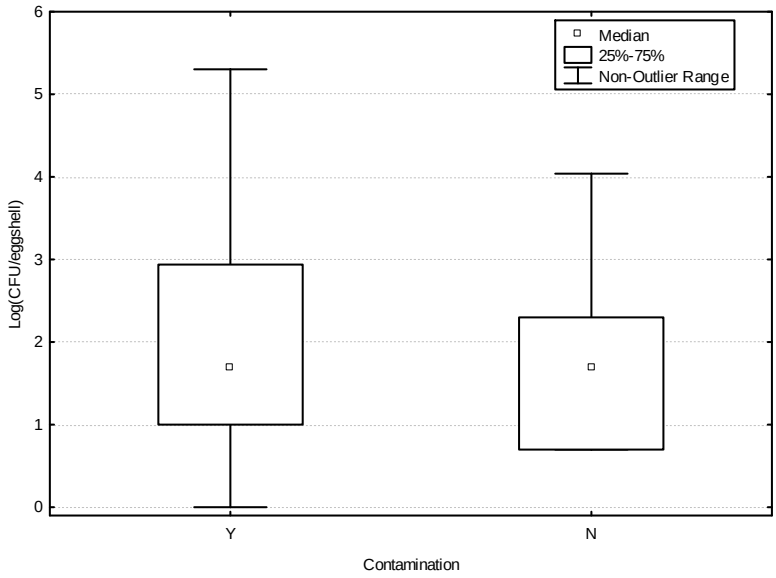

Figure 6.3: Total count of inoculated species on the eggshell of contaminated whole eggs (Y) and non-contaminated whole eggs (N) considering all strains.

3.3 Effect of storage time on eggshell penetration

Independent of the selected strain, the eggshell penetration was observed most frequently at approx day 4 - 5 (Figure 6.4). At day 6 and day 14, respectively, up till 80% and more than 95% of the total eggshell penetration was observed. The histograms (not shown) of the penetration days for each individual strain are comparable; most penetration spots appeared before day 6.

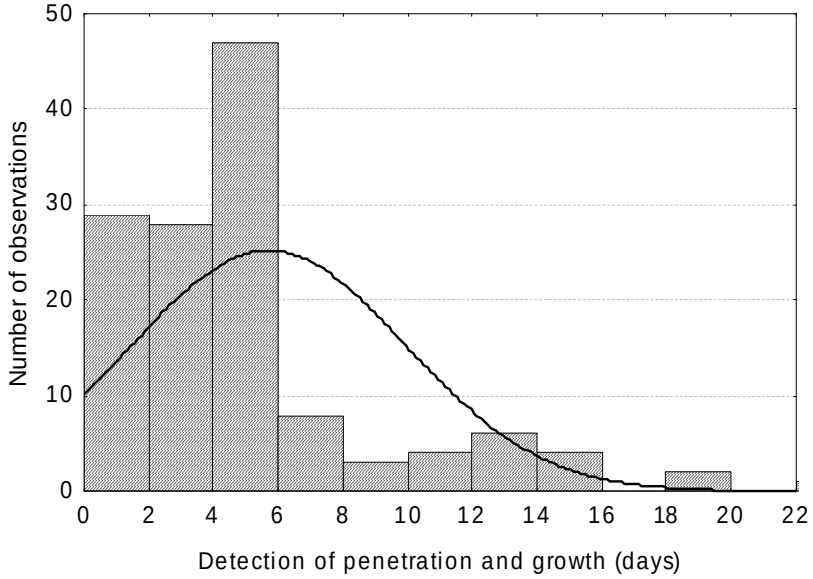

Figure 6.4: Histogram of the penetration day independent of the selected strain (*n* = 131).

3.4 Effect of bacterial strain on eggshell penetration and whole egg contamination

Figure 6.5a shows the percentage of eggshell penetration (agar approach) for all strains used, after 21 days of incubation. *Pseudomonas* sp. and *Alcaligenes* sp followed by *Salmonella* Enteritidis penetrated most frequently the eggshell. They accounted for 60, 58 and 43% of the agar-filled eggs penetration, respectively. Figure 6.5b shows the percentages of whole egg contamination (intact egg approach). The egg contents of whole eggs were most frequently contaminated by *Salmonella* Enteritidis (33%) followed by *Carnobacterium* sp. (17.5%). All strains were able to penetrate in agar-filled eggs (eggshell penetration) as well as to contaminate whole eggs (whole egg contamination). Of the 403 agar-filled eggs, 131 (33%) were penetrated by the selected strains compared to a content contamination of 16% (60 on 385) whole eggs. The fraction albumen and yolk from whole eggs was contaminated for 11% (42 on 385) while 15% (56 on 385) of the eggshells (outside decontaminated) with membranes attached were positive.

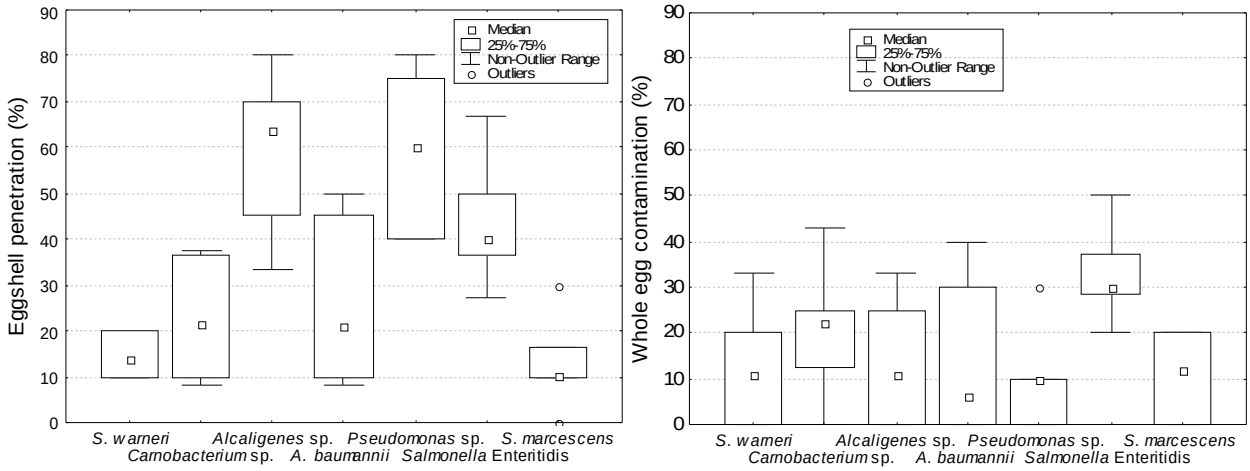

Figure 6.5 a: Percentage of eggshell penetration for each individual bacterial strains.

Figure 6.5 b: Percentage whole egg contamination for each individual bacterial strains.

3.5 Effect of hen age on eggshell penetration, whole egg contamination and eggshell characteristics

Bacterial eggshell penetration and egg content contamination for all 7 selected strains was studied on eggs laid at 34, 46, 69 and 74 weeks of hen age using the agar approach and the intact egg approach (Figures 6.6a and b). The results showed that the bacterial eggshell penetration remained almost constant during the entire laying period. At week 34, 46, 60, 69 and 74 average penetration percentages for all selected strains together were respectively 30, 39, 41, 33 and 37%. The whole egg contamination increased slightly with hen age from

respectively 13%, 13% and 15% in week 34, 46 and 60 till 26% and 20% in week 69 and 74 (not significant; $P = 0.167$).

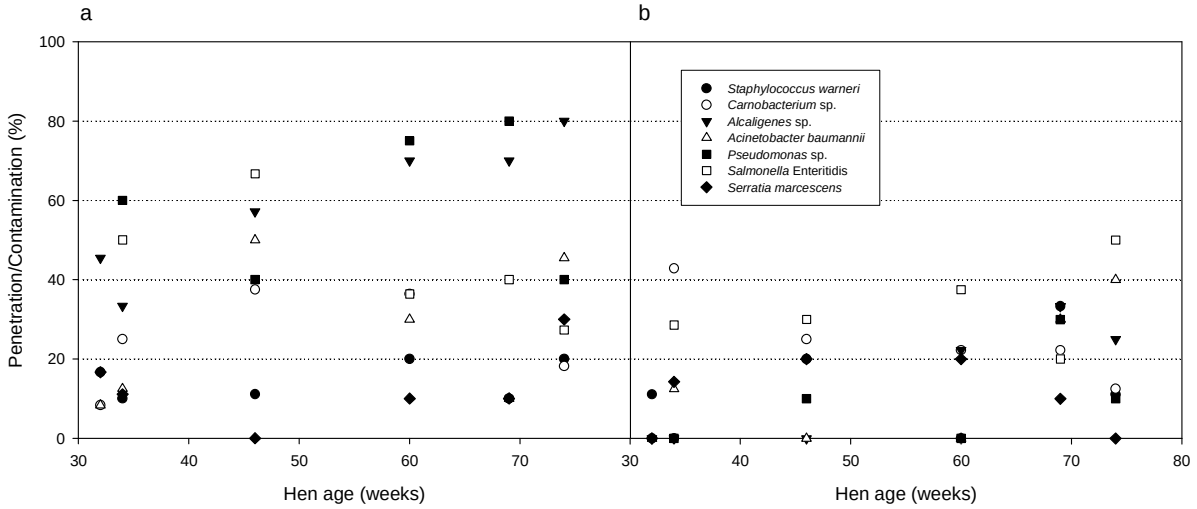

Figure 6.6a: Bacterial eggshell penetration of each selected strain during laying period.

Figure 6.6b: Bacterial whole egg contamination of each selected strain during laying period.

The eggshell characteristics shell thickness and shell area were significantly influenced by hen age, albeit very weak; shell thickness decreased while shell area increased (Figure 6.7).

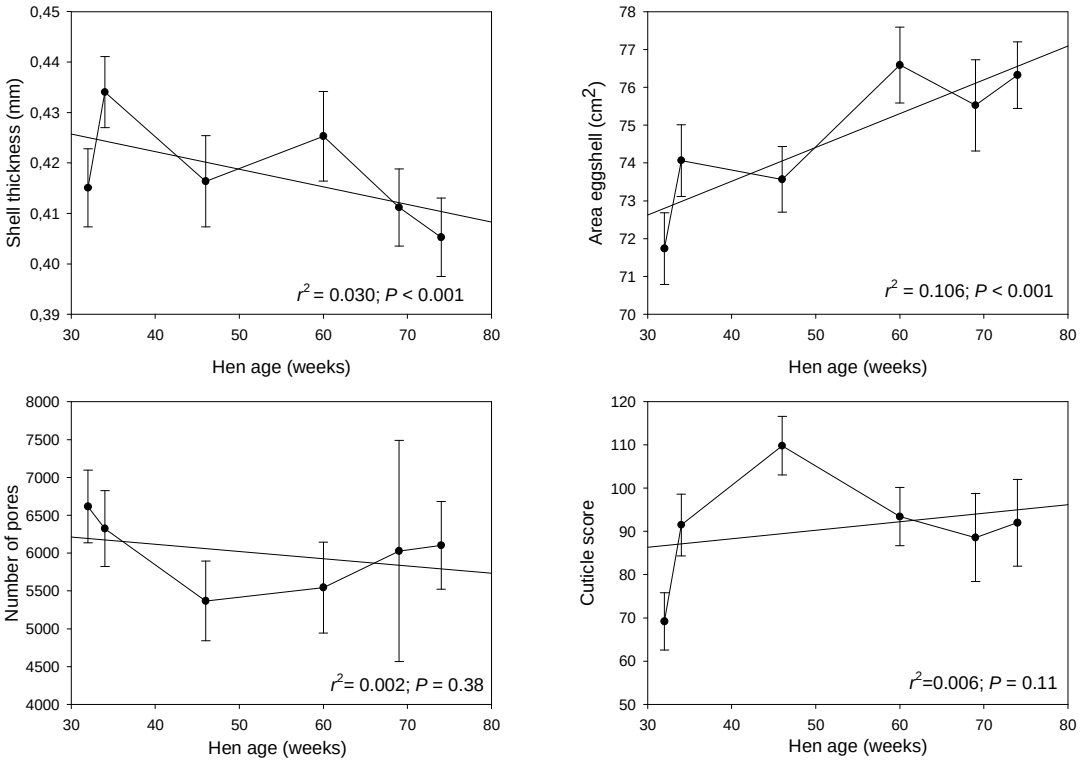

Figure 6.7: Influence of hen age on several egg characteristics. Vertical bars denote 95% confidence intervals. (Full line is linear regression curve, r^2 = correlation coefficient and P = significance slope)

3.6 Whole egg contamination with the different *Salmonella* strains

For the second study; contamination percentages of 18% (MB 1409), 6% (MB 1419), 14% (MB 1535) and 26% (MB 2499) for *Salmonella* Enteritidis isolated respectively from two different egg contents, a deer and a lizard; and of 24% for *Salmonella* Typhimurium (MB 2155) isolated from overshoe of a pig house were found. Fifty intact whole eggs were used in each case (Figure 6.8). Average eggshell contaminations on day 21 were comparable.

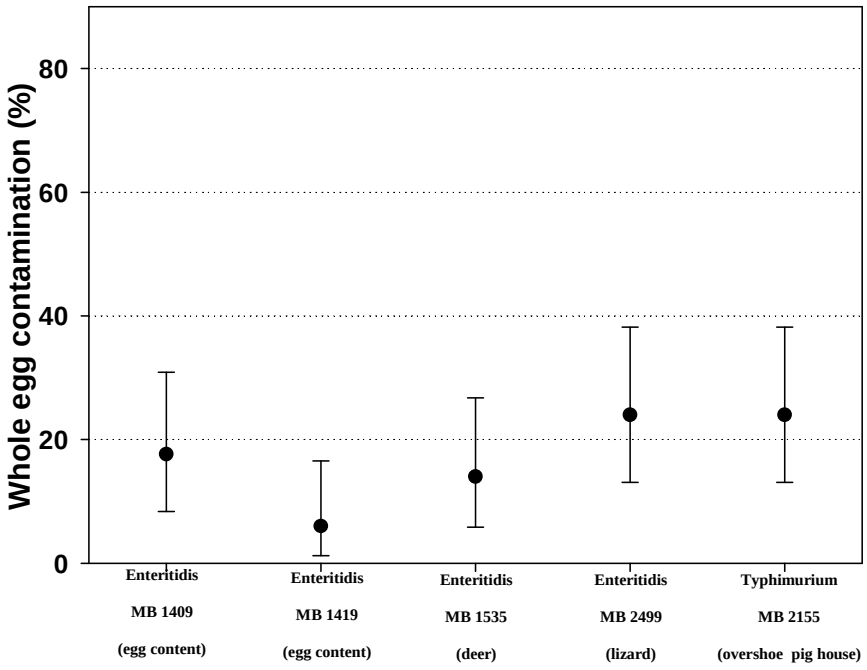

Figure 6.8: Bacterial whole egg contamination for each selected *Salmonella* strain. Vertical bars denote 95% confidence intervals.

4 DISCUSSION

The area of the shell of penetrated eggshells or contaminated whole eggs was not significantly higher compared to non-penetrated shells or non-contaminated whole eggs (Table 6.1 and 6.2). Smeltzer *et al.* (1979b), using the agar method, reported also a shell penetration that was independent of the shell surface area.

In agreement with our results, Williams *et al*. (1968) and Messens *et al.* (2005a) reported that shell thickness did not significantly affect the penetration with *Salmonella* Typhimurium and *Salmonella* Enteritidis respectively. Smeltzer *et al.* (1979b) concluded the same, using agar-filled eggs and the bacterial eggshell flora of nest box and floor eggs. Orel (1959) and Sauter

and Petersen (1969; 1974), using whole eggs, reported the opposite. Eggs with shells of high quality, i.e. high specific gravity (sp.gr.), were more resistant to penetration by *Pseudomonas fluorescens* (Orel 1959; Sauter and Petersen 1969) and *Salmonella* Enteritidis (Sauter and Petersen 1974). The sp. gr. measurements gave an indication of the shell thickness.

The primordial route for bacteria to penetrate intact eggs are the pores with diameters in the range of 6 - 65 µm (Tyler 1953; Tyler 1956), far above the bacterial dimensions. We did not found a correlation between the number of pores and the bacterial eggshell penetration and between the loss of weight at the pores and the whole egg contamination. Fromm and Monroe (1960) and Board and Halls (1973) correlated porosity with bacterial penetration; Reinke and Baker (1966) refuted this view. The studies of Hartung and Stadelman (1963), Nascimento *et al.* (1992) and Messens *et al.* (2005a) also supported that bacterial eggshell penetration is not pore dependent.

The cuticle on the eggshell serves as a water proofing agent and as a barrier of primary importance for particle, bacterial and fungal invasion (Board and Halls 1973). In our study a significant lower cuticle deposition was found on penetrated eggshells compared to non-penetrated eggshells. Alls *et al.* (1964) found that cuticle removal increased microbial contamination from 20 to 60%. Drysdale (1985) found also a significantly higher bacterial contamination in eggs which had a poor cuticle (40%) compared to eggs with a medium or good quality cuticle (26%). The defence of the cuticular layer has on the other hand been questioned by Nascimento *et al.* (1992) and Messens *et al.* (2005a) using agar-filled eggs.

A correlation was found between bacterial eggshell contamination with the inoculated strain(s) on day 21 and shell penetration and whole egg contamination with the strain(s). This corresponds with ample evidence in the literature that eggs with highly contaminated eggshells suffer more from bacterial spoilage or whole egg contamination. Smeltzer *et al.* (1979b) found that floor eggs had a higher incidence of bacterial contamination (15,3%) compared to nest eggs (10.5%). Making comparison between eggs laid in roll away cages (2.6×10^4 CFU/eggshell) and laid in nests (3.4×10^5 CFU/eggshell), Harry (1963) found higher contamination of whole eggs suffering from more bacterial eggshell contamination. Messens *et al.* (2005a) also showed a correlation between counts of *Salmonella* Enteritidis on the eggshell and the probability of eggshell penetration. As different researchers (Board and Halls 1973; Board *et al.* 1979) showed that bacteria such as *Pseudomonas* spp., *Alcaligenes brookeri* and *Streptomyces* can only digest the cuticle when humidity approaches 100%, the minor cuticle deposition we found for all strains at day 21 could not be caused by the higher bacterial loading on penetrated eggshells. The count of inoculated bacteria on day 21 on the

shell of whole eggs was on average 0.6 log CFU/shell lower compared to agar-filled eggs. This may suggest that nutrients available from the agar favour the survival and growth on the shell of agar-filled eggs and/or that the antimicrobial components of the egg content of whole eggs do not stimulate survival or growth.

Using the agar approach *Pseudomonas* sp., *Alcaligenes* sp and *Salmonella* Enteritidis penetrated most frequently (Fig. 6.5a); respectively for 60, 58 and 43% of the inoculated eggshells. The higher shell contamination (on day 21) with *Pseudomonas* sp. and *Alcaligenes* sp. (Table 6.1) can explain the higher fraction of penetrated eggshells. Notwithstanding the comparable eggshell contamination with *Salmonella* Enteritidis and *S. warneri* (both 2.5 log CFU/eggshell) on day 21, penetration prevalence with *Salmonella* was higher (43% versus 18%). It is likely that the motile, non-clustering properties of *Salmonella* favour the eggshell penetration; *Pseudomonas* sp. and *Alcaligenes* sp. also have these properties. Berrang *et al.* (1998), using an agar approach, found 67% penetration with *Salmonella* Typhimurium for eggs sampled at hen ages ranging from week 29 - 56. Eggs were dipped into a 10^4 CFU/ml suspension. Messens *et al.* (2005a), using a inoculation suspension of 10^6 *Salmonella* Enteritidis CFU/ml, found 39% of eggshells penetrated.

Using the intact egg approach in the first study; *Salmonella* Enteritidis followed by *Carnobacterium* sp. seemed to penetrate, survive and eventually grow most frequently (Fig. 6.5b); respectively 33% and 17.5% of the inoculated eggs. Sauter and Petersen (1974) found a contamination average of 47.5% for various salmonellae using whole eggs of poor shell quality (sp. gr. 1.070) and 21.4% and 10.0% for whole eggs of intermediate (sp. gr. 1.080) and excellent shell quality (sp. gr. 1.090), respectively. Eggs were dipped for 3 minutes into solutions containing approx 1.0×10^4 *Salmonella* CFU/ml. Sauter and Petersen (1969) challenged eggs (challenge suspension 1.1×10^6 CFU/ml) with different sp. gr. with *P. fluorescens* and found an incidence of fluorescent spoilage for eggs of high, medium and low levels of shell quality (sp. gr. of 1.085, 1.077 and 1.070 respectively) of 6.3, 19.4 and 29.1% after 8 weeks of storage. In addition, microbiological examination of the eggs that did not show fluorescence by eight weeks indicated that 45% of the eggs also contained viable micro-organisms. In our study 10.5% of the whole eggs were contaminated with *Pseudomonas* sp. Despite the antimicrobial defenses of the membranes and the albumen all selected bacterial strains were able to penetrate the membranes and remain viable during up till 21 days in the albumen. The high prevalence of *Salmonella* Enteritidis and even of the Gram-positive *Carnobacterium* sp. indicates that notwithstanding the antimicrobial aspects of the albumen the survival after penetration of the shell may not be underestimated. Recent research shows a

higher resistance of *Salmonella* Enteritidis to egg albumen compared to other salmonellae; Lu *et al.* (2003) reported the identification of *yafD* as a gene essential for resistance of *Salmonella* Enteritidis to egg albumen. Mayes and Takeballi (1983) reported especially Gram-negative bacteria as *Alcaligenes*, *Pseudomona*s and *Aeromonas* as most common natural contaminants of whole eggs. In our study *Alcaligenes* sp. contaminated 14% of the whole eggs. Notwithstanding *S. warneri* counts on the eggshell on day 21 was higher compared to all other selected strains (Table 6.2); this did not result in higher whole egg contamination prevalence.

Independent of the selected strain, the eggshell penetration was observed most frequently at day 4 - 5 after inoculation of the eggs (Figure 6.4). Taking into account the necessary time for growth of the bacteria on the agar to initiate the appearance of the red spots (formazan) we can conclude most eggshell penetration occurred within 0 - 2 days after inoculation. Williams *et al.* (1968) demonstrated that penetration of the cuticle and the shell by salmonellae occurred almost immediately in some eggs. Messens *et al.* (2005a) found most eggshells being penetrated with *Salmonella* Enteritidis on day 3. Other researchers have demonstrated bacterial penetration in 25 - 60% of inner membranes and in 5 - 15% of albumen in whole eggs on the first day of inoculation (Muira *et al.* 1964; Humphrey *et al.* 1989; Humphrey *et al.* 1991b). Using whole eggs, on day 21 we found 15% of the (outside disinfected) eggshells with membranes being contaminated compared to 11% of the egg contents (albumen and yolk).

Nascimento *et al.* (1992) reported, using an agar approach, an increasing eggshell penetration from 12.9% (beginning of lay) till 25.0% (end of lay) for *Salmonella* Enteritidis (challenge suspension 3×10^3 CFU/ml). In our study (agar approach), eggshell penetration with *Salmonella* Enteritidis even decreased from 50% and 66.7% respectively in week 34 and 46 till 40% and 27%, respectively, in week 69 and 74. This is comparable with Messens et al. (2005a) finding a lower fraction of penetrated eggshell as flock aged, 31.6% of the shells were penetrated at the late end of lay compared to 45.0% at the beginning of lay. Berrang *et al.* (1998), using *Salmonella* Typhimurium, found an upward correlation between number of penetrated eggshells and flock age approaching significance. Our obtained results of all strains (agar approach) showed an almost constant bacterial eggshell penetration during the entire laying period.

The study of Bruce and Johnson (1978) reported for hatching eggs an increasing contamination of whole eggs as flocks became older. Data from Jones *et al.* (2002), using whole eggs, *Salmonella* Enteritidis and *P. fluorescens* (challenge suspension 10^6 CFU/ml),

suggest also that bacterial contamination of air cells, shell membranes and egg contents is more easily achieved in eggs from older hens than from younger hens. In our study whole egg contamination (all strains) slightly increased, respectively, from 13, 13 and 15% in week 34, 46 and 60 till 26 and 20% in week 69 and 74.

Wells (1968) found that old hens lay bigger eggs which have a lower specific gravity and thinner shells. In our study shell thickness also decreased while shell area increased (Figure 6.7). Those two changing eggshell characteristics during flock age did not influence the eggshell penetration. Berrang *et al.* (1998), using an agar approach, did not observe a decline in eggshell quality through flock life, but *Salmonella* Typhimurium penetration patterns varied. They concluded it was likely that other factors than specific gravity and conductance are involved in the bacterial penetration of the eggshell. Messens *et al.* (2005a) found that the variation in shell characteristics were independent of the hen age.

The second study showed no higher resistance of *Salmonella* Enteritidis to egg albumen compared to other salmonellae like *Salmonella* Typhimurium (Figure 6.8). *Salmonella* Enteritidis strains originally isolated from the egg content were also not the primary invaders of the egg content. The first study indicated the potential of *Salmonella* Enteritidis strains to penetrate eggshells and to contaminate whole eggs by the horizontal infection route. Knowing, however, that *Salmonella* Enteritidis is the most frequently isolated *Salmonella* serovar in eggs, the results of the second study do not show any special capacity of egg related *Salmonella* Enteritidis strains compared to other *Salmonella* Enteritidis strains and *Salmonella* serotypes to contaminate whole eggs. This indicates that the frequent egg contamination with *Salmonella* Enteritidis would be mainly due to the transovarian or vertical route, as supported by Humphrey (1994a). The results of this second study also do not support the higher resistance of *Salmonella* Enteritidis to egg albumen as reported by Lu *et al.* (2003). A different percentage of contaminated whole eggs were observed for the same *Salmonella* Enteritidis strain (MB 1409) (an average of 33% during the entire laying period and 32% at the hen age of 46 weeks in the first experiment versus 18% at the hen age of 45 weeks in the second experiment). Probably small differences in experimental conditions or not identified differences in egg content can explain these observations.

5 CONCLUSIONS

The agar approach seemed to be most suited to study the influence of the egg(shell) characteristics on the bacterial eggshell penetration, but it gives no estimation of the

contamination of whole eggs. The intact egg approach gave an estimation of the penetration of the shell followed by the probability of survival and migration in whole eggs. The cuticle seems to be the only analyzed eggshell characteristic influencing the bacterial eggshell penetration; a major cuticle deposition stood for less bacterial penetration. The probability of eggshell penetration is correlated with the eggshell contamination; this is less obvious for the egg content contamination. An average eggshell penetration of 33% is only reduced to an average of 16% whole egg contamination (7 selected strains); indicating the limited antimicrobial aspects of the albumen. Compared to the non-*Salmonella* strains, *Salmonella* Enteritidis was a primary invader of whole eggs. However, egg related *Salmonella* Enteritidis strains have no special capability to contaminate whole eggs by the horizontal infection route compared to other *Salmonella* Enteritidis strains and the *Salmonella* Typhimurium strain.

During storage, eggs are sometimes cooled for a short period (see chapter 2 and 4). It is well known that eggs held at lower temperature have condensate on the shell when moved into a warmer environment (ambient conditions). In chapter 7 the influence of eggshell condensate on the bacterial eggshell penetration and the whole egg contamination is studied using the agar and intact egg approach of the present chapter.

Chapter 7

Influence of eggshell condensation on the bacterial eggshell penetration and the whole egg contamination with *Salmonella* Enteritidis

Redrafted after:

Influence of eggshell condensation on the bacterial eggshell penetration and the whole egg contamination with *Salmonella enterica* serovar Enteritidis

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CHAPTER 7: Influence of eggshell condensation on the bacterial eggshell penetration and the whole egg contamination with *Salmonella* Enteritidis

Abstract

Shells of agar-filled and whole eggs were inoculated with 10^3 - 10^4 CFU Salmonella *Enteritidis per eggshell. The agar-filled eggs were used to study the bacterial eggshell penetration; the whole egg results were used to characterize the contamination of the egg content. Of each group, half of the eggs were stored for 21 days at 20°C and 60% relative humidity (RH); while the other half was first stored for 24 h at 6°C before storage at 20°C. The latter resulted in condensation on the eggshell for 30 min from the moment the eggs were placed at 20°C. Taking into account the three hen ages studied (39, 53 and 67 weeks) an average of 62% of the eggshells with condensate were penetrated compared to 43% for the control group; this difference was statistically significant (*$P < 0.01$*). No significant difference in whole egg contamination was found; 18% of the control eggs were contaminated compared to 22% of the condensate eggs. Remarkable was the significantly higher whole egg contamination of eggs at the end of lay compared to the eggs sampled from the two earlier hen ages. This was probably not due to a higher penetration potential as this was not observed in the corresponding agar-filled eggs. It can be concluded that condensation on the eggshell encouraged the bacterial eggshell penetration, but had a smaller impact on the whole egg contamination.*

1 INTRODUCTION

Salmonella infection resulting from the consumption of contaminated eggs is still a major public health problem. *Salmonella* Enteritidis is responsible for the majority of egg-associated infections. Two possible routes of *Salmonella* contamination of intact eggs have been considered: transovarian or "vertical" transmission of *Salmonella* Enteritidis occurs when eggs are infected during their formation in the hen's ovaries, while horizontal transmission occurs when eggs are subsequently exposed to an environment contaminated with *Salmonella* Enteritidis and the micro-organism penetrates the eggshell. Studies from Sauter and Petersen (1974), Nascimento and Solomon (1991) and we in chapter 6 suggest a relationship between eggshell quality and bacterial eggshell penetration and/or whole egg contamination (horizontal transmission). Harry (1963), Smeltzer *et al.* (1979a) and we in chapter 6 also reported a correlation between the degree of bacterial eggshell contamination and egg infection. Data available on the occurrence of *Salmonella* contaminated eggshells and egg contents are discussed in chapter 1, paragraph 5.2.

A study by Fromm and Margolf (1958) reported that sweating of the eggshell caused an increased bacterial contamination of the egg contents. A more recent study of Ernst *et al.* (1998) reported no increase of the fraction of *Salmonella* Enteritidis positive eggs or the numbers of *Salmonella* Enteritidis present in the egg content, due to eggshell sweating for 30 min. The latter study also mentioned that additional research was needed to determine the relationship between sweating of or condensation on eggshells and bacterial penetration of the shell. In this study the influence of condensate on the bacterial eggshell penetration on the one hand and the whole egg contamination on the other hand was studied.

2 MATERIALS AND METHODS

2.1 Eggs

Eggs from a commercial conventional housing system, with ISA Brown laying hens, were collected at the day of lay at the hen ages of 39, 53 and 67 weeks. Next day (after storage at ambient conditions), eggs were visually inspected by candling and only intact eggs (no cracks, no pin-holes) were included in further analyses.

2.2 Agar method for the assessment of the eggshell penetration

The agar method as described in detail in chapter 6, paragraph 2.3 was used to study and visualize the bacterial eggshell penetration. In short, this method consisted of replacing the egg content by sterile molten Nutrient Agar (NA, Oxoid Basingstoke, UK), containing streptomycin (Sigma-Aldrich, S 6501, St-Louis, USA), cycloheximide (Sigma-Aldrich, C 7698) (preventing yeast and mould growth) and the indicator 2,3,5- triphenyl-tetrazolium-chloride (Difco^{TM} TTC, Becton Dickinson and Company, Sparks, USA). The addition of streptomycin to the agar assured that only the inoculated streptomycin resistant *Salmonella* Enteritidis strain was able to grow on the agar. Where bacterial eggshell penetration occurred, *Salmonella* Enteritidis grew on the agar and reduced the TTC to the red coloured formazan (see chapter 6, Figure 6.1). Candling was performed daily during the first week and three times a week later.

2.3 Inoculation and storage

Agar-filled and whole eggs were inoculated with a streptomycin resistant strain of *Salmonella* Enteritidis (MB 1409, a strain that was isolated from egg contents at our laboratory). Inoculation was performed by immersion as described in chapter 6, paragraph 2.4. This resulted in approx 10^3 - 10^4 CFU *Salmonella* Enteritidis on the eggshell. After drying at ambient conditions the eggs were stored for up to 21 days.

2.4 Determination of the eggshell contamination

At the day of inoculation (day 0) and 21 days later, the eggshell contamination (detection limit 10 CFU/eggshell) with the selected *Salmonella* Enteritidis strain was quantified by adding 10 ml diluent to an agar-filled egg or a whole egg in a plastic bag, and by rubbing the eggshell through the bag to detach the bacteria (see chapter 2, paragraph 3.1). The diluent was next plated by a spiral-enter (Eddy Jet, IUL instruments, Barcelona) on NA with 25 ppm streptomycin. Plates were incubated at 30°C for 72 h.

2.5 Determination of the egg content contamination of whole eggs

The egg content contamination of whole eggs was determined using the method as described in chapter 6, paragraph 2.6.

2.6 Eggshell characteristics

The shell surface area, dynamic stiffness (k_{dyn}), damping of the vibration and resonance frequency were studied on the fresh eggs immediately after candling (detection of cracks, pin-holes …), *i.e.* before the penetration experiment. When the eggshell penetration (agar-filled eggs) experiment was completed, the following eggshell characteristics were determined: shell thickness, number of pores and cuticle score. The dynamic stiffness, damping of the vibration and resonance frequency were measured using a desktop unit to detect eggshell breakage and shell strength, based on vibration measurements (Coucke 1998; De Ketelaere *et al.* 2004). The methods used to determine the other eggshell characteristics are outlined in chapter 6, paragraph 2.7.

As the determination of the whole egg contamination is a destructive method, only the egg(shell) characteristics shell surface area, dynamic stiffness, damping of the vibration and resonance frequency could be measured in this experiment.

2.7 Condensation experiment

At the hen ages of 39, 53 and 67 weeks, in each case 105 agar-filled eggs and 105 whole eggs were inoculated. The first group of eggs was used to study the bacterial eggshell penetration (eggshell and membranes) while with the second group the contamination of the content of whole eggs was studied. On the day of inoculation (day 0); 5 agar-filled eggs and 5 whole eggs were randomly selected to determine the inoculation dose (10^3 - 10^4 CFU *Salmonella* Enteritidis/eggshell). After inoculation, half of the remaining eggs of each group (50) were stored for 21 days in a climate chamber (Termaks KBP 6395 F, Solheimsvinken, Norway) at 20°C and 60% relative humidity (RH). The other half (50) was first stored for 24 h in a refrigerator at 6°C and 70 - 85% RH, immediately followed by a storage of 20 days at 20°C and 60% RH. After placing the latter eggs into the climate chamber, condensation on the eggshell was observed during 30 min.

2.8 Statistical analysis

The bacterial counts were log 10 transformed prior to statistical analysis (Jarvis 1989). Differences in eggshell characteristics as function of the presence of condensate, penetration or contamination were assessed with an analysis of variance. The influence of hen age on eggshell penetration and whole egg contamination was analysed using noncentrality interval estimation and the influence of hen age on the eggshell characteristics was analysed as

outlined in chapter 6; paragraph 2.8. All analyses were done in Statistica 7 (Statsoft, Tulsa, USA). Left and right censored data for bacterial counts on the agar-filled eggs were treated as outlined in chapter 6, paragraph 2.8.

3 RESULTS

3.1 Eggshell characteristics

Table 7.1 shows the mean values with standard deviations (stdev) for each analyzed egg(shell) characteristic for either the egg(shell)s with and without condensate (all weeks; agar-filled eggs). Although the eggs of both groups came from the same lot of sampled eggs (same hen house, hen breed, hen age ...), evaluation of the data showed a minor statistically significant difference ($P < 0.05$) in the shell thickness and a more important statistically significant difference ($P < 0.01$) in cuticle score (Table 7.1, both groups). As the cuticle score is oppositely correlated with the cuticle deposition; control eggs had a significantly lower cuticle deposition. This difference in cuticle deposition was systematic; the difference was found in each sampled week (data not shown). Table 7.1 also compares the egg(shell) characteristics from the penetrated eggs with those from the non-penetrated eggs, both for the control group (Table 7.1, control group) and the condensate group (Table 7.1, condensate group). Both for the control group and for the condensate group, penetrated eggshells contained significantly ($P < 0.05$) more pores than non-penetrated eggshells. A significant ($P = 0.0125$) higher cuticle score was found for the penetrated control eggs compared to the non-penetrated control eggs; for the condensate group this was not observed. This means the average cuticle deposition for penetrated control eggs was lower compared to the non-penetrated control eggs.

Table 7.1: Egg(shell) characteristics of eggs from the control group and the condensate group (agar-filled eggs).

	N	Shell thickness (mm)	Area eggshell (cm^2)	Number of pores (per shell)	Cuticle score	k_{dyn}x100 (N/m)	Damping (%)	Resonance frequency (Hz)
Both groups								
Control	150	0.408±0.034	68.2±3.8	5700±2300	69±24	14400±4800	3.05±1.05	5070±710
Condensate	149	0.417±0.034	68.3±3.9	5700±2300	61±23	13900±3800	3.27±1.18	4960±680
P		*	NS	NS	**	NS	NS	NS
Control group								
Penetrated	65	0.407±0.033	68.3±4.1	6300±2100	74±27	14700±6500	2.95±1.09	5120±910
Not penetrated	85	0.409±0.035	68.2±3.6	5300±2300	64±20	14200±3000	3.13±1.02	5040±510
P		NS	NS	*	*	NS	NS	NS
Condensate group								
Penetrated	93	0.419±0.036	68.1±3.8	6100±2200	61±25	14100±4600	3.19±1.17	4970±830
Not penetrated	56	0.414±0.031	68.6±4.0	5100±2200	60±21	13600±2000	3.41±1.18	4940±340
P		NS	NS	*	NS	NS	NS	NS

Values are means ± stdev; N = number of eggs; NS = not significant; *P < 0.05; **P < 0.01

The eggshell characteristics number of pores and cuticle score were significantly influenced by hen age (Figure 7.1). The number of pores decreased (P < 0.001) with hen age and the cuticle score increased significantly (P < 0.001); meaning the cuticle deposition decreased with hen age.

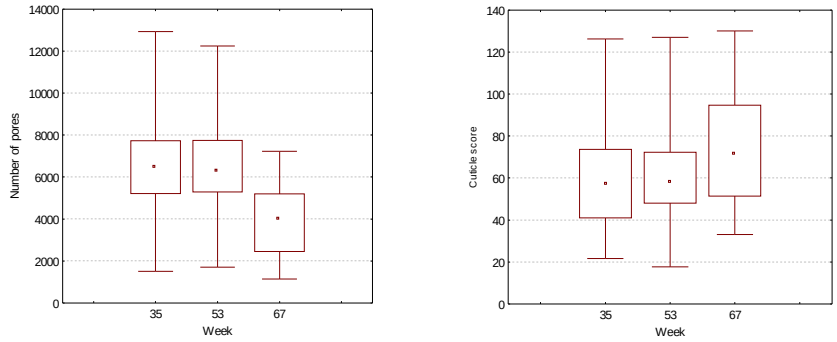

Figure 7.1: Influence of hen age on the eggshell characteristics number of pores and cuticle score.

With regard to the eggs used in the whole egg experiment (egg contamination) (data not shown), no statistically significant differences were found, comparing the same groups as mentioned in Table 7.1 for the shell surface area, dynamic stiffness, damping of the vibration and resonance frequency.

3.2 Eggshell penetration and whole egg contamination

Using eggs of the hens at 39 weeks of age, the bacterial eggshell penetration (agar-filled eggs) increased from 46% (23/50) for the control group to 64% (32/50) for the condensate group. This increase is not statistically significant. The whole egg contamination (whole eggs) did not increase for the group of eggs with condensation; 12% (6/50) of the control eggs were

contaminated compared to 10% (5/50) of eggs which had condensate on the eggshell (Figure 7.2).

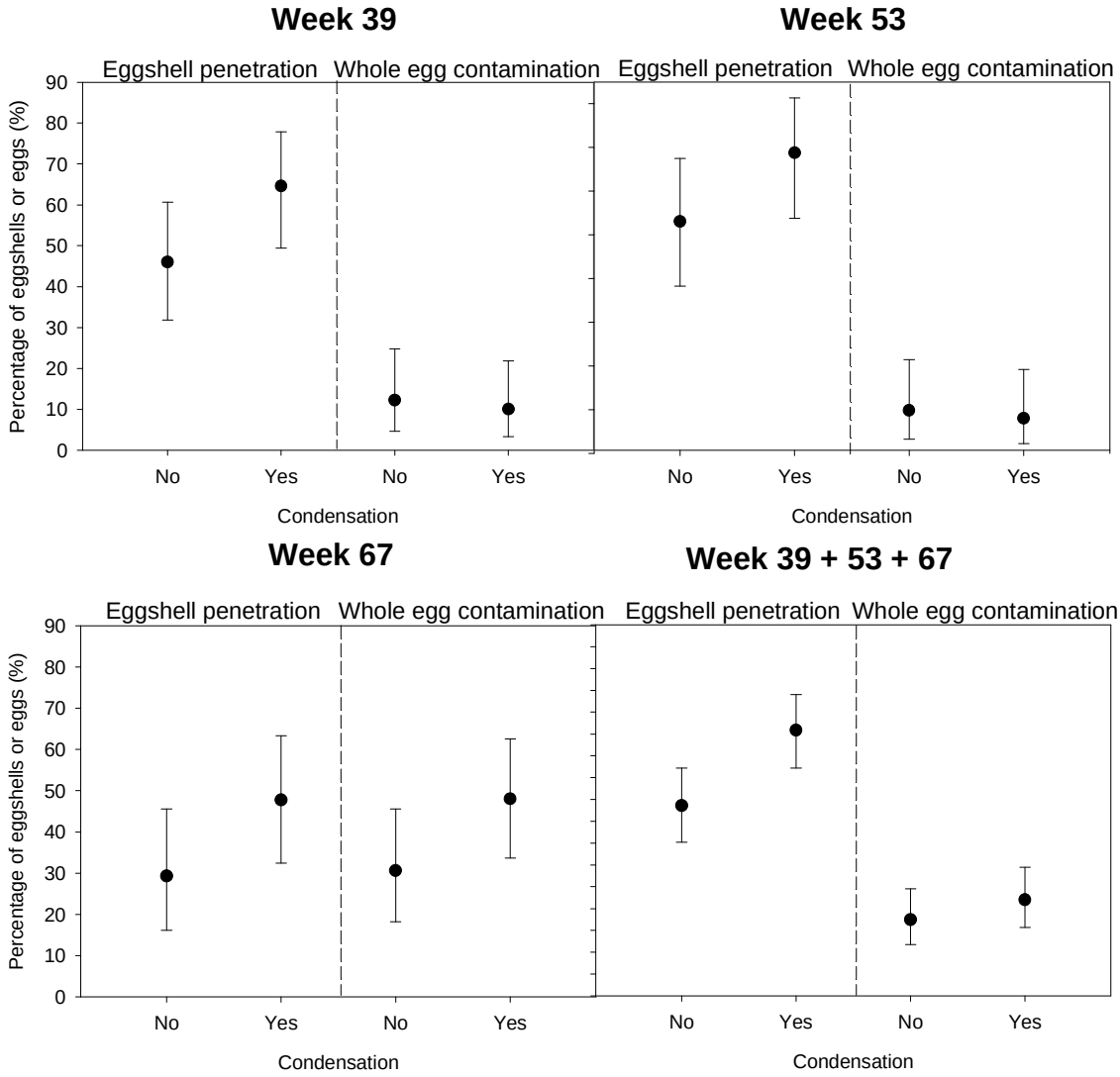

Figure 7.2: Percentage of penetrated eggshells and contaminated whole eggs from egg(shell)s without and with condensate on the shell in function of hen age. Vertical bars denote 0.95 confidence intervals.

With eggs from the hens at the age of 53 weeks, similar results were obtained (Figure 7.2). Bacterial eggshell penetration (agar-filled eggs) increased (not statistically significant) from 53% for the control group to 69% for eggshells with condensate. The whole egg contamination (whole eggs) was similar for control eggs (10% contaminated) compared to condensate eggs (8% contaminated).

At the end of lay (week 67) a lower proportion of bacterial eggshell penetration (agar-filled eggs) was found; 48% of the eggshells with condensate were penetrated compared to only 29% for the control group (not significant different) (Figure 7.2). Oppositely the whole egg

contamination was higher compared to the previous two hen ages; 31% of the control eggs were contaminated compared to 48% of the condensate eggs (Figure 7.2) (difference statistically not significant). The increase of contamination of the whole eggs with condensate from 10% and 8%, respectively, at week 39 and 53 till 48% at week 67 was statistically significant ($P < 0.001$).

Taking into account the three hen ages, 62% (93 on 149 eggshells) of the eggshells with condensate were penetrated compared to 43% (65 on 150 eggshells) for the control group; this difference is statistically significant ($P < 0.01$) (Figure 7.2). No significant difference in whole egg contamination was found; 18% control eggs (27 on 150 whole eggs) were contaminated compared to 22% (33 on 150 whole eggs) for the whole eggs which had condensate on the eggshell.

3.3 Effects of storage time on eggshell penetration (agar-filled eggs)

The day of eggshell penetration was not significantly influenced by condensation; both groups (control and eggshells with condensate) were on average (week 39, 53 and 67) penetrated on approximately day 4; respectively after 3.6 and 4.2 days (Figure 7.3).

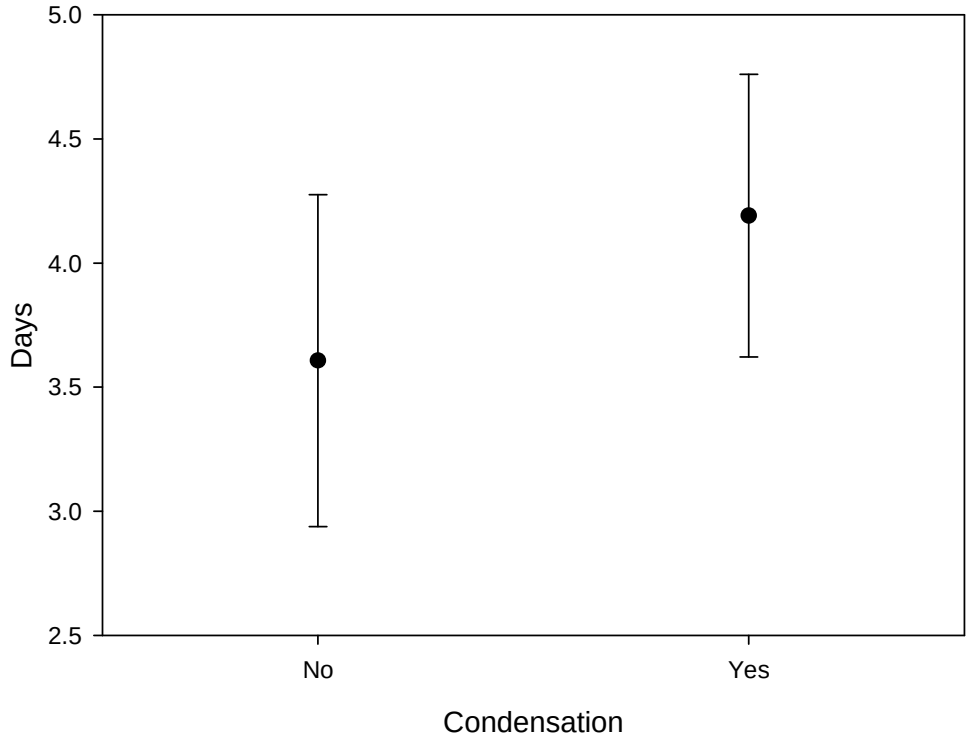

Figure 7.3: Moment eggshell penetration was observed for control eggshells versus eggshells with condensation (agar-filled eggs). Vertical bars denote 0.95 confidence intervals.

3.4 Bacterial survival of *Salmonella* Enteritidis on the eggshell of agar-filled eggs and whole eggs.

Figure 7.4 shows a significantly higher ($P < 0.001$) average count of the inoculated *Salmonella* Enteritidis strain, still present on the eggshells of agar-filled eggs at day 21 for the condensate group compared to the control group; 2.59 log CFU/eggshell versus 1.95 log CFU/eggshell; average eggshell contamination of respectively 149 and 150 agar-filled eggs.
No difference in eggshell contamination of the whole eggs was found between both groups at day 21. Of the 150 whole eggs of the control group 135 eggs were contaminated with < 10 CFU/eggshell (detection limit) while 134 eggshells of the 150 whole eggs with condensate had a comparable low contamination of < 10 CFU/eggshell. The 15 remaining whole eggs (control group) had an average contamination of 1.96 log CFU/eggshell (stdev of 1.00 log CFU/eggshell) compared to an average of 2.47 log CFU/eggshell (stdev of 1.00 log CFU/eggshell) for the condensate group ($n = 16$). This difference in eggshell contamination between the remaining eggs is also not significant ($P = 0.16$).

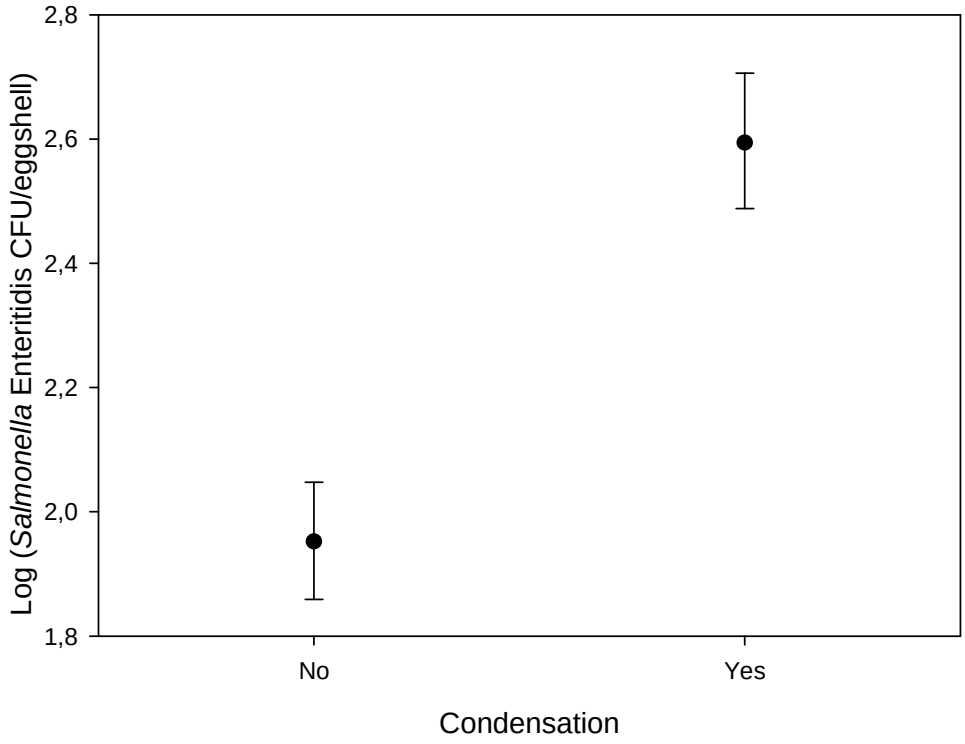

Figure 7.4: Relationship between condensate on the eggshell and *Salmonella* Enteritidis contamination on the shell of agar-filled eggs at the end of storage (21 days). Vertical bars denote 0.95 confidence intervals.

4 DISCUSSION

In studies of Alls *et al.* (1964), Drysdale (1985) and us (chapter 6), a major cuticle deposition stood for less bacterial penetration and/or contamination. In the study of this chapter, this was also true for the agar-filled control eggs. In absence of condensate a significantly higher ($P < 0.05$) cuticle deposition (lower cuticle score) was found for the non-penetrated eggshells compared to the penetrated eggshells. For the condensate group however, no difference in cuticle score between penetrated and non-penetrated eggs was found. Notwithstanding the major cuticle deposition (lower cuticle score) of the eggshells with condensate, a higher eggshell penetration was found for the agar-filled eggs with condensate compared to the control group. These results indicate that the major cuticle deposition formed a less important barrier, possibly due to the presence of condensate. Although the eggs of both groups came from the same lot of sampled eggs, a systematic difference in cuticle deposition between the control and condensate eggs was found. As the cuticle deposition was examined when the penetration (agar-filled eggs) experiment was completed, the higher cuticle deposition (lower cuticle score) of the condensate eggs could be due to the absorption of water from the condensate or due to other unknown reasons. Simons and Wiertz (1970) observed that the cuticle showed thinning during egg storage as a result of drying out. As the shell thickness does not affect penetration (see chapter 6, Williams *et al.* (1968), Smeltzer *et al.* (1979a) and Messens *et al.* (2005a)), the minor difference in shell thickness between control and condensate eggs did not interfere the results of this study.

The significantly higher eggshell contamination (agar approach) with the inoculated *Salmonella* Enteritidis strain at day 21, on the eggshells which had 30 min condensate, was striking. The presence of condensate on the eggshell, after cold storage, must have positively influenced the bacterial survival on the eggshell and this also indirectly affected eggshell penetration. This corresponds with the literature that eggshell penetration is related with the degree of bacterial contamination on the eggshell. Messens *et al.* (2005a) found a high correlation between shell contamination with *Salmonella* Enteritidis and its shell penetration. We also found in chapter 6 for each of seven selected species, originating from egg contents, a correlation between the bacterial eggshell contamination and the occurrence of eggshell penetration.

The moment of eggshell penetration was not significantly influenced by cold storage (6°C, higher RH of 70 - 85%) of the agar-filled eggs for one day (condensate group). Only a slightly earlier penetration time (day 3.6) for the control eggs was found, which can be due to the

faster growth of *Salmonella* Enteritidis on the agar at 20°C compared to 6°C at the first day of storage.

A comparison between eggshell characteristics from penetrated eggs with non-penetrated eggs, both for the control group and for the condensate group, showed that penetrated eggshells contained slightly ($P < 0.05$) more pores compared to non-penetrated eggshells. This indicates that the porosity was slightly correlated with bacterial penetration as shown in previous studies (Fromm and Monroe 1960; Board and Halls 1973). On the contrary, Reinke and Baker (1966), Hartung and Stadelman (1963), Nascimento *et al.* (1992), Messens *et al.* (2005a) and we (chapter 6) supported that bacterial eggshell penetration is not pore dependent. The fact that some pores do not extend through the thickness of the shell but end abruptly (Silyn-Roberts 1983) and the presence of cuticular capping and plugs often present on/into pores and preventing microbial penetration (Board and Halls 1973) may contribute to these conflicting opinions.

In accordance with our study, Messens *et al.* (2005a) and we in chapter 6 also did not found a significant influence of flock age on the eggshell penetration. The trend found by Messens *et al.* (2005a) towards a lower percentage of penetrated eggshells with *Salmonella* Enteritidis (agar-filled eggs) as the flock ages (45.0% at the beginning of lay till 31.6% at the late end of lay), was confirmed by our study in this chapter; respectively from 46% in week 39 till 29% in week 67 (agar-filled control eggs). On the other hand, Nascimento *et al.* (1992), also using agar-filled eggs, reported an increasing eggshell penetration from 12.9% (beginning of lay) till 25.0% (end of lay) for *Salmonella* Enteritidis. In our study the eggshell characteristics cuticle deposition and number of pores decreased significantly throughout the flock age. The lower number of pores could explain the lower penetration; oppositely a lower cuticle deposition should encourage eggshell penetration.

Contrary to the agar-filled eggs, the eggshells of whole eggs in the condensate group were not significantly higher contaminated with *Salmonella* Enteritidis compared to the control whole eggs. Analyzing the results of all weeks together, no significant difference in whole egg contamination was found between both groups of eggs. The higher potential of eggshell penetration observed for agar-filled eggs with condensate did not result in a higher contamination of the egg content. The *Salmonella* counts on the shell of whole eggs at day 21 were significantly lower compared to the agar-filled eggs. Nutrients either available from the agar of the agar-filled eggs favour and/or antimicrobial components of the egg content of whole eggs do not stimulate the survival and growth of *Salmonella* Enteritidis on the eggshell, as suggested before in chapter 6. Taking into account all weeks, the whole egg contamination

was also significantly lower compared to the bacterial eggshell penetration. This can be explained by the lower survival of *Salmonella* Enteritidis on the eggshell and the antimicrobial defences of the albumen inside the whole eggs that must have prevented the whole egg contamination. The high impact of the antimicrobial properties of the albumen was also shown by Jones *et al.* (1995); despite having a *Salmonella* incidence of 7.8% on eggshells (7 on 90 eggshells), no *Salmonella* was found in 180 egg contents of the same sampling.

The whole egg contamination found at the end of lay was higher than at the previous two hen ages. This higher contamination cannot be explained by a higher penetration potential because it was not observed in the agar-filled eggs from the same batch. In chapter 6 we also found a slight increase in whole egg contamination (all strains) at the end of lay. In a study of Jones *et al*. (2002) the contamination with *Salmonella* Enteritidis also increased; from 30% in week 34 till 50% in week 74 (see also chapter 6). Fajardo *et al*. (1995) reported 43% of whole eggs positive for *Salmonella* Enteritidis after incubation of the inoculated eggs, from 72-week-old hens, for 48 h at 32°C. According to Jones *et al.* (2002) shell and egg quality decreases as hen ages, resulting in a better ability of micro-organisms to infect the egg. In our study no significant difference in shell quality (dynamic stiffness, damping of the vibration and resonance frequency) of the whole eggs from different age groups was observed.

Ernst *et al.* (1998), using intact eggs (hen age not mentioned) that had been stored (4°C) for 32 days, found no significant difference in egg content contamination with *Salmonella* Enteritidis due to sweating: 2.8% (1/36) of unsweated eggs and 5.7% (2/35) of sweated eggs were contaminated. This prevalence of contamination approaches ours, using eggs from hens at the age of 39 and 53 weeks. Using cracked eggs (small line checks) a similar conclusion was obtained; 77% unsweated cracked eggs were contaminated versus 64% sweated cracked eggs. In their study moisture on the eggshell was obtained by placing inoculated eggs in sterile plastic bags and overnight storage at 2 - 4°C followed by storage at 32°C and about 95% RH. Using this protocol, eggs were observed to sweat continuously for 3 h. In an early study of Fromm and Margolf (1958), bacteria were more likely to be present in albumen or yolk of eggs allowed to sweat for 1, 3 or 5 h. Four groups of eggs were used; clean unwashed, dirty unwashed, clean washed, dirty washed. The procedure to obtain sweating differed again from those we used; eggs first stored for 0, 1, 4, 8 or 12 days at 10 - 12°C and 80% RH were moved to 22 - 24°C and 80 - 85% RH for 1, 3 or 5 h and returned to storage in the refrigerator (10 - 12°C and 80% RH) until day 12. All eggs were analyzed for bacterial contamination at day 12. The higher incidence of contamination of the sweated eggs, could probably be due to

the negative pressure in the eggs; by placing the eggs after sweating again in the refrigerator, bacterial loaded moisture could be drawn through the shell pores, resulting in the contamination of the egg content (Haines and Moran 1940).

5 CONCLUSIONS

It can be concluded that condensation on the eggshell has encouraged the bacterial eggshell penetration with *Salmonella* Enteritidis but had a smaller and not significant impact on the whole egg contamination. The higher survivals of the pathogen on the eggshells of agar-filled eggs with condensate might explain the higher penetration of those eggshells. The low impact of condensation on the whole egg contamination can be explained by the equal survival of *Salmonella* Enteritidis on the eggshell of whole eggs with and without condensation, and by the antimicrobial defences of the albumen. The higher whole egg contamination found at the end of lay compared to the previous two hen ages was striking.

General discussion, conclusions and perspectives

General discussion, conclusions and perspectives

The present study showed that, with the exception of heavily soiled shells (*e.g.* ground eggs), bacterial contamination of the eggshell can not be judged by visual evaluation of the eggshell. Before, Board and Tranter (1995) mentioned also only for heavily soiled eggs a correlation between the level of bacteria and the appearance of the shell. Hence, a method to assess the general bacterial contamination of the eggshell of consumption eggs through the production chain was needed and developed. The total count of aerobic and Gram-negative bacteria on the eggshell can be used to assess the bacterial eggshell contamination and to detect critical points for contamination in the egg production chain. Washing of the eggshell was the most suited method for recuperation of the bacteria from the eggshell.

Our pilot studies (chapter 3) showed that eggs laid in the nest boxes of the furnished cages have a similar general bacterial eggshell contamination compared to eggs from the conventional cages. Mallet *et al.* (2004) found that eggs from furnished cages laid outside the nests, in the litter area or in the cage, had a higher bacterial eggshell contamination with aerobic bacteria compared to nest eggs. Therefore a good design of furnished cages, as suggested by Wall et *al.* (2002), should prevent eggs from being laid outside the nests. Our pilot studies showed that the type of nest-floor material (wire floor or artificial turf) used in the furnished cages did not consistently influence the general bacterial shell contamination. A 1.0 log higher contamination with total aerobic flora was found on the nest eggs collected from the aviary system compared to the conventional and furnished cages. Comparable results in pilot studies were obtained by Protais *et al.* (2003a). This difference in contamination was confirmed by us in the commercial systems, although less pronounced; 0.38 versus > 1.0 log. Since very recently the first commercial furnished cage productions are available in Belgium, further research on the initial eggshell contamination in commercial circumstances is recommended.

The higher initial bacterial eggshell contamination was also reflected in a higher total bacterial count in the air of the alternative (non-cage) systems. In the pilot and commercial studies a positive correlation ($r^2 = 0.66$, $P < 0.001$ and $r^2 = 0.77$, $P = 0.099$ respectively) was found between the concentration of total bacteria in the air of the poultry houses and the initial bacterial eggshell contamination. Comparable to Protais *et al.* (2003b), we found averages of 4.4 log CFU/m^3 for the conventional cages compared with > 5.3 log CFU/m^3 in

the aviary housing. The poorer air quality of the alternative systems is due to the freedom of movement of the birds and the more complex environment with litter and manure. A reduction of the air quality and the hygienic status in the alternative systems will not be obvious. Although various technologies were proven successful for reducing airborne dust, including misting with an oil spray, water mists, extra ventilation and air ionization, their industrial application will not be evident. Other measures such as providing enough pop holes giving access to the outer area for open run systems, removal of litter from the nest area, increasing the available air volume per bird can be more quickly applied. The upcoming introduction of a 'winter garden', which is a screened-in porch providing much fresh air, is another possibility and an actual research item. It is generally believed that the contamination of the surfaces making contact with the eggs immediately after lay is important for the contamination of the egg(shell) (Bruce and Drysdale 1994; Board and Tranter 1995). Research concerning the hygiene of egg contact surfaces (nest floor, egg belt, …) and the cleaning and disinfection possibilities in the different housing systems is therefore an important focus for the future. This research will result in important information about the benefit of interim cleaning and disinfection of certain egg contact surfaces in connection to eggshell contamination. Beside the general bacterial flora, the determination of the number of *Enterobacteriaceae* on the egg contact surfaces could provide a better estimate for cross contamination with *Salmonella* in the different housing systems.

Placing enough and well-designed nest boxes besides training of the birds in the alternative housings can help to reduce the amount of the highly contaminated ground eggs. With eggshell counts up to 7 log CFU total aerobic flora and 4 log CFU Gram-negative bacteria these eggs can not be used as consumption eggs. It has to be stressed that extra nest boxes placed were ground eggs accumulate need to be well designed. Our work has indicated that poor designed extra nest boxes placed on the ground also delivered highly contaminated eggshells.

In all our experiments a comparable or even significantly lower initial contamination with Gram-negative bacteria on the eggshell was found in the alternative housings compared to the cage housings. In recent research (De Reu, unpublished results) counts of *Enterobacteriaceae* on eggshells from eggs from commercial aviary housing systems also tend to be lower compared to commercial furnished cages. Possibly the higher initial contamination of the alternative eggs with Gram-positive bacteria oppressed in some cases the adhesion of Gram-negative bacteria. Literature shows that Gram-negative bacteria are best equipped to overcome the antimicrobial defences of the egg (Mayes and Takeballi 1983). The equal or

even lower contamination of the eggshell in alternative housing systems with Gram-negative bacteria makes the hygienic argument as contra-indication for the introduction of alternative housing systems less relevant. Based on these findings and knowing that the study of the UK Food Standards Agency (Anon. 2004d) did not show significant differences in *Salmonella* spp. contamination due to the production system, we have the opinion that in practice observed differences between cage eggs and non-cage eggs are limited concerning the microbiological product quality and negligible concerning food safety aspects. Collection of monitoring data on the surveillance of zoonotic bacteria and especially *Salmonella* spp. for the different egg types is an important goal for the future and necessary for a good opinion on the food safety issue. However, one has to be aware of the large amount of data necessary to statistically sustain this research considering the very low frequency of *Salmonella* positive egg contents even from flocks known to be infected with *Salmonella* (Kinde *et al.* 1996; Schlosser *et al.* 1999). Another possibility is to study the horizontal transfer of *Salmonella* to hens in the different housing systems. These infection pathways can be different due to differences in cleaning and disinfection between succeeding flocks, contact with water, feed, litter and manure, contact between hens, contact with the outsite environment, ….

Notwithstanding the difference in initial eggshell contamination due to the different housing system, storing of eggs can reduce those differences at the retail level. The results of the different commercial chains showed that storing of shell eggs, whether temporary refrigerated or not, for 9 days or more, causes a significant decrease in bacterial eggshell contamination for both analyzed parameters, total count of aerobic bacteria and Gram-negative bacteria. The results of the pilot studies and the commercial chains also showed that, independently of the housing system, shell contamination was not influenced by hen age. This makes eggs from older hens not more sensitive to bacterial eggshell contamination.

Next to the initial eggshell contamination, only one critical point for introducing bacterial eggshell contamination through the commercial production chains was found; a so called ‘accumulator’. The accumulation of eggs on a short conveyor belt together with the type of conveyor, a metal grid which can contain more dirt and egg content compared to (double) roller conveyor belts, were the main reasons for the increase in bacterial load on the eggshell. Those types of conveyor belts must be avoided if possible and cleaned and/or disinfected regularly.

A possibilty to reduce the bacterial eggshell and conveyor belt contamination is offered by UV disinfection (Kuo *et al.* 1997b; Chavez *et al.* 2002; Coufal *et al.* 2003). Our study with a

commercial UV disinfection system reduced the natural flora with 0.9 log and recent eggshell contamination even with 4 log. UV disinfection can be used as a cheaper and safer alternative for egg washing to reduce bacterial eggshell contamination of clean eggs. As removal of dirt is also an important goal during egg washing; the UV disinfection system can be combined with hygienic double rollers and feather removers to reduce the dirt on the eggshell. Beside a high resolution camera can be used to separate the remaining eggs with dirt on the eggshell (Mertens 2004). The fact that all operations are done in dry conditions is advantageous to eggs washing.

The microbial ingress into the egg content by the horizontal route was examined by looking at the eggshell penetration and the egg content contamination separately. Eggshell penetration was studied using agar-filled eggs while egg content contamination was studied with whole eggs. Only intact eggs (no cracks) were used.

Microbial ingress of 7 phylogenetically diverse bacterial species was studied simultaneously; *Staphylococcus warneri, Acinetobacter baumannii, Alcaligenes* sp., *Serratia marcescens, Carnobacterium* sp., *Pseudomonas* sp. and *Salmonella* Enteritidis. The experiments with agar-filled eggs indicate that the Gram-negative, motile and non-clustering bacteria penetrated the eggshell most frequently. All 7 selected bacteria were able to penetrate the eggshell; resulting in an average eggshell penetration of 33% (average of 7 selected bacteria). An average of 16% egg content contamination (whole eggs) was found; with the highest survival for *Salmonella* Enteritidis (33%) followed by *Carnobacterium* sp. (17.5%).

Notwithstanding *Salmonella* Enteritidis contaminated the egg content (whole eggs) most frequently compared to the non-*Salmonella* strains, we are convinced that this result has to be interpreted in a broader experimental context. We consider the contamination % of the same *Salmonella* Enteritis strain MB 1409 in the different independent studies presented in the chapters 6 and 7 as too variable (10 - 33%) to conclude that the *Salmonella* strain is a primary invader of whole eggs. In addition egg related *Salmonella* Enteritidis strains did not show a special capability to contaminate whole eggs by the horizontal infection route compared to other *Salmonella* Enteritidis strains and *Salmonella* serotypes, as shown in chapter 6. Knowing that *Salmonella* Enteritidis is the most frequently isolated *Salmonella* serovar in eggs, our results do not contra-indicate that the frequent egg contamination with *Salmonella* Enteritidis would be mainly due to the transovarian or vertical route as supported by Humphrey *et al.* (1991b) and Cogan and Humphrey (2003). The decrease in human salmonellosis cases caused by *Salmonella* Enteritidis that was observed during the recent

years can be a result of intervention in the vertical contamination route by the forced vaccination campaigns.

Of all different studied eggshell characteristics, shell surface area, shell thickness, number of pores and cuticle deposition; only the latter influenced the bacterial eggshell penetration of the 7 selected bacterial species. This was also confirmed in the condensation experiment using control eggs inoculated with *Salmonella* Enteritidis and by studies of other research workers (Alls *et al.* 1964; Drysdale 1985). A major cuticle deposition stood for less bacterial penetration. Therefore damage, *e.g.* during egg-washing, of this important physical barrier for egg invading organisms must be avoided. Hen breed, storage conditions, limitation of stress and eventually other factors like feed can also contribute to a major cuticle deposition (Ball *et al.* 1975; Sparks 1985). In the presence of condensate however the cuticle formed a less important barrier for eggshell penetration.

On the other hand, our experiments on whole egg contamination showed contamination was not influenced by one of the analysed eggshell characteristics.

Comparable to Messens *et al.* (2005a) penetrated agar-filled eggs showed a significantly higher count on the egg surface compared to non-penetrated eggshells. In the studies of the egg content contamination (whole eggs) this was less obvious. Eggshell condensation also encouraged the bacterial eggshell penetration (agar-filled eggs) with *Salmonella* Enteritidis but had a smaller and not significant impact on the egg content contamination (whole eggs). The higher survival of the pathogen on the eggshells of agar-filled eggs with condensate explains the higher penetration of those eggshells. The low impact of condensation on the whole egg contamination can also partly be explained by the equal survival of *Salmonella* Enteritidis on the eggshell of whole eggs with and without condensation.

All selected bacterial species were able to remain viable in the albumen. This indicates that notwithstanding the antimicrobial aspects of the albumen, survival after penetration of the shell may not be underestimated (growth not investigated). However the average eggshell penetration (7 bacterial strains) was found to be 33%, while the average egg content contamination was 16%. This reduction emphasizes the importance of the antimicrobial aspects of the membranes and the albumen for the egg content contamination. The lower impact of eggshell condensation on the whole egg contamination can possibly also be partly explained by those antimicrobial aspects.

In the study with the 7 selected bacteria as well in the condensation experiment using *Salmonella* Enteritidis, the influence of hen age on the bacterial eggshell penetration was not significant. This was comparable with studies of Messens *et al*. (2005a). Using the 7

phylogenetically diverse bacterial species, the whole egg contamination slightly increased (not significantly) with hen age. Jones *et al.* (2002) came to the same conclusion. This was even more stressed in our condensation experiment where a significantly higher whole egg contamination with *Salmonella* Enteritidis was found at the end of lay. Notwithstanding the cuticle deposition slightly but significantly decreased with hen age, we are not convinced that this was the major reason for the higher egg content contamination observed at the end of lay. Alterations in shell membranes and/or albumen might be more important. Kröckel *et al.* (2003) also found that resistance of the egg (albumen) against microbial growth decreased with hen age and was even affected by the genetic origin of hens. According to the research group it is not known yet which albumen components relevant as barriers for microbial growth are influenced by hen age. The focus for possible further research will definitely be the study of the possible alteration in the membrane bounding to the shell, the membrane penetrability and the albumen resistance of eggs from older hens, combined with their influence on the bacterial egg content contamination. The selection of laying hens with higher anti-microbial albumen properties, as suggested by Vidal *et al.* (2003), will be an other important challenge.

Different factors influencing the horizontal infection route of eggs were studied in this PhD work. In literature very little information is available on the relative contribution of horizontal and vertical transmission of bacterial contamination to the egg content. A preliminary study on the occurrence of vertically and horizontally contaminated whole eggs was performed (data not shown).

From 14 commercial laying hen production facilities (cage, furnished cage and alternative housings) in each case approx 80 eggs were sampled at the hen house (conveyor belt or nest boxes) the morning of lay. Half of the eggs (approx 40) were used to determine the egg content contamination at the day of sampling (by enrichment); the other half of the eggs (approx 40) was stored at room conditions up till 21 days after egg laying, followed by the determination of the egg content contamination. The proportion of contamination was respectively 2.7% (15/554) immediately after lay and 3.4% (18/532) after 21 days storage. Comparing these contamination proportions the importance of horizontal transmission of intact eggs must be put into perspective. Although the contamination we observed can be due to vertical transmission, the probability of a possible contamination during our experimental procedure can not be excluded. We have however taken extreme precautions. Dipping the eggs in 30% hydrogen peroxide solution for 10 s, followed by sprinkling the egg with 75%

ethanol and burning off the alcohol during approx 5 s seemed to be the most suitable method to eliminate all and especially spore-forming bacteria from the eggshell. In the case the observed egg content contamination would be due to an experimental bias, we consider its probability equal for eggs tested directly after lay and after 21 days of storage so that our conclusion about the relative low importance of horizontal transmission stays relevant. These preliminary results are the basis for further research.

Our research indicates a relative low importance of horizontal transmission of intact eggs stored under optimal conditions. Also we consider that the egg content contamination was not influenced substantially by the bacterial eggshell contamination. Nevertheless these observations, we are convinced that reducting the general bacterial eggshell contamination is an important tool in preventing egg content contamination in practice. Considering whole egg contamination, the importance of eggshell cracks may not be underestimated. Avoiding cracks in eggshells is therefore very important. Using inoculated eggs, Ernst *et al.* (1998) found a huge increase in egg content contamination with *Salmonella* Enteritidis due to cracked eggs (hair-cracks); 2.8% intact eggs were contaminated versus 77% cracked eggs. In practice Hamilton *et al.* (1979) found 8 - 10% cracked eggs while during this PhD study at retail level, 5.7% (33 on 580 eggs) cracked eggs were found. The risks involved with eggshell condensation must be reduced. No doubt moisture can facilitate eggshell penetration and with an additional positive temperature differential the egg contents contracts and can draw water loaded with bacteria through the open pores or cracks. As we found all 7 phylogenetically divers bacterial species remaining viable in albumen and findings in literature sometimes report substantial multiplication possibilities, possible hazards must be reduced.

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Summary

Summary

In **chapter 1** a literature review was given; discussing the formation and the components of the egg, the mechanisms of microbial contamination of intact eggs, the type of contaminating flora of eggs with special attention for *Salmonella*, the egg production chain with the different housing systems for laying hens, and some aspects on egg washing.

In **chapter 2** is shown that washing eggs in sterile plastic bags with diluent is an efficient sample preparation method for the determination of the bacterial contamination on eggshells. Total count of aerobic and Gram-negative bacteria on the eggshell were used to detect critical points for contamination in the egg production chain. The number of eggs to be sampled at a point of the production chain was determined on a statistical basis and fixed on 40 for non-graded eggs and on 20 for graded eggs. In two production chains, one cage production and one organic production system, critical points for contamination were identified. The most critical point for the cage production system was a short conveyor belt at the entrance of the candling, grading and packaging area, for the organic production system it was the initial contamination at the nest boxes. With the exception of heavily soiled shells, like shells from eggs collected from the ground (ground eggs), there is a poor correlation between the level of bacterial contamination and the visual eggshell contamination. A positive correlation was found between the initial bacterial eggshell contamination and the concentration of bacteria in the air of the poultry houses.

The influence of the housing system on the initial bacterial contamination of the eggshell was studied in **chapter 3**. Two long-term experiments were performed. The bacterial eggshell contamination, as expressed by total count of aerobic and Gram-negative bacteria, was periodically analyzed for eggs from a conventional cage, a furnished cage with nest boxes containing artificial turf or grids as nest-floor material and an aviary housing system. For these experiments no systematic differences were found between the conventional cage and furnished cage. The type of nest-floor material in the nest boxes of the furnished cages also did not systematically influence the bacterial shell contamination. A possible seasonal influence on the eggshell contamination with a decrease in the winter period (up to > 0.5 log CFU/eggshell) of total count of aerobic and Gram-negative bacteria was observed in the first experiment. The contamination with total aerobic flora was higher (> 1.0 log) on eggs derived

from the aviary housing system compared to the conventional and the furnished cage systems. For Gram-negative bacteria this was not the case. During the entire period of both experiments, independent of the housing system, shell contamination was not influenced by hen age or period since placing the birds in the houses. For the total count of aerobic bacteria a positive correlation (r^2 = 0.66; P < 0.001) was found between the concentration of total bacteria in the air of the poultry houses and the initial bacterial eggshell contamination.

The bacterial eggshell contamination of consumption eggs in different commercial housing systems; two conventional cages, one organic aviary system and one barn production, were compared in **chapter 4**. The total count of aerobic bacteria and the total count of Gram-negative bacteria on the eggshell were used to detect critical points for introducing bacterial eggshell contamination and to study the progress of the eggshell contamination in the egg production chains.

The critical points for the bacterial eggshell contamination were the accumulation of eggs on a short conveyor belt, the initial eggshell contamination in the alternative housing systems and the extra nest boxes placed on the ground. A high bacterial load of ground eggs (> 6.3 log CFU total aerobic flora/eggshell) was observed.

On average a significant higher (P < 0.001) initial eggshell contamination with total count of aerobic bacteria was found for eggs from the alternative housing systems compared to the conventional systems; respectively 5.46 compared to 5.08 log CFU/ eggshell. However, the initial contamination with total count of Gram-negative bacteria on the eggshells was significantly lower (P < 0.001) in the alternative housings; 3.31 compared to 3.85 log CFU/eggshell. A moderate and not significant (r^2 = 0.77; P = 0.099) positive correlation was found between the initial bacterial eggshell contamination and the concentration of bacteria in the air of the poultry houses.

Storing shell eggs, whether temporary refrigerated or not, for 9 days or more, resulted in a significant decrease in bacterial eggshell contamination for both bacterial variables.

The effect of UV irradiation on the bacterial load of shell eggs and of a roller conveyor belt was studied in **chapter 5**. The natural bacterial load on the eggshell of clean eggs was significantly reduced by a standard UV treatment of 4.7 s; from 4.47 to 3.57 log CFU/eggshell. For very dirty eggs no significant reduction was observed. Eggs inoculated with *Escherichia coli* and *Staphylococcus aureus* (4.74 and 4.64 log CFU/eggshell respectively) passed the conveyor belt and were exposed to UV for 4.7 and 18.8 s. The

reduction of both inoculated bacteria on the eggshell was comparable and significant for both exposure times (3 and 4 log CFU/eggshell, respectively). *E. coli* was reduced but still detectable on the conveyor rollers. The internal bacterial contamination of eggs filled up with diluent containing *E. coli* or *S. aureus* was not influenced by UV irradiation. In conclusion; the penetration of UV into organic material appears to be poor and UV disinfection can be used as an alternative for egg washing of clean eggs.

In **chapter 6** trans-shell infection routes and whole egg contamination of 7 selected bacterial strains; *Staphylococcus warneri, Acinetobacter baumannii, Alcaligenes* sp., *Serratia marcescens, Carnobacterium* sp., *Pseudomonas* sp. and *Salmonella* Enteritidis, recovered from egg contents, were studied. A first objective was to correlate bacterial eggshell penetration with various eggshell characteristics and the identity of phylogenetically diverse bacterial strains. An agar approach was used to assess the eggshell penetration. A second objective was to assess the contamination of whole eggs with the bacterial strains; whole intact eggs were used in this case. The intact shells of agar-filled and whole eggs were inoculated with 10^3 - 10^4 CFU of the selected strains. Inoculated eggs were stored for 3 weeks at 20°C and 60% relative humidity. Bacterial eggshell penetration was regularly monitored and whole egg contamination was analyzed after 3 weeks. Contrary to the cuticle deposition, the eggshell characteristics shell surface area, shell thickness and number of pores did not influence the bacterial eggshell penetration. The whole egg contamination was not influenced by neither the area of the eggshell or the porosity of the eggshell. The results of the agar approach indicate that the Gram-negative, motile and non-clustering bacteria penetrated the eggshell most frequently; *Pseudomonas* sp. (60%) and *Alcaligenes* sp. (58%) were primary invaders followed by *Salmonella* Enteritidis (43%). All selected strains were able to penetrate; penetration was observed most frequently after approx 4 - 5 days. In comparison with the non-*Salmonella* strains, *Salmonella* Enteritidis was a primary invader of whole eggs in the first study: the membranes and/or the content of 32% of the whole eggs were contaminated. Penetrated eggshells and contaminated whole eggs showed a significantly higher bacterial contamination on the eggshell compared to respectively not penetrated eggshells and not contaminated whole eggs (general results of all strains). The influence of hen age on bacterial eggshell penetration and egg content contamination was not significant.
The whole egg contamination with four different *Salmonella* Enteritidis strains and one *Salmonella* Typhimurium strain was studied as well. Contamination percentages ranged from

6% - 26%, with no special capacity of egg related *Salmonella* Enteritidis strains compared to other *Salmonella* Enteritidis strains and the *Salmonella* Typhimurium strain.

In **chapter 7** the influence of eggshell condensation on the bacterial eggshell penetration and the whole egg contamination with *Salmonella* Enteritidis was studied. Shells of agar-filled and whole eggs were inoculated with 10^3 - 10^4 CFU *Salmonella* Enteritidis per eggshell. The agar-filled eggs were used to study the bacterial eggshell penetration; the whole egg results were used to characterize the contamination of the egg content. Of each group, half of the eggs were stored for 21 days at 20°C and 60% relative humidity (RH); while the other half was first stored for 24 h at 6°C before storage at 20°C. The latter resulted in condensation on the eggshell for 30 min from the moment the eggs were placed at 20°C. Taking into account the three hen ages studied (39, 53 and 67 weeks) an average of 62% of the eggshells with condensate were penetrated compared to 43% for the control group; this difference was statistically significant ($P < 0.01$). No significant difference in whole egg contamination was found; 18% of the control eggs were contaminated compared to 22% of the condensate eggs. Remarkable was the significantly higher whole egg contamination of eggs at the end of lay compared to the eggs sampled from the two earlier hen ages. This was probably not due to a higher penetration potential as this was not observed in the corresponding agar-filled eggs. It can be concluded that condensation on the eggshell encouraged the bacterial eggshell penetration, but had a smaller impact on the whole egg contamination.

In **Conclusions and perspectives** the major conclusions of this work are summarized and some recommendations to limit the egg content contamination are discussed. Also the first results of actual research on the real impact of the vertical and horizontal infection route of shell eggs are mentioned.

Beside the critical points for initial eggshell contamination with total aerobic bacteria in the alternative (non-cage) housing systems, only one other critical point for introducing eggshell contamination through the chain was found. Further improvements in the design of alternative housing systems must reduce the impact of some critical points. Bacterial eggshell penetration (agar-filled eggs) was positively correlated with the degree of bacterial eggshell contamination. Notwithstanding the supposed relative low importance of horizontal transmission of intact eggs stored under optimal conditions, and despite the less obvious influence of the amount of bacterial eggshell contamination on the egg content contamination

(whole eggs), we are convinced that the reduction of the general bacterial eggshell contamination is an important tool in preventing egg content contamination in practice.

Samenvatting

Samenvatting

Hoofdstuk 1 betreft de literatuurstudie; de vorming en de bestanddelen van het ei, de wijze waarop intacte eieren microbiologisch kunnen gecontamineerd worden, het type bacteriologische flora dat eieren kan besmetten met speciale aandacht voor Salmonella, de productieketen van consumptie-eieren met aandacht voor de diverse types huisvestingssystemen voor leghennen en tenslotte enkele aspecten omtrent wassen van eieren worden erin toegelicht.

In **hoofdstuk 2** wordt aangetoond dat wassen van eieren in een steriele plastiek zak met verdunningoplossing een geschikte monstervoorbereiding is voor de bepaling van de algemene bacteriologische belasting van de eischaal. De telling van het totaal aantal aërobe en Gramnegatieve bacteriën op de eischaal werd gebruikt voor de opsporing van kritische punten voor de introductie van eischaalcontaminatie in de productieketen van schaaleieren. Het aantal te bemonsteren eieren per staalnamepunt werd statistisch onderbouwd en vastgelegd op 40 eieren voor niet-geschouwde en 20 voor geschouwde eieren. In twee verschillende productieketens, een conventionele legbatterij en een biologische volière huisvesting, werden de kritische punten bepaald. Het meest kritische punt voor de introductie van eischaalcontaminatie in de legbatterij was een korte metalen ketenmat voorafgaand aan de schouwkamer. Voor het biologische legbedrijf bleken de legnesten het meest kritische punt. De studie kon, met uitzondering van sterk bevuilde eieren zoals vb. grondeieren, geen correlatie aantonen tussen de visuele vuilschaligheid en de bacteriologische belasting van de eischaal. Tenslotte werd een positief verband gevonden tussen de bacteriologische belasting van de stallucht en de belasting van de schaal van eieren geraapt in de stallen.

De invloed van het huisvestingssysteem voor leghennen op de initiële bacteriologische belasting van de eischaal werd bestudeerd in **hoofdstuk 3** en dit gedurende twee volledige legronden. De bacteriologische belasting van de eischaal met totaal aantal aërobe en Gramnegatieve bacteriën werd periodiek bepaald voor eieren geraapt in een conventionele legbatterij, in verrijkte kooien met legnesten voorzien van matten of roosters op de bodem en in een volière huisvesting. Gedurende de twee legronden werden geen systematische verschillen in eischaalcontaminatie gevonden tussen de conventionele legbatterij en de verrijkte kooien. Het type legnestmateriaal had eveneens geen systematische invloed op de

eischaalbelasting. Tijdens de eerste legronde waren de eieren tijdens de winterperiode lager belast met totaal aantal aërobe en Gramnegatieve bacteriën (> 0.5 log kve/eischaal lager). De belasting van de eischaal met totaal aantal aërobe bacteriën lag systematisch hoger (> 1 log) voor eieren afkomstig uit de volière huisvesting ten opzicht van de conventionele of verrijkte kooi huisvesting. Dit was niet het geval voor de Gramnegatieve bacteriën. Er werd gedurende beide legronden geen invloed van de henleeftijd op de bacteriologische belasting van de eischaal vastgesteld. Tenslotte werd een positieve correlatie (r^2 = 0.66; P < 0.001) gevonden tussen de bacteriologische belasting van de stallucht met totaal aantal aërobe kiemen en de eischaalbelasting.

De bacteriologische belasting van de eischaal van consumptie-eieren afkomstig van diverse commerciële huisvestingssystemen (twee conventionele legbatterijen, één biologische productie en één scharrelbedrijf) werden vergeleken in **hoofdstuk 4**. De bepaling van het totaal aantal aërobe en Gramnegatieve bacteriën op de eischaal werd gebruikt voor de opsporing van kritische punten voor de introductie van eischaalcontaminatie en voor de studie van het verloop van de eischaalcontaminatie in de productieketen.
Kritische punten voor de bacteriologische belasting van de eischaal waren het laten samenkomen van vele eieren op een korte transportband, de initiële contaminatie van de eischaal in alternatieve huisvestingssystemen en de bijkomende legnesten geplaatst op de grond. Voor grondeieren werd een hogere bacteriologische belasting van de eischaal gevonden (> 6.3 log kve aërobe kiemen/eischaal).
Er werd gemiddeld een significant hogere (P < 0.001) initiële belasting van de eischaal met totaal aantal aërobe bacteriën gevonden voor eieren afkomstig van de alternatieve huisvestingssystemen in vergelijking met de conventionele huisvesting; respectievelijk 5.46 tegenover 5.08 log kve/eischaal. Daartegenover lag de initiële contaminatie van de eischaal met Gramnegatieve bacteriën significant lager (P < 0.001) in de alternatieve huisvestingen; 3.31 tegenover 3.85 log kve/eischaal. Er werd een beperkte niet significante (r^2 = 0.77; P = 0.099) positieve correlatie aangetoond tussen de initiële contaminatie van de eischaal en het aantal bacteriën in de lucht van de leghennenstal.
Het bewaren van consumptie-eieren, al dan niet tijdelijk gekoeld, voor 9 dagen of meer, zorgt voor een significante daling van de eischaalcontaminatie met beide microbiologische variabelen.

Het effect van UV belichting op de bacteriologische belasting van consumptie-eieren en transportbanden werd bestudeerd in **hoofdstuk 5**. De natuurlijke bacteriologische belasting van de eischaal van propere eieren werd significant gereduceerd door een standaard UV belichting van 4.7 s; respectievelijk van 4.47 tot 3.57 log kve/eischaal. Bij sterk bevuilde eieren werd geen significante daling vastgesteld. Eieren kunstmatig besmet met *Escherichia coli* en *Staphylococcus aureus* (respectievelijk met 4.74 en 4.64 log kve/eischaal) werden op de transportband geplaatst en UV belicht voor 4.7 en 18.8 s. De reductie van beide geïnoculeerde bacteriën was vergelijkbaar en significant voor beide belichtingstijden (respectievelijk 3 en 4 log kve/eischaal). *E. coli* werd gereduceerd maar kon nog steeds aangetoond worden op de transportband. De besmetting van de inhoud van eieren opgevuld met verdunningsvloeistof met hetzij *E. coli* hetzij *S. aureus* werd niet beïnvloed door UV belichting. Samenvattend werd in het hoofdstuk aangetoond dat de penetratie van UV licht in organisch materiaal beperkt is en dat UV disinfectie een alternatief kan zijn voor het wassen van propere eieren.

In **hoofdstuk 6** werd de bacteriële penetratie van de eischaal en de contaminatie van de ei-inhoud van eieren bestudeerd gebruik makend van 7 uit de ei-inhoud geïsoleerde en geselecteerde bacteriële stammen; *Staphylococcus warneri, Acinetobacter baumannii, Alcaligenes* sp., *Serratia marcescens, Carnobacterium* sp., *Pseudomonas* sp. en *Salmonella* Enteritidis. Een eerste doelstelling was het correleren van de bacteriële penetratie van de eischaal met diverse eischaalkarakteristieken en met de identiteit van fylogenetisch verschillende bacteriële stammen. Met behulp van met agar opgevulde eieren werd de penetratie van de eischaal bestudeerd. Een tweede doelstelling was het inschatten van de contaminatiegraad van de ei-inhoud met de diverse geselecteerde bacteriële stammen; in deze studie werd gebruik gemaakt van intacte eieren. De niet beschadigde eischalen van met agar opgevulde en intacte eieren werden kunstmatig besmet met 10^3 – 10^4 kve van de geselecteerde stam. De geïnoculeerde eieren werden vervolgens gedurende 3 weken bewaard bij 20°C en 60% relatieve luchtvochtigheid. De bacteriële penetratie van de eischaal werd op geregelde tijdstippen beoordeeld en de contaminatie van de ei-inhoud werd na 3 weken nagegaan. In tegenstelling tot de afzetting van de cuticula, bleken de eischaalkarakteristieken oppervlakte van de eischaal, dikte van de schaal en aantal poriën geen invloed te hebben op de bacteriële penetratie van de eischaal. De contaminatie van de ei-inhoud werd noch beïnvloed door de oppervlakte van de eischaal noch door zijn porositeit. De resultaten van de met agar opgevulde eieren tonen aan dat de Gramnegatieve, beweeglijke en niet trosvormende

bacteriën de eischaal het vaakst penetreren; *Pseudomonas* sp. (60%) en *Alcaligenes* sp. (58%) waren de belangrijkste indringers gevolgd door *Salmonella* Enteritidis (43%). Alle geselecteerde stammen waren in staat de eischaal te penetreren; doorgaans werd de penetratie vastgesteld na 4 – 5 dagen. In vergelijking met de niet-*Salmonella* stammen bleek vooral *Salmonella* Enteritidis het meest in staat om de inhoud van intacte eieren te besmetten: membranen en/of ei-inhoud van 32% van de intacte eieren waren besmet. Er werd een significant hogere bacteriologische belasting van de eischaal vastgesteld voor gepenetreerde eischalen en gecontamineerde intacte eieren tegenover niet-gepenetreerde eischalen en niet-gecontamineerde intacte eieren (resultaten van alle stammen samen). Er werd bovendien geen significante invloed van de henleeftijd op de bacteriële penetratie van de eischaal en de contaminatie van de ei-inhoud vastgesteld.

De besmetting van de ei-inhoud van intacte eieren met vier verschillende *Salmonella* Enteritidis stammen en één *Salmonella* Typhimurium stam werd eveneens bestudeerd. De contaminatie van de ei-inhoud varieerde van 6% - 26%. Eigerelateerde *Salmonella* Enteritidis stammen waren niet meer in staat om de ei-inhoud te besmetten dan de overige *Salmonella* Enteritidis stammen en de *Salmonella* Typhimurium stam.

In **hoofdstuk 7** werd de invloed van condens op de eischaal, op de bacteriële penetratie van de eischaal en de contaminatie van de ei-inhoud van intacte eieren met *Salmonella* Enteritidis bestudeerd. Eischalen van met agar opgevulde eieren en intacte eieren werden kunstmatig besmet met 10^3 – 10^4 kve *Salmonella* Enteritidis per eischaal. De met agar opgevulde eieren werden gebruikt voor de studie van de eischaal penetratie terwijl intacte eieren werden gebruikt voor het inschatten van de contaminatie van de ei-inhoud. Van elke groep eieren werd de helft van de eieren gedurende 21 dagen bewaard bij 20°C en 60% relatieve luchtvochtigheid; de overige helft werd eerst gedurende 24 uur bewaard bij 6°C gevolgd door de bewaring bij 20°C. Dit laatste zorgde voor de aanwezigheid van een laagje condens op de eischaal gedurende 30 minuten op het moment dat de eieren bij 20°C geplaatst werden. Rekening houdend met de resultaten bekomen op de drie henleeftijden (39, 53 en 67 weken) werd een gemiddelde eischaalpenetratie van 62% vastgesteld bij de eieren met condens tegenover 43% voor de controlegroep; dit was een statistisch significant verschil ($P < 0.01$). Er werd geen significant verschil in contaminatie van intacte eieren aangetoond; 18% van de controle-eieren waren gecontamineerd tegenover 22% van de intacte eieren met condens. Opmerkelijk was wel de significant hogere besmetting van de ei-inhoud van intacte eieren geraapt op het einde van de leg, in vergelijking met de twee jongere henleeftijden. Deze

besmetting werd vermoedelijk niet veroorzaakt door een hogere graad van eischaalpenetratie, aangezien dit niet kon aangetoond worden bij de overeenkomstige met agar opgevulde eieren. Besluitend kan gesteld worden dat condens op de eischaal de penetratie van de eischaal bevorderde maar een beperktere impact had op de contaminatie van de ei-inhoud.

In '**Conclusions and perspectives**' werden de belangrijkste besluiten van dit doctoraatswerk samengevat en worden enkele suggesties ter reductie van de contaminatie van de ei-inhoud besproken. Eveneens werden er de eerste resultaten over de reële impact van de verticale en horizontale besmetting van de ei-inhoud besproken.

Naast de kritische punten voor de initiële bacteriologische belasting van de eischaal met totaal aantal aërobe kiemen in de alternatieve (zonder kooi) huisvestingssystemen, werd slechts één ander kritisch punt voor de introductie van eischaalcontaminatie in de keten aangetoond. Een verdere verbetering van de design van de alternatieve huisvestingssystemen moet toelaten om de impact van enkele kritische punten verder te reduceren. De bacteriologische penetratie van de eischaal (agar opgevulde eieren) was gecorreleerd met de graad van bacteriologische belasting van de eischaal. Niettegenstaande bij bewaring onder optimale omstandigheden de horizontale besmetting van intacte eieren vermoedelijk beperkt is en niettegenstaande de minder uitgesproken invloed van de bacteriële eischaalbelasting op de besmetting van intacte eieren, zijn we er toch van overtuigd dat in praktijkomstandigheden een reductie van de eischaalcontaminatie belangrijk is voor het beperken van de contaminatie van de ei-inhoud.

Curriculum vitea

Curriculum vitea

Koen De Reu werd geboren op 4 juni 1966 te Gent en groeide op te Brugge. In 1984 behaalde hij het diploma Wiskunde aan het Koninklijk Atheneum I te Brugge. In 1990 promoveerde hij tot Ingenieur voor de scheikunde en de landbouwindustrieën.

Na zijn legerdienst was hij vanaf 1 februari 1992 werkzaam als assistent op het toenmalige Rijkszuivelstation. Hij was er tot begin 2000 verantwoordelijk voor de wetenschappelijke begeleiding van de Vlaamse interprofessionele organismen betrokken bij de bepaling van de kwaliteit en de samenstelling van de rauwe melk. In de periode 1992 - 1993 was hij eveneens verantwoordelijk voor het Fysisch-chemische routinelaboratorium van het Rijkszuivelstation, om zich vanaf 1994 ook in te zetten als kwaliteitsverantwoordelijke met het oog op het bekomen van een BELTEST accreditatie voor diverse laboratoria van het Rijkszuivelstation. Deze accreditatie werd op 26 september 1995 een feit.

In 2000 ruilde hij de job als verantwoordelijke voor de wetenschappelijke begeleiding in voor de functie als laboratoriumverantwoordelijke van het geaccrediteerde microbiologische routinelaboratorium en bleef verder kwaliteitsverantwoordelijke van de BELTEST geaccrediteerde kwaliteitsafdeling van het ondertussen van naam veranderde Rijkszuivelstation in Departement voor de kwaliteit van dierlijke producten en transformatietechnologieën (DVK). Als afgeleide van het microbiologische routinelaboratorium werden na vele jaren dienstverlening nu ook de eerste stappen in het wetenschappelijke onderzoek gezet. Vanaf 1 maart 2001 werd hij o.a. verantwoordelijk voor het project ‘Kwaliteitsbewaking in de productieketen van eieren als middel tot reductie van de bacteriële infectie – S5999 en S6133’ gefinancierd door de Federale Overheidsdienst Volksgezondheid, Veiligheid Voedselketen en Leefmilieu (Betoelaagd Onderzoek). Dit project werd eveneens de basis voor het opstellen van dit doctoraat. Naast het wetenschappelijk onderzoek en de dienstverlenende taken als laboratorium- en kwaliteitsverantwoordelijke is hij eveneens consultant bij de accreditatie van de andere laboratoria van het Centrum voor Landbouwkundig Onderzoek (nu Instituut voor Landbouw- en Visserijonderzoek - Gent) en technische auditeur microbiologie (voorheen Fysica-Chemie) voor de Nederlandse Raad voor Accreditatie. Tenslotte is hij eveneens lesgever bij het Instituut voor Permanente Vorming.

Momenteel is hij op onderzoeksvlak verantwoordelijk voor de uitvoering van het onderzoeksproject “Vergelijking van verrijkte kooien versus alternatieve huisvestingssystemen voor leghennen met betrekking tot sanitaire status en dierenwelzijn - S-6164” gefinancierd door de eerder vermelde Federale Overheidsdienst.

In het dagelijkse leven is Koen vake van twee prachtige zonen Jarne (4 jaar) en Jitze (2 jaar). Samen met hun moeke Jessy tracht hij hun gezinsleven zo avontuurlijk mogelijk in te vullen met voornamelijk uitstapjes, reizen, scouting en muziek.

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   “Melkcontrole met infraroodtechnieken”

4. KAHO-Sint-Lieven studieavond “Statistische procescontrole in de praktijk”, Gent, Belgium, 9 May 2000.
   “Statistische procesbeheersing in een geaccrediteerd laboratorium”

5. Symposium “Veilig produceren – veilig consumeren” georganiseerd door Hogeschool Gent Dept. BOIT., Gent, Belgium, 14 April 2002.
   “Kwaliteitscontrole en risicobeheersing in de zuivelsector”

6. 6th Workshop for the National Reference Laboratories on Milk and Milk products, AFFSA, Maisons-Alfort, France, 3 - 4 June 2003.
   “Hygienic parameters, toxins and pathogen occurrence in retail raw milk cheeses, raw farm milk, direct marketing raw milk farm products and imported Feta cheeses”

7. 17th Forum for Applied Biotechnology, Gent, Belgium, 18 - 19 September 2003.
   “Quality assurance in the egg production chain to reduce the bacterial contamination of the eggshell”

8. XVIth European Symposium on the Quality of Poultry Meat & Xth Symposium on the Quality of Eggs and Egg Products, Saint-Brieuc, Ploufragan, France, 23 - 26 September 2003.
   "Bacterial eggshell contamination in the egg production chain and in different housing systems"

9. XXIIth World's Poultry Congress, Istanbul, Turkey, 8 - 13 June 2004.
   "Assessment of the eggshell penetration by different bacteria, including *Salmonella* Enteritidis, isolated from the egg content of consumption eggs"

10. Symposium Sectie Levensmiddelenmicrobiologie van de Nederlandse Vereniging voor Microbiologie "Microbiologisch onderzoek van levensmiddelen: eigen werk", Wageningen, The Netherlands, 22 June 2004.
    "Bepaling van de doordringing van de eischaal door diverse bacteriën, waaronder *Salmonella* Enteritidis"

11. COST 923 Expert meeting: WG3 StudyGroup "Hygiene", Thessaloniki, Greece, 14 - 18 July, 2004.
    "Quality assurance in the egg production chain of consumption eggs to reduce the bacterial contamination of the eggshell"

12. 19th International ICFMH symposium – Food Micro 2004, Portoroz, Slovenia, 12 - 16 September 2004.
    "Eggshell factors influencing eggshell penetration and intact egg contamination by different bacteria, including *Salmonella* Enteritidis".

13. COST 923 meeting Multidisciplinary Hen Egg Research, Barcelona, Spain, 18 - 19 October 2004.
    "Bacterial eggshell contamination in the egg production chain, reduction of the contamination and bacterial eggshell penetration and whole egg contamination".

14. Symposium "GGO's in de praktijk", Departement voor Plantenveredeling en Plantengenetica, Merelbeke, Belgium, 16 December 2004.

"GGO analyses in de praktijk: ISO 17025 accreditatie"

15. Symposium from The Animal Science Group, Wageningen UR "Should hens be kept outside", Nijmegen, The Netherlands, 18 - 20 April 2005.
"Bacterial eggshell contamination in conventional cages, furnished cages and aviary housing systems for laying hens"

16. XVIIth European Symposium on the Quality of Poultry Meat & XIth Symposium on the Quality of Eggs and Egg Products, Doorwerth, The Netherlands, 22 - 26 April 2005.
"Influence of eggshell condensation and heat stress for laying hens on the bacterial eggshell penetration and the whole egg contamination with *Salmonella enterica* serovar Enteritidis"

17. Symposium van het DVK-CLO "Evolutie van analytische technieken in het voedingslaboratorium", Merelbeke, Belgium, 23 September 2005.
"Evolutie in de BELTEST accreditaties van het DVK-CLO"

Congresses and symposia with poster

1. Second International Sonthofen Symposium – IDF - Quality and economic efficiency in dairy and food laboratories, Sonthofen, Germany, 20 - 22 May 1996.
"Scientific guidance of the Belgian and Luxembourgian laboratories in charge of the official determination of milk composition."

2. 18th International ICFMH Symposium - Food Micro 2002, Lillehammer, Norway, 17 – 23 August 2002.
"Quality assurance in the egg production chain to reduce the bacterial contamination."

3. Tenth Conference on Food Microbiology, Luik, Belgium, 23 – 24 June 2005.
"The role of bacterial species and strains in the whole egg contamination by horizontal transmission."

Congresses and symposia without contribution

Different other congresses and symposia were participated without contribution.

Major other oral contributions

1. "Wetenschappelijke begeleiding van de Vlaamse Provinciale Comités en de Vereniging voor de Melkkwaliteit", 15 March 1993, Bokrijk, Belgium, op de jaarvergadering ingericht door het Provinciaal Comité voor de Melkkwaliteit Limburg.

2. "Results of Belgian experiments with cold samples", 11 March 1994, Lier, Belgium, on the "International Bactoscan workshop".

3. "Werking, instelling en borging van BactoScan 8000" 28 October 1996, Lier, Belgium, op de Workshop BactoScan 8000.

4. "Accreditatie op het DVK", 5 December 2000, Melle, Belgium, op de CLO-Gent studiedag "Accreditatie: wanneer en hoe?".

5. "Vergelijking gevolgde methoden totaal kiemgetal – plaatmethode", 22 februari 2001, Melle, Belgium, op de "Workshop totaal kiemgetal (plaatmethode) voor Belgische departementale laboratoria" georganiseerd door het DVK-CLO.

6. "Problematiek van antibioticagebruik op melkveebedrijven", 28 May 2001, Bokrijk, Belgium, op de jaarvergadering ingericht door het Provinciaal Comité voor de Melkkwaliteit Limburg.

7. "Accreditatie in de praktijk", 1 June 2001, Merelbeke, Belgium, op de studiedag "VLARISUB" georganiseerd door het DFE-CLO Merelbeke.

8. “Technische aspecten bij de uitvoering van de bepaling van coliformen”, 13 March 2002, Melle, Belgium, op de “Workshop coliformen voor de Belgische Zuivelsector” georganiseerd door het DVK-CLO.