THE EFFECT OF DIFFERENT HOUSING SYSTEMS ON SALMONELLA AND ANTIMICROBIAL RESISTANCE IN LAYING HENS

SEBASTIAAN VAN HOOREBEKE

Thesis submitted in fulfillment of the requirements for the degree of Doctor in Veterinary Sciences (PhD), Faculty of Veterinary Medicine, Ghent University, 2010

Promoters:
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This research was funded by the EU FP6, under the contract 035547 (Safehouse project)

Schilderij kaft: Koen Pattyn

Printed by: Ryhove Plot-it
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SUMMARY  

SAMENVATTING  

CURRICULUM VITAE  

DANKWOORD  

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LIST OF ABBREVIATIONS

BGA brilliant green agar
BPW buffered peptone water
CI confidence interval
CLSI Clinical and Laboratory Standards Institute
DANMAP Danish Integrated Antimicrobial Resistance Monitoring and Research Program
EFSA European Food Safety Authority
EU European Union
EUCAST European Committee on Antimicrobial Susceptibility Testing
FAVV Federaal Agentschap voor de Veiligheid van de Voedselketen
GEE generalized estimating equations
MDR multidrug resistance
MSRV modified semisolid rappaport vassiliadis agar
OR odds ratio
PT phage type
SPSS Statistical Package for the Social Sciences
subsp. subspecies
VEPEK Vereniging voor Pluimvee, Eieren en Konijnen
XLD xylose lysine decarboxylase
CHAPTER 1

GENERAL INTRODUCTION
PREFACE

Nowadays, consumers have more and more concerns about modern food production systems, especially with respect to animal welfare, the protection of the environment and food safety issues. Since these three aspects are strongly linked to each other, implementing regulations or new standards to improve one aspect may negatively influence other aspects, creating potential conflicts between regulatory aims (de Passillé and Rushen, 2005).

Perhaps one of the best known manifestations of consumers awareness of animal welfare is the growing discontent with the conventional industrial egg production, resulting in an aversion to eggs originating from hens housed in conventional battery cages (Appleby, 2003). This finally lead to the European ban on conventional battery cages (Council Directive 1999/74/EC). From 2012 onwards, laying hens must be housed in enriched cages or non-cage housing systems such as aviaries, floor-raised and free-range systems.

At the same time food safety has become a very important consumer demand. Eggs used for human consumption should be free from both microbiological and chemical contaminants. An important zoonotic pathogen typically associated with eggs and egg products is *Salmonella Enteritidis* (Davies and Breslin, 2001; Namata et al., 2008). *S. Enteritidis* is, together with *S. Typhimurium*, the most commonly isolated serotype in human cases of salmonellosis (WHO, 2006; EFSA, 2007) in Europe.

Another aspect of food safety gaining importance over the last 15 years is the emergence of antimicrobial resistance in food production animals and the spread of resistant bacteria from animals to humans. To fully assess the size of the problem in animal production, standardized and continuous surveillance programs are necessary to monitor the occurrence and persistence of antimicrobial resistance in food animals (Aarestrup, 2004; Wallmann, 2006; WHO, 2008). Antimicrobial resistance in pathogenic and indicator bacteria in broilers, pigs and cattle has been extensively explored and described. However, epidemiological data on the prevalence of antimicrobial resistance in laying hens is still scarce. Since it has been described for laying hens that the incidence of bacterial diseases tends to increase in non-cage systems, possibly resulting in higher antibiotic usage, it is necessary to further explore this topic.

In this thesis, the influence of different housing systems for laying hens on the prevalence of *Salmonella* and antimicrobial resistance in indicator bacteria will be explored.
1. LAYING HEN HUSBANDRY
   a. Production chain

   ![Diagram of the egg industry](image)

   **Figure 1:** Diagram of the egg industry (adapted from Defra, 2004 and VEPEK, 2009)

   The egg production chain is characterised by a strongly integrated nature, meaning there is a strong entanglement between all links of the supply chain. In the flowchart below, the different stages are schematically represented (Figure 1).

   At the top, there are the farms with the parent breeding hens. They produce breeding eggs that are delivered to the hatcheries. In the hatcheries, day-old layer chicks are produced. These chicks are transported to the rearing farms. The young pullets stay there till 18 weeks of age after which they are brought to the egg producing farms. A proportion of the finally produced eggs is transported to the packing station. From here the eggs go the retail. The other eggs are not sold as whole table eggs but are processed in the industry and incorporated
in other products, such as sauces, biscuits, frozen fried eggs, pasteurized whole eggs, albumen and yolks...

Usually, the productive life span of laying hens is around 14 months but depending on the price of the eggs versus the cost of feed and replacement pullets, the producer can decide to recycle the current flock by induced moulting to achieve a second, but shorter, laying cycle (North and Bell, 1990; Holt, 2003). Induced moulting is thought to be economically advantageous, especially in a market situation with higher egg prices, declining values of hen carcasses and availability of fewer processors willing to process them (Holt, 2003; Webster, 2003). Feed withdrawal to achieve body weight loss, followed by a rest period and then restimulation of egg production has since long been the typical industry method for induced moulting (Webster, 2003), although for welfare reasons this has been forbidden in the EU (Yousaf and Chaudhry, 2008). Besides welfare issues, it has also been described that the process of induced moulting has many adverse influences on the presence and shedding of *Salmonella* in laying hens (Holt and Porter; 1993; Ricke, 2003; Golden et al., 2007).

Although the egg production chain is very strictly organized from top to bottom, it is no isolated entity. There are very frequent and intense contacts with other segments of poultry production in specific and the agricultural sector in general, which implies risks for the spread of diseases. To illustrate this, the contact structure of the Belgian poultry sector is presented in Figure 2 with the number of incoming and outgoing contacts per month between the different segments of the poultry sector.
b. Housing of laying hens

i. History of the battery cages

For centuries, free-range housing and indoor floor housing were the most common methods of laying hen husbandry. This changed rapidly when the keeping of laying hens in cages boomed in the USA during the 1930’s and 1940’s. During the following decades this housing system for laying hens became the most used system worldwide (Bell, 1995; Duncan,
2000; Tauson, 2005). Originally, cages were introduced for single laying hens. Later on, several hens were placed in a cage and group sizes from three to eight hens were typically used. The term ‘battery cage’ is derived from the large number of cages in a laying hen house which is called a battery of cages (Keeling, 2002; Appleby et al., 2004).

The housing in battery cages had numerous advantages: it gave way to automatization, it was less labour-intensive for the laying hen keepers and it allowed keeping more hens on the same surface. Besides these economical and ergonomical advantages it also implied much higher hygienic standards because the hens were now separated from their own faeces, there was significant less interference from the environment and there was a reduced risk of mortality due to cannibalism, feather pecking and aggression (Duncan, 2000; Savory, 2004).

In spite of all these advantages, it is no surprise that from the beginning concerns were raised about the adverse consequences of battery cage housing for the welfare of the laying hens, especially because of the very restricted space and the impossibility to perform their natural behaviour.

ii. The EU ban on battery cages

The European Union took a leading role in the debate on laying hens’ welfare by adopting Council Directive 1999/74/EC, stating that from January 1st 2012 onwards the housing of laying hens in conventional battery cages will be forbidden in all EU member states. However, it took some time before a consensus on the main points was reached.

The welfare of laying hens was brought to the public attention as early as 1964, when Ruth Harrison published her book ‘Animal Machines’. The book focused principally on the welfare of animals kept under intensive production systems, such as the battery cages for laying hens, and it also raised questions about the safety of eating products from animals kept under such conditions (Fraser, 2001). The public concern, triggered by this book and others, made that the welfare of farm animals has been under discussion ever since.

To meet this concern, in 1976 the Council of Europe introduced the Convention on the Protection of Animals kept for farming purposes, with the objective to improve the care, husbandry and housing of farm animals, especially those in intensive systems (Anon., 1976). However, the recommendations in the Convention were only described in very general terms. To counter this, the Council organized a Standing Committee whose task was to work out some more specific requirements (Appleby, 2003).
The EU was an important player in the process, first by becoming an active party in the Convention in 1978 and then by financing background scientific work on poultry welfare in the farm animal welfare coordination program from 1979 (Tarrant, 1983; Appleby, 2003). All this resulted nearly a decade later in a Directive (Anon., 1986) laying down minimum standards for the protection of hens in battery cages, being one of the first Europe-wide statutes that actually specified how animals were to be kept. It stated that by the 1st of January 1988, all newly built battery cages had to provide at least 450 cm² per hen, and these standards had to apply to all cages by January 1995. The strength of this Directive was that EU member states were forced to implement the provisions into their national legislation (Appleby, 2003).

In 1992, because of ongoing public pressure, a new draft of an EU-Directive was formulated by the European Commission, although this did not influence the situation in the field, partially because of the huge scepticism of the egg producing industry. It was not until March 1998 that the European Commission brought out a new proposal for an EU Directive, stating that hens should be provided with a nest, litter and perching facilities (Anon., 1998). It was for the first time that the term ‘enriched cage’ was mentioned, defined as ‘a battery cage equipped with litter, perches and a ‘nestbox’.

Directive 1999/74/EC, laying down minimum standards for the protection of laying hens, restricts the housing of laying hens in the EU to three different categories: unenriched cages, enriched cages and alternative systems. The Directive states that the so-called unenriched cages (i.e. conventional battery cages) must be phased out by January 1st 2012. In the meantime, cages have to provide 550 cm² per hen since 2003. In newly built houses, hens cannot be housed in conventional battery cages since 2003.

All member states had to implement Directive 1999/74/EC into their national legislation before 1 January 2002. Belgium however only implemented it in October 2005 (Anon., 2005a). Because each country can be stricter than the minimum requirements imposed by the Directive, there are a few exceptions on the time scheme. Sweden for instance banned battery cages from 1999 onwards; Germany did so from the 1st of January 2010.

iii. Alternatives for conventional battery cages

From 2012 onwards, the housing of laying hens in the EU will be restricted to enriched cages and to the so-called alternative systems. The main characteristics of these systems are summarized below and in Table 1.
Table 1: Characteristics of enriched cages and alternative housing systems according EU Directive 1999/74/EC

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Enriched cages</th>
<th>Alternative systems</th>
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<tr>
<td><strong>Area per hen</strong></td>
<td>Min. 750 cm$^3$ of which 600 cm$^2$ is usable area$^1$</td>
<td>Max. 9 hens/m$^2$</td>
</tr>
<tr>
<td></td>
<td>Total cage area min. 2000 cm$^2$</td>
<td></td>
</tr>
<tr>
<td><strong>Height</strong></td>
<td>Usable area$^1$: min. 45 cm</td>
<td>Max. 4 levels</td>
</tr>
<tr>
<td></td>
<td>Other area: min. 20 cm</td>
<td>Min. 45 cm between the levels</td>
</tr>
<tr>
<td><strong>Floor</strong></td>
<td>Slope max. 14 % or 8°</td>
<td>Must support the forward facing claws</td>
</tr>
<tr>
<td><strong>Length feeders</strong></td>
<td>Min. 12 cm per hen</td>
<td>Linear feeders: min. 10 cm/hen</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Circular feeders: min. 4 cm/hen</td>
</tr>
<tr>
<td><strong>Water supply</strong></td>
<td>Appropriate to group size</td>
<td>Continuous drinking through: 2.5 cm/hen</td>
</tr>
<tr>
<td></td>
<td>Min. 2 nipples or cups within the reach of each hen</td>
<td>Circular drinking through: 1 cm/hen</td>
</tr>
<tr>
<td><strong>Claw-shortening devices</strong></td>
<td>Suitable devices</td>
<td>No</td>
</tr>
<tr>
<td><strong>Nest$^2$</strong></td>
<td>Yes</td>
<td>Yes, min. 1 nest/7 hens</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Group nest: min. 1 m$^2$ for max. 120 hens</td>
</tr>
<tr>
<td><strong>Litter$^3$</strong></td>
<td>Yes, such that pecking and scratching are possible</td>
<td>Yes, min. 250 cm$^2$/hen</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Min. 1/3 of the ground surface</td>
</tr>
<tr>
<td><strong>Perches</strong></td>
<td>Yes, min. 15 cm/hen</td>
<td>Yes, min. 15 cm/hen</td>
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$^1$ usable area: an area at least 30 cm wide with a floor slope not exceeding 14 %, with headroom of at least 45 cm.

$^2$ nest: a separate space for egg laying for an individual hen or for a group of hens

$^3$ litter: any friable material enabling the hens to satisfy their ethological needs
Chapter 1

*Enriched cage*

Enriched cages must provide 750 cm$^2$ per hen, a nestbox, perches and litter such that pecking and scratching are possible (Figure 3). Enriched cages exist in a wide variety of group sizes, ranging from small (< 15 hens), over medium (15 – 30 hens) to large (> 30 hens) (LayWel, 2006). Enriched cages are also called modified or furnished cages. Although the latter term is recommended because it is thought to give the most accurate description (Appleby, 2004; LayWel, 2006), throughout this study the term ‘enriched’ will be used in accordance with the terminology of the Directive.

![Figure 3: Enriched or furnished cage](image)

The term ‘alternative’ refers to a wide variety of non-cage housing systems. All these systems are operated from inside and entered by the laying hen keepers. Because of the possible confusion caused by the word ‘alternative’ it has been stated that the term ‘non-cage’ is more appropriate (Blokhuis et al., 2007). Therefore the term ‘non-cage’ will be used throughout this thesis. The following non-cage systems will be described: aviaries, floor-raised systems, free-range systems and free-range organic systems.

*Aviary system*

Aviaries consist of the ground floor plus 1 to 4 levels of perforated platforms, from which manure cannot fall on the hens below (Figure 4). The headroom between the levels must be at least 45 cm. The maximum stocking density is 9 hens/m$^2$ usable area.

![Figure 4: Aviary system](image)
**Floor-raised system**

Floor-raised or ‘barn’ systems are single-level systems, meaning that there is only one level for the birds at any point in the house. The maximum stocking density must not exceed 9 hens/m² usable area. Each hen must have at least 250 cm² of littered area, the litter occupying at least 1/3 of the ground surface. Floor-raised systems may be combined with a covered outdoor facility, the so-called ‘Wintergarden’ (Figure 5). This outdoor area is connected to the hen house and has a concrete floor, usually covered with litter.

![Figure 5: ‘Wintergarden’](image)

**Free-range system**

Free-range systems are also single-level systems but they are always combined with uncovered outdoor facilities (Figure 6). There must be several popholes giving direct access to the outer area extending along the entire length of the building. The outdoor run must be of an area appropriate to the flock size and it must be equipped with shelter from inclement weather and predators.

![Figure 6: Free-range system](image)

**Free-range organic**

The organic laying hen husbandry is with regard to the design of the house similar to free-range systems. However, there are some very important additional requirements. The maximum stocking density must not exceed 6 hens/m² usable area, instead of 9 in the other systems. The maximum capacity of the hen house is restricted to 3,000 birds. Hens should
have access to an outdoor run for at least 1/3 of their productive lifespan and each hen should at least have 4 m$^2$ of usable outdoor area.

In conclusion, it is clear that the above mentioned ban on conventional battery cages aims to improve the welfare of laying hens (Wall et al., 2004). Although the influences of these alternatives for conventional battery cages on laying hen welfare, productivity and user-friendliness have been extensively evaluated and discussed (Abrahamsson and Tauson, 1995; Tauson et al., 1999; Tauson, 2002; Rodenburg et al., 2005; 2008), it has also initiated the question whether there aren’t any adverse consequences of this decision on the spread and/or persistence of infectious diseases in a flock. After all, one of the biggest advantages of conventional battery cages is that, because hens are separated from their faeces, the risk for disease transmission through faeces can be minimized (Duncan, 2000). For instance the intensive and frequent hen-to-hen contacts and the presence of litter in non-cage systems jeopardize the biosecurity and increase the risk for specific diseases to develop and spread. For several infections such as *E. coli*, erysipelas and pasteurellosis an increase in incidence has been observed after the move to non-cage systems (Hafez, 2001; Permin et al., 2002). This leads to the question whether the same effect is to be expected for zoonotic pathogens such as *Salmonella*. 
2. THE ROLE OF EGGS IN HUMAN SALMONELLOSIS

a. What is Salmonella?

In 1884, G. Gaffky was the first to culture Salmonella (Le Minor, 1994). Two years later, D.E. Salmon gave his name to this bacterium when he succeeded, together with T. Smith, in isolating Salmonella Choleraesuis from a pig (Salmon and Smith, 1886). Today, the genus Salmonella encompasses a large taxonomic group with nearly 2500 recognized serotypes or serovars (Heyndrickx et al., 2005). The most common way to classify isolated Salmonella bacteria is serotyping according to the Kauffmann-White scheme, based on the presence of H (flagellar), capsular (Vi) and O (somatic) antigens (Popoff and Le Minor, 1997). Phage typing has been, and still is, a very important tool for the phenotypical characterization of Salmonella strains, especially in outbreak investigations (Ward et al., 1987; Vieu, 2003; Baggesen et al., 2010). Other ways of typing Salmonella strains are pulsed field gel electrophoresis (Murase et al., 1995), random amplification of polymorphic DNA (Hilton et al., 1996) and ribotyping (Grimont and Grimont, 1986).

From an epidemiological point of view, Salmonella serovars can also be divided into groups based on their particular host specificity and pathogenesis (Kingsley and Bäumler, 2000). A first group consists of the host-restricted serovars. These serovars are capable of causing severe systemic disease in a very limited number of related species. Typical examples of these serotypes are S. enterica subsp. enterica serovar Gallinarum in poultry and S. enterica subsp. enterica serovars Typhi and Paratyphi A in humans. A second group are the so-called host-adapted serovars which are prevalent in one particular host species but which can also cause disease in other host species (e.g. S. enterica subsp. enterica serovar Dublin, causing severe disease in cattle but it may also cause disease in humans). Finally there is the large group of the un-restricted or ubiquitous serovars (e.g. S. enterica subsp. enterica serovar Enteritidis and S. enterica subsp. enterica serovar Typhimurium). These serovars only seldom cause systemic infections in healthy adults but they are capable of colonising the gastrointestinal tract of a broad range of animals. The intestinal colonisation and often high levels of faecal shedding in food producing animals allow these serovars to enter the food chain, resulting from time to time in human cases of salmonellosis (Uzzau et al., 2000; Wallis and Barrow, 2006).

Salmonella constitutes a genus of Gram-negative bacteria belonging to the family of the Enterobacteriaceae, implying that strains belonging to this family are straight rods which
generally are motile because of the presence of peritrichous flagella; they ferment glucose, often with gas production; they reduce nitrate into nitrite and they are oxidase negative (Grimont et al., 2000). Metabolic characteristics of the genus *Salmonella* are that urea is not hydrolysed; tryptophan and phenylalanine are not deaminated; acetoin is not produced; lactose, adonitol, sucrose, salicin and 2-ketogluconate are not fermented; H$_2$S is produced from thiosulphate; and lysine and ornithine are decarboxylated.

Until now, the focus of the European *Salmonella* control programs has been on *S. enterica* subsp. *enterica* serovar Enteritidis (S. Enteritidis in short) and *S. enterica* subsp. *enterica* serovar Typhimurium (S. Typhimurium), since these are the most commonly isolated serovars in cases of human salmonellosis. In the next chapters, mainly *Salmonella* Enteritidis will be discussed because of its typical relationship with eggs and egg products. Humans acquire *Salmonella* Typhimurium via a much more diverse range of vectors such as pork (Soumpasis and Butler, 2009), beef and poultry meat products (Dechet et al., 2006; Ethelberg et al., 2007) and unpasteurized dairy products (Cody et al., 1999; Villar et al., 1999).

b. Infection pathway

i. Pathogenesis in chickens

The usual route of infection in chickens is the oral uptake of *Salmonella* bacteria from the birds’ environment. Contaminated feed and water for instance are important sources of *Salmonella* infections in chickens (Cox et al., 1991; Heyndrickx et al., 2002). The vertical transmission of *Salmonella* can also be an important issue in poultry: neonate chicks can get infected by infected breeder hens and their eggs (Barrow, 1999; Poppe, 2000; Liljebjelke et al., 2005). Once a few animals are infected, the infection can spread throughout the flock through animal to animal contact (Byrd et al., 1998; Gast and Holt, 1999), through vectors such as rodents and flies (Kinde et al., 2005; Carrique-Mas et al., 2009) or by airborne transmission (Lever and Williams, 1996; Nakamura et al., 1997; Holt et al., 1998).

As in the pathogenesis of other bacteriological intestinal infections, the adhesion of the *Salmonella* bacteria to the surface of the intestinal epithelial cells of the host is a very important step in the interaction between the pathogen and the host (Finlay and Falkow, 1989). This process occurs mainly through fimbriae, although some outer membrane proteins seem to play a role as well (Fadl et al., 2002; Kingsley et al., 2002).
Once attached, *Salmonella* stimulates its own uptake by the intestinal epithelial and other cell types using a type III secretion system, encoded by the *Salmonella* Pathogenicity Island I (SPI-1) (Darwin and Miller, 1999; Zhou and Galán, 2001). This is the invasion phase of the pathogenesis. In chickens, this occurs mainly in the caeca (Desmidt et al., 1997; 1998). Shortly after invasion, the intestinal epithelial cells will produce pro-inflammatory cytokines (Kaiser, 1994; Klasing, 1998), attracting heterophilic granulocytes and macrophages (Van Immerseel et al., 2002). The *Salmonella* bacteria will be phagocytised and are able to survive and replicate in the macrophages. This also requires a type III secretion system regulated by SPI-2. Through the spread of these infected macrophages, the bacteria will be able to reach other internal organs such as the liver, spleen and the reproductive tract (Barrow and Lovell, 1991; Barrow, 1999). This is the systemic phase of infection.

**ii. Clinical symptoms in chickens**

*Salmonella* infections in chickens often cause only very limited symptoms or even no symptoms at all. Two exceptions are *Salmonella* Gallinarum and *Salmonella* Pullorum, serovars causing severe illness in chickens, with high morbidity and mortality in the flock (Erbeck et al., 1993; Wong et al., 1996; Shivaprasad, 2003).

Young chicks can show symptoms of disease after infection with *S. Enteritidis* or *S. Typhimurium*. The chicks may exhibit symptoms including anorexia, adipsia, general depression, huddling together in small groups, immobility, ruffled feathers, white diarrhoea and stained or pasted vents (Marthedal, 1977; McIlroy et al., 1989). Normally no typical clinical symptoms occur in infected flocks of mature laying hens, although seldom a limited increase in mortality may be observed (Humphrey et al., 1991; Kinde et al., 2000).

In general, the egg production in naturally infected laying hen flocks remains in the normal range (Muller and Korber, 1992; Awadmasalmeh and Thiemann, 1993). However, under experimental conditions a decreased egg production has been reported after oral (Gast and Beard, 1992; Gast, 1994) or intravenous (Guard-Petter, 1998; Okamura et al., 2001) infections of laying hens with *S. Enteritidis*.

**iii. Mechanisms of egg contamination and numbers of infected eggs**

As already mentioned above, consumption of contaminated eggs or egg products is the main source of infection with *S. Enteritidis* for humans (Delmas et al., 2006; EFSA, 2006).
The mechanisms of egg contamination have been extensively described by Gantois et al. (2009). Below, only a short description of the main mechanisms of egg contamination will be presented.

Generally, eggs can get contaminated by *Salmonella* bacteria via horizontal or vertical transmission. Horizontal transmission means that the eggs get contaminated by penetration of *Salmonella* through the egg shell during or after oviposition (Messens et al., 2005; De Reu et al., 2006), with the bacteria coming from the colonized gut or from contaminated faeces. The second possibility is vertical transmission, with direct contamination of the different egg components before oviposition (Keller et al., 1995; Miyamoto et al., 1997; Okamura et al., 2001). In this scenario the *Salmonella* bacteria originate from infection of the reproductive organs.

For long, the percentage of eggs that are actually infected in an infected flock of laying hens has been the topic of debate. Under experimental conditions, egg contamination rates from *Salmonella* infected hens ranging from 0 % to 27.5 % have been reported, depending on the inoculation route, the infection dose and the number of animals used (Keller et al., 1995; Okamura et al., 2001). In naturally infected laying hen flocks, there is agreement that this percentage varies strongly (Humphrey et al., 1989), although most studies indicate that it does not exceed 3 % (Kinde et al., 1996; Schlosser et al., 1999).

Statistical techniques and quantitative risk assessment models are another way to estimate the number of infected eggs. Going through the literature, estimates ranging from 1 out of 12,000 up to 1 out of 30,000 infected eggs can be found (Ebel and Schlosser, 2000; Hope et al., 2002).

The number of contaminated eggs can also be determined at retail level. The Egg Survey report of the Food Standards Agency (2004) studied the proportion of eggs contaminated with *Salmonella* on the retail sale level by purchasing boxes of 6 eggs from a cross-section of retail outlets throughout the UK. It showed that in 0.34 % (95 % CI 0.17 – 0.62 %) of the 4,753 boxes tested at least one egg out of the 6 was positive for *Salmonella*. A smaller study with similar aims did not detect any *Salmonella* in the contents of 100 dozens eggs (Schutze et al., 1996).

The number of infected eggs in a flock is logically related with the number of infected hens in that flock and the timing of egg production relative to infection: eggs produced soon after infection are much more likely to harbour *Salmonella* (Gast and Beard, 1990). However,
it is very hard to gain accurate information on the within-flock prevalence of *Salmonella* in laying hen flocks. This lack of knowledge complicates the quantitative assessment of several management and preventive measures at the level of the primary production.

c. Human *Salmonella* infections

   i. Evolution in time

Already for many decades salmonellosis is recognised worldwide as an important foodborne disease, even becoming more common because of the globalization of the food supply and changes in lifestyle (Todd, 1997). The most recent data on zoonoses of the European Food Safety Authority indicate that *Salmonella* is the second most important foodborne disease in the EU, only preceded by *Campylobacter* (EFSA, 2010). In 2008, there were 131,468 confirmed cases of human salmonellosis in the 27 EU member states. In the European Community, the number of human salmonellosis cases shows a statistically significant decreasing trend in the past 5 years (Figure 7). However, this is only the case in 10 out of the 27 member states. In several countries no significant difference could be observed during this period, whereas in 7 member states even a significant increase was observed.

![Figure 7: Evolution in number of confirmed cases of human salmonellosis in the EU (EFSA, 2010)](image)

In 2008, *S. Enteritidis* was the most commonly reported serovar in the EU (58.0 % of all confirmed cases), followed by *Salmonella* Typhimurium (21.9 %) (EFSA, 2010). It is worth mentioning that the number of *S. Enteritidis* cases tends to decrease on the EU level, increasing the relative proportion of *S. Typhimurium* cases. Specific for Belgium, a
downward trend in numbers of human salmonellosis cases can be observed, with 3,944 confirmed cases in 2008 (National Reference Centre for *Salmonella* and *Shigella*, 2009). Since the peak of 15,774 cases in 1999, a decrease in number of human salmonellosis cases in Belgium has been observed with a considerable drop from 2005 onwards, strongly associated with a drastic decrease of *S*. Enteritidis cases (Collard et al., 2008). It is thought that the vaccination of commercial laying hens against *Salmonella* has played a huge role in this evolution. In Belgium, *S*. Typhimurium has become the most commonly isolated serotype, accounting for 57.78 % of the cases. *S*. Enteritidis came second with 20.89 % (National Reference Centre for *Salmonella* and *Shigella*, 2009).

**ii. Relationship between eggs and human infections**

Although humans can get infected by *Salmonella* through a wide range of food products, both from animal and non-animal origin, eggs and egg products are generally considered as the major sources of infection with *S*. Enteritidis, the most common cause of human salmonellosis worldwide (Rodriguez et al., 1990; Angulo and Swerdlow, 1999; Poppe, 2000; Patrick et al., 2004; Namata et al., 2008). An illustration of the strong relationship between eggs and human *Salmonella* (Enteritidis) infections is the observation that the increase in number of *S*. Enteritidis isolates in humans is directly related with an increase in number of isolates from eggs and chicken meat (Lee, 1974; Rabsch, 2000). The studies of McIlroy and Mc Craken (1990) and Van Duijkeren et al. (2002) both showed a drastic increase in *S*. Enteritidis isolates from chickens in the United Kingdom and The Netherlands respectively during the 1980’s, coinciding with the worldwide rise of *S*. Enteritidis outbreaks in humans (Hogue et al., 1997). The opposite is also true: reducing *Salmonella* flock prevalence results in a directly proportional reduction in human health risk (Altekruse et al., 2003).

**iii. Clinical symptoms and economic relevance of salmonellosis in humans**

The incubation period of a *Salmonella* infection in humans varies from 5 hours to 7 days (Plym Forshell and Wierup, 2006), but clinical signs usually begin 12 to 36 hours after consumption of contaminated food (Omwandho and Kubota, 2010). Diarrhoea, abdominal pains, vomiting, headache and fever are the most common symptoms (Steinert et al., 1990; Anon., 1992). The duration of illness varies from 4 to 10 days but faecal shedding can last 4
to 7 weeks in adults and children respectively (Buchwald and Blaser, 1984). Treatment with antimicrobials does not seem necessary in cases without complications because of the risk of prolonging the carrier state (Dixon, 1965; Aserkoff and Bennett, 1969). Especially in the population of the so-called YOPI’s (i.e. the young, the older ones, the pregnant and the immune deficient), septicaemia may occur leading to meningitis, arthritis, osteomyelitis, pneumonia and pericarditis. Occasionally a *Salmonella* infection might even result in death (Hohmann, 2001; McCabesellers and Beattie, 2004). Subclinical infections and/or carriers also occur and investigations found that 7 % to 66 % of infected humans are subclinical carriers (Plym Forshell and Wierup, 2006; and references therein).

Looking at the age distribution (Figure 8), it is clear that the highest number of confirmed cases per 100,000 population can be found in the group of the 0 to 4 years old. This is 3 times higher than in the group of the 5 to 14 year old and 6 to 9 times higher than in the other age categories. A slight increase can be seen in the group of 65 years and older (EFSA, 2010).

![Figure 8: Age-specific distribution of confirmed cases of human salmonellosis (EFSA; 2010)](image)

Human *Salmonella* infections also have a significant economical impact (e.g. direct medical and non-medical costs, temporary absence from school or work...). A study in The Netherlands estimated the costs for *Salmonella* infections during 1999 at 4 million € (van den Brandhof, 2004). Roberts et al. (2003) concluded that hospital costs were the highest for *Salmonella* compared to other infectious intestinal diseases. An American study (Bryan and Doyle, 1995) estimated that the costs per case of human salmonellosis ranges from
approximately US $ 40 for uncomplicated cases to US $ 4.6 million for those ending with hospitalization and death.

In this context, it should be mentioned that presumably the true prevalence of human *Salmonella* infections is much higher, as many cases of salmonellosis are not reported because the ill person either does not visit a physician, no sample is obtained for laboratory tests or the laboratory findings are not registered in national data bases (Rabsch et al., 2001). Voetsch et al. (2004) estimated that for each laboratory-confirmed case of *Salmonella*, there are 38 cases that are not ascertained through surveillance.
Chapter 1

3. EPIDEMIOLOGY OF *SALMONELLA*

   a. Prevalence of *Salmonella* in laying hens: the EFSA baseline study

   In order to provide a scientific basis for setting *Salmonella* reduction targets on commercial large-scale laying hen farms a good indication of the prevalence was needed. Therefore the European Commission ordered an EU-wide *Salmonella* baseline study (Commission Decision 2004/665/EC) (Anon., 2004a) co-ordinated by DG SANCO and the European Food Safety Authority (EFSA). In each member state the survey, carried out by the official authorities, implied that a random group of commercial laying hen farms, stratified by the total capacity of the farms, was sampled. Only farms with a capacity of more than 1000 laying hens were sampled. From each farm only one randomly selected flock was sampled within nine weeks of depopulation. From this flock, 5 pooled faeces samples and 2 mixed dust samples were collected. A flock was considered positive if in one or more of the seven samples *Salmonella* could be detected. All specifications of this survey were prescribed by the technical document of the European Commission DG SANCO (Anon., 2004b).

   The objectives of this baseline study were five-fold: a) to estimate the prevalence of *Salmonella* in commercial laying hen flocks, both at the EU level and the level of the individual members states b) to estimate the prevalence of *S. Enteritidis* and *S. Typhimurium* at farm level c) to investigate the serovar distribution, both at the EU level and the level of the individual members states d) to determine potential risk factors for the presence of *Salmonella* on laying hen farms and e) to evaluate the precision and the accuracy of the sampling design with regards to the prevalence estimates (EFSA, 2007).

   The final dataset contained data from 23 EU member states and Norway, 5,310 laying hen farms in total. For several reasons, no data from Malta and Slovakia were included. All samplings took place between October 2004 and September 2005. *Salmonella* could be detected on 30.8 % of the laying hen farms in the EU, although large differences between individual member states could be observed with prevalences ranging from 0 % to 79.5 %. Specifically looking at *S. Enteritidis* and/or *S. Typhimurium*, 20.4 % of the laying hen farms were positive at the EU level, ranging from 0 % to 62.5 % (EFSA, 2007). The observed prevalences of all participating countries are presented in Table 2. The three most commonly found serovars were *S. Enteritidis*, *S. Infantis* and *S. Typhimurium*, with *S. Enteritidis* counting for 60 % of the isolates on the positive farms.
The housing of laying hens in battery cages turned out to be a risk factor. However, confounding with other variables such as the flock size could not be ruled out. Vaccination against *Salmonella* on the other hand turned out to be a protective factor, although this beneficial effect could mainly be observed in countries with a high *Salmonella* prevalence (EFSA, 2007).

In the framework of the baseline study, 141 Belgian farms were sampled, resulting in a *Salmonella* prevalence of 37.6%. *S. Enteritidis* and/or *S. Typhimurium* were found on 27.7% of the farms.
<table>
<thead>
<tr>
<th>Country</th>
<th>No. of flocks</th>
<th>% pos.</th>
<th>CI 95%</th>
<th>% pos.</th>
<th>CI 95%</th>
</tr>
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<tr>
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<td>337</td>
<td>15.4</td>
<td>12.7-18.5</td>
<td>10.7</td>
<td>8.4-13.4</td>
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<tr>
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<td>31.4-44.1</td>
<td>27.7</td>
<td>22.1-33.9</td>
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<tr>
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<td>25</td>
<td>28.0</td>
<td>21.7-33.0</td>
<td>8.0</td>
<td>3.7-12.3</td>
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<tr>
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<td>65.6</td>
<td>61.3-68.2</td>
<td>62.5</td>
<td>58.0-65.2</td>
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<tr>
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<td>1.6-4.3</td>
<td>1.6</td>
<td>0.8-3.0</td>
</tr>
<tr>
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<td>18.2</td>
<td>-*</td>
<td>9.1</td>
<td>-</td>
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<tr>
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<td>0.0-1.6</td>
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<td>0.0-1.6</td>
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<tr>
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<td>14.6-20.2</td>
<td>8.0</td>
<td>6.2-10.3</td>
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<td>25.7-32.3</td>
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<td>0.0-1.3</td>
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<td>7.8</td>
<td>5.9-10.4</td>
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<td><strong>29.8-31.8</strong></td>
<td><strong>20.4</strong></td>
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<td>0.0-0.8</td>
<td>0.0</td>
<td>0.0-0.8</td>
</tr>
</tbody>
</table>

*No confidence interval for Estonia and Luxembourg since all farms in these member states were sampled.
b. Prevalence of *Salmonella* in laying hens: the Belgian situation

i. Breeding parents flocks

Since Regulation No. 1003/2005 (Anon., 2005b) became valid, implying that the prevalence of five *Salmonella* serovars of public health importance (i.e. *S. Enteritidis*, *S. Typhimurium*, *S. Hadar*, *S. Infantis* and *S. Virchow*) in breeding flocks of more than 250 hens may not exceed 1 %, the prevalence of the targeted serovars in Belgian breeding flocks decreased from 4.2 % in 2004 to 0.9 % in 2008, which is also the average at the EU level (EFSA, 2007; EFSA, 2010). It is however remarkable that 7.3 % of the breeding flocks were positive for serovars other than the five targeted ones, which is above the 1.8 % at the EU level (EFSA, 2010).

ii. Laying hen flocks

In laying hen flocks, the prevalence of *S. Enteritidis* and *S. Typhimurium* decreased dramatically from 27.2 % in 2004 to 3.7 % in 2008, which is below the mean prevalence of 5.9 % at the EU level (EFSA, 2007; EFSA, 2010). The drop in *Salmonella* prevalence was most pronounced in 2005, an observation which has been attributed to the vaccination of the laying hens: although the vaccination of laying hens against *Salmonella* only became mandatory in 2007, the Belgian Federal Agency for the Safety of the Food Chain already strongly recommended vaccination since early 2005, and many companies implemented this measure (Collard et al., 2008). As is the case in Belgian breeding flocks, also during the rearing and the production period more flocks are found positive for serovars other than *S. Enteritidis* and *S. Typhimurium* than the EU average (8.0 % vs. 2.3 %) (EFSA, 2010).

c. Risk factors for the presence of *Salmonella* in laying hen flocks

In addition to the detection of *Salmonella* in laying hen flocks and the monitoring in time, also the prevention of *Salmonella* contamination of laying hens is of the utmost importance. This requires knowledge on the risk factors associated with its presence on laying hen farms. Several risk factors for the prevalence of *Salmonella* in laying hens have been identified in observational and experimental studies.
The main risk factors are:

- The system in which the hens are housed has often been described as a risk factor but the published studies show contradictory results, ranging from a preventive effect of the conventional battery cage system (Mollenhorst et al., 2005) over no influence (Schaar et al., 1997) up to a higher risk of *Salmonella* in battery cages in comparison to non-cage system (Mølbak and Neimann, 2002; EFSA, 2007). When interpreting these results, three things have to be taken into account. First, there were large differences in sample size and methodology used between all studies. Secondly, none of the mentioned studies was specifically set up to determine the influence of housing systems on the *Salmonella* prevalence in laying hen flocks. Finally, factors such as farm and flock size and vaccination status of the flock presumably play a confounding role. All this makes it very hard to draw a consistent conclusion on the influence of the housing system on the prevalence of *Salmonella* in laying hen flocks.

- Stress is shown to have an immunosuppressive effect in laying hens (El-Lethy et al., 2003; Humphrey, 2006), which can have negative consequences with respect to *Salmonella* infection and shedding. There are several moments in the laying hen’s life where the bird is subjected to stress: moving from the rearing site to the egg producing plant (Hughes et al., 1989), the onset of lay (Jones and Ambali, 1987; Humphrey, 2006), final stages of the production period, thermal extremes (Thaxton et al., 1974; Marshally et al., 2004) or transportation to the slaughterhouse (Beuving and Vonder, 1978). The stress associated with induced moulting potentially has a harmful influence on the *Salmonella* status of the flock (Holt, 2003).

- The persistence of *Salmonella* on a farm is also a significant risk factor, even to the extent that it is thought that the major part of *Salmonella*-infections on laying hen farms are not newly introduced on the farm but are the result of re-introduction of the pathogen from the farm’s environment (van de Giessen et al., 1994; Gradel et al., 2004; Carrique-Mas et al., 2009b). This observation underlines the importance of an adequate cleaning and disinfection policy. Nevertheless, because of their intrinsically complicated structures, laying hen houses are notoriously difficult to clean and disinfect (Wales et al., 2006).
The role of rodents, flies, beetles and wild birds as vectors in the transfer of Salmonella has been extensively discussed (Guard-Petter, 2001; Davies and Breslin, 2001; 2003; Kinde et al., 2005; Stenzel et al., 2008; Carrique-Mas et al., 2009a). Another very important pest in laying hens’ houses is the poultry red mite (Dermanyssus gallinae). It has been shown under experimental conditions that mites could play a role in the persistence of Salmonella in laying hens, either by transferring the bacterium from hen to hen or by hens consuming contaminated mites leading to a persisting infection (Valiente-Moro et al., 2007; Valiente-Moro et al., 2009). Whether this is also the case under field conditions and in non-battery cage housing systems still remains unknown.

Finally, the use of vaccination against Salmonella has beyond doubt a significant protective influence on shedding of Salmonella in laying hen flocks since the currently available vaccines reduce both the shedding and colonization of the reproductive tract, leading to a decrease in the number of internally contaminated eggs (Feberwee et al., 2001; Woodward et al., 2002; Gantois et al., 2006).

d. *Salmonella* control programs in laying hen flocks

   i. Legislative background

The dramatic increase in human *S. Enteritidis* cases worldwide since the 1980’s has been associated with the rise of *S. Enteritidis* in poultry between the 1960’s and the 1980’s (Hogue et al., 1997; Rabsch et al., 2000). Because of this important role of poultry in the epidemiology of human salmonellosis, the European Parliament issued Council Directive 92/117/EEC (Anon., 1992). The requirements of this directive were three-fold. Member states should a) monitor for zoonotic agents b) take steps to reduce the risk of introducing *Salmonella* on the farm and c) control *Salmonella* in flocks of parents breeding hens. Although this directive was a fair attempt to tackle the swift rise of human salmonellosis in the EU, the benefit to human health was rather limited. One of the reasons for this is that the main focus was on the control of the vertical transmission of *Salmonella*, with very strict measures at the level of the grand parent and parent breeding flocks. However, both for invasive and non-invasive serotypes the horizontal transmission between animals is at least as important as the vertical transmission. Therefore the top-down approach pushed forward in this directive was by far insufficient to control *Salmonella* on the level of the laying hen farms.
In order to fine-tune the measures of this first directive, the EU parliament adopted Regulation No. 2160/2003 which stated that proper and effective measures must be taken in the member states to detect and control *Salmonella* at all relevant stages of production, processing and distribution, particularly at the level of primary production (Anon., 2003a). Each type of measure at each stage has some importance in the reduction of *Salmonella*, but no measure is successful on its own. A subsequent directive (2003/99/EC) stated that zoonotic agents and their antimicrobial resistance must be strictly monitored (Anon., 2003b). The emphasis of the European approach in controlling *Salmonella* contamination of eggs is on the prevention and monitoring during the live-phase, i.e. the rearing of the laying hens and the egg production at the laying hen farms.

One of the spearheads of the above mentioned legislation is that EU member states need to establish national *Salmonella* control programs (NCP’s). Minimal requirements for NCP’s in commercial laying hen flocks were laid down in Regulation No. 1091/2005 (Anon., 2005c). They comprise that a) antimicrobials cannot be used to control *Salmonella* b) in member states where the prevalence of *S. Enteritidis* in commercial laying hens is higher than 10 %, vaccination against *S. Enteritidis* is mandatory and c) live vaccines can only be used during the rearing period of the pullets and the manufacturer has to provide a method to distinguish the vaccine from field strains of *S. Enteritidis*.

The surveillance data, available since the application of Directive 92/117/EEC, made it possible to set reduction targets for the prevalence of *Salmonella* in parents breeding flocks. These reduction targets were laid down in Regulation No. 1003/2005 (Anon., 2005b), implying that the prevalence of 5 *Salmonella* serovars of public health importance in breeding flocks of more than 250 hens may not exceed 1 %. One of the measures to achieve this is the mandatory sampling of all breeder flocks and hatcheries since 1 January 2007. If *S. Enteritidis* or *S. Typhimurium* is detected, the eggs from these flocks can no longer be hatched and the hens are culled or subjected to sanitary slaughter. If *S. Infantis*, *S. Virchow* or *S. Hadar* is detected, the farmer has to draw up a specific action plan in order to eliminate infection and prevent dissemination. Non-members states of the EU supplying hatching eggs or live poultry for breeding to the EU must have submitted a *Salmonella* control program which is considered equivalent to the EU provisions.

In a next step, annual reduction targets for *S. Enteritidis* and *S. Typhimurium* in all commercial laying hen flocks (both pullets and egg producing chickens) were set by Regulation No. 1168/2006 (Anon., 2006). The reduction targets differ from member state to
state, depending on the result of each country in the EFSA baseline study from 2004 - 2005. For the countries with a S. Enteritidis and S. Typhimurium combined prevalence lower than 10 %, an annual reduction of 10 % was required. When the combined prevalence was 10-19 %, 20-39 % and higher than 39 % the annual reduction targets were set at 20 %, 30 % and 40 % respectively.

This regulation also gives detailed information on the sampling programs for laying hen flocks. Rearing flocks should be sampled a) at day old (which means on arrival of the chicks from the hatchery) and b) 2 weeks before entering the egg laying phase using 2 pairs of boot swabs in non-cage rearing systems per house or 60 faeces samples of 1 g in cage reared pullets. Laying hen flocks must be sampled every 15 weeks during the entire production cycle from the age of 22 - 26 weeks onwards. From laying hens in cage systems, 2 pooled faeces samples must be collected, flocks in non-cage housing systems have to be sampled using 2 pairs of boot swabs. All obtained samples have to be analyzed in an officially approved laboratory, following the ISO 6579:2002 (Annex D) method. In addition to this, farms with a capacity of >1000 hens have to be sampled by the official authorities meaning that from each farm yearly 1 randomly selected flock has to be tested using 2 pooled faeces samples or 2 pairs of boot swabs and 1 mixed dust sample. A flock is regarded as infected with Salmonella if at least 1 sample from the laying hens or 1 relevant environmental sample tests positive.

Furthermore, Regulation No. 1237/2007 laid down restrictions concerning the trade of table eggs from flocks which are infected with S. Enteritidis or S. Typhimurium, saying that from February 2009 onwards, eggs from any flock infected with these 2 serovars will be banned from the market, unless they are treated in a manner that guarantees that all Salmonella bacteria are destroyed. Because of the considerable economical implications, the regulation gives the farmer the opportunity to dispute positive sampling results. This can be achieved by the testing, at the producer’s expense, of either a) seven faecal/environmental samples b) 4.000 eggs from the affected flock or c) 300 hens tested for the presence of Salmonella in their caeca and ovaries. When the results of these additional tests are negative, the original findings become invalid.

ii. Detecting Salmonella in laying hen flocks: the chicken or the egg?

The detection of Salmonella in laying hen flocks is one of the cornerstones of the national Salmonella control programs imposed by the EU. Detecting and monitoring the presence of Salmonella allows taking necessary and sometimes corrective steps (e.g. adequate
cleaning and disinfection, rodent control, vaccination...) to combat *Salmonella* and at the same time it generates useful information on the prevalence of *Salmonella* and its evolution in time on the population level.

However, the efficiency of such control programs is highly dependent on the accuracy of the chosen sampling method (Fletcher, 2006) and the sensitivity of the culture methods (Carrique-Mas and Davies, 2008). The total number, volume and frequency of the collected samples all influence the sensitivity of a sampling protocol. Especially in the case of a low within-flock prevalence higher numbers of samples are necessary but there are obviously several logistic and economical issues that need to be taken into account when setting up a control program. One of the major merits of the EU in its crusade against *Salmonella* is the harmonisation and coordination of the different monitoring and control programs that were installed in the different members states by drawing up Regulation No. 1168/2006, stating that laying hen flocks must be sampled throughout the production cycle using 2 pooled faeces samples (in cage flocks) or 2 pairs of boot swabs (in non-cage systems).

Besides the sampling protocol imposed by Regulation No. 1168/2006, a wide variety of different methodologies for the sampling of *Salmonella* have been used in different countries and different time periods. Summarized, the investigation of *Salmonella* in poultry at the primary production stage may involve either the collection of samples from the laying hens themselves such as eggs, blood, cloacal swabs and caecal samples or the collection of material from the environment in the poultry house such as faeces, dust and litter (Carrique-Mas and Davies, 2008a).

Since eggs are the main vectors for human *Salmonella* (Enteritidis) infections, it seems plausible to focus the sampling of laying hen flocks on the bacteriological detection of *Salmonella* in eggs. However there are some major drawbacks making the testing of eggs very labour-extensive and expensive (Carrique-Mas and Davies, 2008b). Firstly, there is a very low rate of egg contamination, even in flocks known to be infected with *S. Enteritidis*. Secondly, the number of *Salmonella* bacteria deposited in contaminated eggs is believed to be low, making the bacteriological detection challenging. Finally, the fact that in many countries commercial laying hens are vaccinated against *Salmonella* further hampers the bacteriological testing of eggs as a useful methodology because of the strongly reduced shedding of the pathogen by vaccinated hens.
The use of caecal and ovarial samples from hens post-mortem is regarded as a very accurate method to detect *Salmonella* in laying hen flocks (Nief and Hoop, 2008) although practical and economical factors impose limitations on the user friendliness (Barnhart et al., 1993). According to Regulation No. 1237/2007, the caeca and/or ovaries of 300 hens in a flock can be analyzed when a positive test result from the standard control program is disputed by the farmer.

Serology has been used in *Salmonella* control programs, for instance in Denmark (Wegener et al., 2003). Combined with bacteriology it increases the sensitivity of the testing protocol. However, serology is no longer a usable tool in many countries because of the vaccination of laying hen flocks against *Salmonella* and the cross-reactivity between vaccine induced and natural antibodies.

Cloacal swabs have the advantage that they directly originate from the laying hen, giving information on the current *Salmonella* status of the flock. However, the low sensitivity is a very important disadvantage because of the intermittent shedding of the bacteria by infected hens (Van Immerseel et al., 2004) and the very limited amount of faecal material tested (Bichler et al, 1996). To circumvent this, a large number of cloacal swabs should be taken in a flock but this implies practical problems and causes agitation among the birds.

One of the most commonly used methods is the testing of fresh faeces. This can be done by collecting naturally pooled fresh faecal material or by collecting and pooling individual piles of fresh faeces. Wales et al. (2006) stated that the inclusion of larger volumes of mixed faecal material from a larger number of hens enhances the detection of *Salmonella*. On the other hand, many authors also suggested that there might be a dilution effect when positive and negative faecal material is mixed for culture (Kivelä et al., 2007; Arnold et al., 2010; Singer et al., 2009).

The intensive sampling of the laying hen’s environment is also regarded as an effective method to detect *Salmonella* in laying hen flocks (Aho, 1992; Musgrove et al., 2005; Carrique-Mas et al., 2009). From time to time the relevance of environmental sampling has been disputed because the detection of *Salmonella* in a sample from the poultry house’s internal environment may originate from other sources such as wildlife and feed, rather than reflecting the actual infection status of the flock. Despite these remarks there is general agreement on the value of environmental sampling and the chance to gain interesting
information on the infection status of the flock can even be increased by including vectors such as rodents in the sampling (Kinde et al., 2005; Wales et al., 2007). A wide range of environmental samples have been described such as dust (Davies and Wray, 1996; Davies and Breslin, 2003), litter (Kingston, 1981), boot/sock swabs (Skov et al., 1999; McCrea, 2005), drag swabs (White et al., 1997; Castellan et al., 2004) and hand-held gauze swabs (Davies and Wray, 1996; Zewde et al., 2009).

The efficiency of a certain sampling method is also dependant on other factors such as the vaccination status of the flock and the stage of lay. It has been thought that there is more chance to detect *Salmonella* in a flock as the flocks becomes older, although little is known about the underlying mechanisms (Garber et al., 2003; EFSA, 2007). Vaccines claim to reduce the shedding of *Salmonella*, but this possibly also reduces the detection of infected flocks because of the lower number of shed organisms and the lower within-flock prevalence in vaccinated flocks (Van Immerseel et al., 2004). This raises the question whether the currently used sampling methodologies in the *Salmonella* monitoring programs of several EU member states are accurate enough to detect *Salmonella* in low prevalence flocks.
4. **ANTIMICROBIAL RESISTANCE IN LAYING HENS**

Another ‘hot topic’ is the origin and spread of antimicrobial resistance, both in animals and humans. In this context, the transfer of antimicrobial resistance from food producing animals to humans is, along with the microbiological integrity of the food, gaining weight as an important aspect of food safety.

More and more concerns have raised on the systematic use of antimicrobials in animal food production. The emergence of antimicrobial resistance initiated by this use compromises animal health and welfare because of therapy failure. In addition to this, there are also some possible adverse consequences for public health because of the spread of antimicrobial resistance from animals to humans, leading to treatment failure in human infectious diseases (EFSA, 2009; Jordan et al., 2009).

There are several pathways by which the presence of antimicrobial resistance in food derived from animals could have potential human health implications a) antimicrobial resistant zoonotic pathogens (e.g. *Salmonella*) in contaminated food cause a human infection that requires antibiotic treatment and therapy is compromised b) antimicrobial-resistant bacteria non-pathogenic to humans are selected in the animals and when contaminated food is ingested, the bacteria transfer resistance determinants to other commensal and potential pathogenic bacteria in the human gut and c) antibiotics remain as residues in food products, allowing the selection of antibiotic-resistant bacteria after the food is consumed (Piddock, 1996). Another route via which resistant bacteria can be transmitted is through direct contact. Similar resistance patterns have been described in animals and the farmers and slaughterhouse workers handling and processing them, indicating circulation of bacterial genetic material between the animals and humans (Ozanne et al., 1987; Nijsten et al., 1994; van den Bogaard et al., 2001; 2002). From this point of view, the transfer of resistance from animals to humans can be seen as an occupational disease (Cole et al., 2000).

To fully assess the size of the problem of antimicrobial resistance in food animal husbandry, the EU adopted Directive 2003/99/EC (Anon., 2003b), implying that member states must monitor and report on antimicrobial resistance in *Salmonella* and *Campylobacter* isolates from animals and food. However, there are still some major drawbacks. A first weakness is that in many of these programs the monitoring of antimicrobial resistance is restricted to pigs, cattle and broilers (Aarestrup, 2004). Laying hens are either not sampled, or
the data from hens are merged with the results of the broilers. This can be explained by the fact that in laying hens the use of antimicrobials is relatively sparse, especially during their productive life on the egg-producing plant, giving the impression that laying hens are of negligible importance within the whole issue. Nonetheless, the consumption of eggs and -to a lesser extent- meat of culled hens can pose a public health risk. Secondly, the obligation to monitor and report only applies for zoonotic pathogens, the monitoring of resistance data from indicator bacteria is voluntary.

All this explains why the published data for laying hens primarily report about antimicrobial resistance in zoonotic pathogens such as *Salmonella* and *Campylobacter* spp. or from bacteria with a clinical relevance for laying hens such as Avian Pathogenic *E. coli* and *Pasteurella multocida* (Gyles, 2008; Huang et al., 2009).

When comparing results of the monitoring of resistance in zoonotic and pathogenic bacteria, some possible sources of bias are present such as varying selection criteria used over time and varying participation among veterinarians. Moreover, some infections are more likely to generate isolates than others and isolates from some infections are more likely to be sent for susceptibility testing. In many cases, treatment will already have been started when isolates are collected and sent to the laboratory. Furthermore, inclusion of only clinical isolates could lead to an overestimation of the occurrence of resistance because veterinarians will often only send samples after they have experienced treatment failure (Aarestrup, 2004; Bywater, 2005; Wallmann, 2006). Therefore, the monitoring of resistance in indicator bacteria is of utmost importance in surveillance programs.

Indicator bacteria are generally regarded as useful tools to monitor antimicrobial resistance since they can be isolated from healthy animals, giving a hint of the level of resistance in a particular population and they are a potential source of resistance genes that can spread horizontally to zoonotic and other bacteria through the food chain (Winokur et al., 2001; Wang et al., 2006). In addition, they acquire antimicrobial resistance faster than other commonly found bacteria, implying that changes in the resistance of these species serve as a good indicator of resistance in potentially pathogenic bacteria (Aarestrup, 2004; WHO, 2007; Miranda et al., 2008). Commensal *Escherichia coli* and *Enterococcus faecalis* are internationally used as respective Gram-negative and Gram-positive indicator bacteria because of their common presence in the animal and human intestinal tract (Wray and Gnanou, 2000; Sørum and Sunde, 2001; de Jong et al., 2009). Besides this, both *Escherichia*
coli and Enterococcus faecalis are associated with disease in humans. For E. coli, urinary tract infection, sepsis/meningitis and enteric/diarrheal disease are the 3 general clinical syndromes associated with human infection (Nataro and Kaper, 1998; Stenutz et al., 2006). E. faecalis causes 80% to 90% of human enterococcal infections (Teixeira and Facklam, 2003). The most commonly observed human infections by enterococci are urinary tract infections, pelvic and intra-abdominal wound infections, bacteriaemia and endocarditis.

Till now, the number of studies describing antimicrobial resistance in indicator bacteria from healthy laying hens or shell eggs is very limited. Moreover, differences in size and methodology make it difficult to gain a clear insight into the situation in laying hens. An overview of the studies describing antimicrobial resistance in healthy laying hens is presented in Table 4.

The move to housing systems different from the conventional battery cages, as imposed by the EU, can potentially influence antimicrobial resistance patterns. Both for poultry and other animal species it has been suggested that the move from conventional indoor production towards free-range and organic production exerted a beneficial effect on the levels of antimicrobial resistance in zoonotic and indicator bacteria (Avrain et al., 2003; Thakur and Gebreyes, 2005; Ray et al., 2006, Schwaiger et al., 2008; 2010, Young et al., 2009). On the other hand it has been shown that the move to non-cage housing systems for laying hens resulted in an increased incidence of particularly bacterial diseases (Fossum et al., 2009; Kaufmann-Bart and Hoop, 2009), which could potentially lead to increased antibiotic usage. It is therefore necessary to investigate the impact of these new laying hen housing systems on the prevalence of antimicrobial resistance and to monitor antimicrobial resistance development in laying hens.
Table 4: Overview of the studies describing antimicrobial resistance in healthy laying hens

<table>
<thead>
<tr>
<th>Reference</th>
<th>Study country</th>
<th>Bacterium</th>
<th>Sample type</th>
<th>No. of isolates (no. of farms)</th>
<th>Methodology of testing</th>
<th>Cut-off</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yoshimura et al. (2000)</td>
<td>Japan</td>
<td><em>E. faecalis</em> and <em>E. faecium</em></td>
<td>Faecal droppings</td>
<td>222 (34)</td>
<td>MIC</td>
<td>JSC</td>
</tr>
<tr>
<td>Musgrove et al. (2006)</td>
<td>USA</td>
<td><em>E. coli</em></td>
<td>Shell eggs</td>
<td>194 (N.A.)</td>
<td>MIC</td>
<td>CLSI</td>
</tr>
<tr>
<td>Kojima et al. (2009)</td>
<td>Japan</td>
<td><em>E. coli</em></td>
<td>Fresh faecal samples</td>
<td>530 (N.A.)</td>
<td>MIC</td>
<td>JSC and CLSI</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>E. faecalis</em></td>
<td>Fresh faecal samples</td>
<td>251 (N.A.)</td>
<td>MIC</td>
<td>JSC and CLSI</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>E. faecium</em></td>
<td>Fresh faecal samples</td>
<td>159 (N.A.)</td>
<td>MIC</td>
<td>JSC and CLSI</td>
</tr>
<tr>
<td>Schwaiger et al. (2008)</td>
<td>Germany</td>
<td><em>E. coli</em></td>
<td>Cloacal swabs</td>
<td>533 (20)</td>
<td>MIC</td>
<td>DIN</td>
</tr>
<tr>
<td>Schwaiger et al. (2010)</td>
<td>Germany</td>
<td><em>E. faecium</em></td>
<td>Cloacal swabs</td>
<td>84 (20)</td>
<td>MIC</td>
<td>DIN</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>E. raffinosus</em></td>
<td>Cloacal swabs</td>
<td>439 (20)</td>
<td>MIC</td>
<td>DIN</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>E. faecalis</em></td>
<td>Cloacal swabs</td>
<td>328 (20)</td>
<td>MIC</td>
<td>DIN</td>
</tr>
</tbody>
</table>

CLSI: Clinical laboratory Standards Agency  
DIN: Deutsches Institut für Normung  
JSC: Japanese Society for Chemotherapy  
N.A.: not available
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CHAPTER 2

SCIENTIFIC AIMS
Salmonella is one of the most common bacterial causes of human disease in the European Union. Eggs and egg products remain one of the major sources of infection for humans.

The EU decision to ban conventional battery cages was based on the concerns about laying hen welfare. Whether this move to alternative non-cage systems will also influence the prevalence and persistence of zoonotic pathogens, such as Salmonella, in the flock has not yet been thoroughly examined.

For several animal species such as pigs and cattle it has been described that the farming type (e.g. organic) may influence the levels of antimicrobial resistance. By contrast, for laying hens, because of the lack of epidemiological data on antimicrobial resistance, very little is known about the influence of different housing systems on the occurrence of antimicrobial resistance.

Therefore, the specific aims of this study were:

- To determine the between- and within-flock prevalence of Salmonella in laying hen flocks housed in conventional battery cages and non-cage housing systems.
- To identify risk factors for the presence of Salmonella in laying hen flocks with specific emphasis on the effect of the different housing systems
- To determine the prevalence of antimicrobial resistance in indicator bacteria in laying hens
- To examine how the laying hen housing system influences the levels of antimicrobial resistance
CHAPTER 3

FAECAL SAMPLING UNDER-ESTIMATES THE ACTUAL PREVALENCE OF SALMONELLA IN LAYING HEN FLOCKS


Adapted from Zoonoses and Public Health (2009) 56: 471-476
ABSTRACT

In all EU member states, *Salmonella* monitoring in poultry flocks is obligatory. In these monitoring programs a limited number of pooled faeces and / or dust samples are collected to determine whether *Salmonella* is present in the flocks or not. Whether these limited sampling protocols are sufficiently sensitive to detect expected low within-flock prevalences of an intermittently shed pathogen is not yet clear. In this paper, a comparison is made between different sampling procedures for the assessment of the between and within-flock prevalence of *Salmonella* in laying hens. In total, 19 flocks were sampled. All the sampled flocks were found negative for *Salmonella* based on the results from the official *Salmonella* control program. Using an extended on-farm sampling methodology, *Salmonella* could not be detected in any of the flocks. After transportation of the hens to the laboratory and subsequent analysis of cloacal swabs and caecal contents, *Salmonella* Enteritidis was detected in laying hens from 5 out of 19 farms. The observed within-flock prevalence ranged from 1 – 14 %. Based on the results of this study, it can be expected that, depending on the sampling procedure, different estimates of the prevalence of *Salmonella* can be obtained and the proportion of *Salmonella* infected flocks is underestimated based on the results of the official monitoring program.

**Key words:** *Salmonella*; laying hens; sampling; prevalence
INTRODUCTION

In the European Union (EU), *Salmonella* is currently the second most important foodborne pathogen (European Food Safety Authority, 2007). Outbreaks affecting only a few individuals are most common but on some occasions outbreaks involving large numbers of cases are observed (Bell and Kyriakides, 2002). Outbreaks of human salmonellosis in the EU are predominantly caused by *Salmonella* Enteritidis and Typhimurium (European Food Safety Authority, 2007). Contaminated poultry meat and eggs are the main sources of *Salmonella* Enteritidis for humans (Davies and Breslin, 2001; Namata et al., 2007). Pork and pork-related products are the main vectors for transfer of *Salmonella* Typhimurium to humans (Van Pelt et al., 2000; Jansen et al., 2007). Since many years, *Salmonella* Enteritidis is the main cause of human salmonellosis, both in Europe and in North-America, and this is mainly due to consumption of contaminated eggs (Angulo and Swerdlow, 1998; Delmas et al., 2006; EFSA, 2006). Recently, a decrease in human *Salmonella* Enteritidis infections has been noted in different countries (Cogan and Humphrey, 2003; Collard et al., 2008). This can most likely be attributed to a number of measures that have been taken since the mid to late 1990s, such as improved biosecurity and hygiene on breeder and laying hen farms, either or not in combination with vaccination of commercial laying hens (EFSA, 2006; Mossong et al., 2006; Wales et al., 2007).

The EU baseline study on the prevalence of *Salmonella* in laying hens, carried out in 2004-2005, has demonstrated a variable level of infection of laying hen holdings in the different EU member states. The European Regulation No. 2160/2003 makes strict sampling schemes mandatory in the EU member states in order to provide follow-up data on the level of flock contamination: every flock of at least 1000 commercial laying hens has to be sampled every 15 weeks with a first sampling at the age of 24 ± 2 weeks. The following samples need to be collected during the production cycle: 2 pooled faeces samples of 150 g each (on farms with a conventional battery cage or a furnished cage housing system) or 2 pairs of overshoes (on farms with an aviary, floor-raised or free-range housing system) (Regulation No. 1168/2006). It is unclear however whether this sampling scheme is able to detect low *Salmonella* contamination levels and therefore provides accurate follow-up data on the level of laying hen holding contamination in the EU.

The aim of the study described below is to evaluate whether a more intensive sampling methodology results in different estimates of the within and between flock prevalence in commercial layer flocks.
MATERIALS AND METHODS

Selection of the sampled farms

Flocks were selected based upon a list of contact addresses of registered laying hen farms provided by the official Belgian Identification & Registration authorities. The only inclusion criterion used was the flock size (>1000 hens). In total, 220 farms with a minimal capacity of 1000 laying hens were registered. The farms which were in the last month of the production cycle were contacted by telephone. Participation was voluntary. All of the contacted farms volunteered to participate. In total 19 flocks from 19 different farms were sampled, comprising conventional battery cage flocks and flocks housed in alternative housing systems. On farms with more than one house, only one house was randomly selected to be sampled. When several flocks were present in a given house, only one flock was selected at random. The size of the selected flocks varied between 3500 and 29000 hens. All sampled flocks were vaccinated against Salmonella and screened negative for Salmonella by the official monitoring program which means that the flocks were sampled every 15 weeks starting from 6 weeks after arrival of the hens on the farm (following EU Regulation No. 1168/2006).

Moment of sampling

Participating laying hen farms were sampled one week prior to depopulation. The age of the sampled hens ranged from 70 to 82 weeks. The samplings were performed between June 2007 and May 2008.

Number and sample type taken on-farm

On-farm sampling consisted of 5 pooled faeces samples, 1 mixed dust sample and 40 cloaca swabs of 40 randomly selected laying hens. Depending on the housing system, the pooled faeces samples were taken as follows:

- Caged flocks: 5 samples of mixed fresh faeces were taken from dropping belts, scrapers or deep pits depending on the type of cage (conventional battery cage or furnished cage). Each pooled faeces sample consisted of approximately 250 g.
- Floor-raised, free-range and organic farms: for each of the 5 pooled faeces samples 60 piles of fresh faeces were collected from the floor and the slats. Each pooled faeces sample consisted of approximately 250 g.
The mixed dust sample was collected with a gloved hand on various places in the house such as exhaust fan baffles, adjacent ledges, beams, partitions, pipes or underneath cages. Enough dust was collected to fill a 250 ml jar. Both the pooled faeces samples and the mixed dust sample were placed in a sterile recipient. Gloves were changed in between collection of each pooled faeces sample and the mixed dust sample. The cloaca swabs were taken from hens that could be caught without causing too much agitation. Care was taken that hens were selected evenly throughout the house. Forty hens in the flock were selected and from each hen a cloaca swab was taken by inserting a cotton-tipped swab approximately 5 cm into the cloaca, taking care to avoid contact with the surrounding feathers and skin. Afterwards, the swabs were placed in tubes containing Ames medium. The sample size of 40 swabs was calculated in function of an expected within-flock prevalence of 20 %, an accepted error of 10 %, a confidence level of 90 % and a flock size of 10000. All samples were placed in an appropriate leak-proof bag and outer container and transported to the lab under ambient conditions and were incubated for bacteriological analysis (see further) within 12 hours post-sampling.

From each flock, 100 hens were caught. The selection of 100 samples is based on the formula used for the detection of disease (WinEpiscope 2.0). Based on this formula it is shown that for the detection of a disease with a minimal prevalence of 3 % and a desired level of confidence of detection of 95 % one needs a sample of 98 in a flock of 5000 animals or more. The caught hens were placed in cleaned and disinfected transport boxes. Each transport box was disinfected with a commercial available disinfectant proven to be effective against Salmonella spp. (Virocid®). Separations of clean disposable cardboards prevented contact between hens and faeces in the different boxes. The boxes containing the hens were transported to the Faculty of Veterinary Medicine of Ghent University in a cleaned and disinfected van. Transportation time ranged from 30 to 90 minutes. All possible hygienic measures were taken during transportation of the hens to avoid contamination of the hens by the environment: After each transport, the transport boxes were cleaned (first dry and afterwards with water). Each transport box was disinfected with a commercial available disinfectant proven to be effective against Salmonella spp. (Virocid®, CidLines, Belgium). The van in which the hens were transported was also cleaned and disinfected. During transport, clean disposable cardboards were used to avoid contamination of the hens of one box with faeces of the hens of the neighboring boxes.
**Number and sample type taken after transport**

Immediately after arrival at the Faculty of Veterinary Medicine, all hens were labeled and a cloaca swab of each hen was taken in the same way as described above. After sampling, each hen was euthanized by intravenous embutramid injection (T61®, Intervet, Belgium). Subsequently, the hens were necropsied and both caeca were aseptically removed. Both caeca of each hen were homogenized and pooled for further processing. Bacteriological analysis of all samples started on the day of sampling.

**Bacteriological analysis of samples**

All samples were analyzed using a modification of ISO 6579:2002, as recommended by the Community Reference Laboratory for *Salmonella* in Bilthoven, The Netherlands. From each pooled faeces sample, 25 g was added to 225 ml of buffered peptone water (BPW) (Oxoid, Basingstoke, Hampshire, UK). Of the mixed dust sample, there was twice 10 grams of mixed dusty material separately added to 90 ml of BPW. Cloaca swabs were placed in 9 ml of BPW. Pooled faeces samples and dust samples were mixed in a stomacher bag for 1 minute. All samples were incubated for $18 \pm 2$ h at $37 \pm 1$ °C. Next, 3 droplets of the pre-enrichment culture were inoculated onto a modified semi-solid Rappaport-Vassiliadis (MSRV) (Difco; Becton Dickinson) agar plate containing 0.01 g l$^{-1}$ novobiocine and incubated for $2 \times 24 \pm 2$ h at $42 \pm 1$°C. Suspect white culture from the border of the growth zone was plated on Brilliant Green Agar (BGA; Oxoid) and Xylose Lysine Deoxycholate agar (XLD; Oxoid), followed by incubation for $24 \pm 2$ h at $37 \pm 1$°C. Presumed *Salmonella* colonies on BGA and XLD were biochemically confirmed using ureum agar, triple sugar iron agar and lysine-decarboxylase broth. Serotyping of *Salmonella*-isolates according to the Kauffmann-White scheme was performed at the Scientific Institute of Public Health (Brussels, Belgium).

**Statistical analysis**

Both for the between-as for the within-flock prevalence, 95 % confidence intervals have been calculated using the formula for the calculation of confidence intervals (Thrusfield M., 1995). The Pearson’s $\chi^2$-test was used to determine significant differences between the different sampling methods ($P < 0.05$).
RESULTS

A detailed overview of the bacteriological analysis of samples is presented in Table 1. *Salmonella* could not be detected in any of the pooled faeces samples and the mixed dust samples. *Salmonella* could also not be detected using on-farm sampling of 40 individual hens by taking cloaca swabs. After transportation of the animals, *Salmonella* was detected in laying hens of 5 out of 19 farms, both in cloaca swabs and in the caeca. All of the isolated strains belonged to the *Salmonella* Enteritidis serotype. Serotyping and antimicrobial susceptibility testing was performed to make sure that it was not the live vaccine strain that was isolated.

In positive flocks, estimations of the within-flock prevalence differed depending on the sample type. The within-flock prevalence determined using cloacal swabs was always below 4%, whereas the within-flock prevalence based on the bacteriological examination of the caeca varied between 5 and 14%. In all hens that were positive for *Salmonella* after bacteriological analysis of cloaca swabs, the bacterium was also found in the caecal samples.

<table>
<thead>
<tr>
<th>Farm</th>
<th>Housing type</th>
<th>Bacteriological analysis</th>
<th>Cloacal swabs after transport</th>
<th>Caeca</th>
<th>Phage types of S. Enteritidis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Battery</td>
<td></td>
<td>3 (0 - 6.33)</td>
<td>6 (1.37 - 10.63)</td>
<td>PT 1</td>
</tr>
<tr>
<td>2</td>
<td>Organic</td>
<td></td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Battery</td>
<td></td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Free-range</td>
<td></td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Floor-raised</td>
<td></td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Floor-raised</td>
<td></td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Floor-raised</td>
<td></td>
<td>3 (0 - 6.19)</td>
<td>10 (4.39 - 15.61)</td>
<td>PT 21</td>
</tr>
<tr>
<td>8</td>
<td>Battery</td>
<td></td>
<td>1 (0 - 2.94)</td>
<td>14 (7.24 - 20.76)</td>
<td>PT 12</td>
</tr>
<tr>
<td>9</td>
<td>Free-range</td>
<td></td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Free-range</td>
<td></td>
<td>4 (0.18 - 7.82)</td>
<td>7 (2.02 - 11.98)</td>
<td>PT 1, PT 35</td>
</tr>
<tr>
<td>11</td>
<td>Organic</td>
<td></td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Free-range</td>
<td></td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Battery</td>
<td></td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Free-range</td>
<td></td>
<td>0</td>
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<td></td>
</tr>
<tr>
<td>15</td>
<td>Battery</td>
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<td></td>
</tr>
<tr>
<td>16</td>
<td>Floor-raised</td>
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</tr>
<tr>
<td>17</td>
<td>Organic</td>
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<td>Floor-raised</td>
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<td>0</td>
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</tr>
<tr>
<td>19</td>
<td>Organic</td>
<td></td>
<td>2 (0 - 4.74)</td>
<td>5 (0.74 - 9.26)</td>
<td>PT 11</td>
</tr>
</tbody>
</table>
DISCUSSION

Bacteriological analyses of faecal samples as requested in European Regulation No. 2160/2003 most likely under-estimate the actual prevalence of *Salmonella* in laying hen flocks. Indeed, all 19 flocks were screened negative for *Salmonella* by the official monitoring program, using analysis of faecal samples. Even the increased on-farm sampling, combining bacteriological analysis of 40 cloaca swabs, 1 mixed dust sample and 5 pooled faeces samples, did not result in the detection of *Salmonella*, although several studies have shown that sampling of dust is a good method to detect a *Salmonella* infection on the farm (Davies and Breslin, 2001; Mahé et al., 2008). After transportation, *Salmonella* Enteritidis was found in laying hens of 5 farms. Both cloacal swabs and caecal homogenates were found positive in a low number of sampled animals.

Based on analysis of these samples the between-flock prevalence can be estimated at 26.3% (7.47 – 45.13). This is comparable to the between-holding prevalence estimate of *Salmonella* Enteritidis for Belgium (27.7%) determined during the EU-wide baseline study performed in 2004-2005 at a time when the vast majority of laying hens in Belgium were not vaccinated (EFSA, 2006). In the EU-wide baseline study a flock was identified as being *Salmonella* positive if at least one out of the 5 pooled faeces or 2 dust samples was positive. When using the same decision criterion in this study we did not find any flock positive which would result in an estimated between-flock prevalence of 0%. Also the estimates of the within-flock prevalence in the positive flocks were largely depending upon the sample types considered. The results of the analysis of individual cloaca swabs taken at the farm were all negative, whereas in the infected flocks a limited number of cloacal swabs taken after transport were positive (within-flock prevalence estimate never exceeded 4%). In the flocks where some cloaca swabs were found positive, analysis of caecal homogenates yielded more *Salmonella* positive animals (estimated within-flock prevalence between 5 and 14%). First it should be taken in account that the differences between the results of the different sampling procedures could be attributed to the fact that the number of samples taken after transport is larger than the number of samples taken on the farm. Furthermore these differences may be explained by the intermittent excretion of *Salmonella* by infected animals (Van Immerseel et al., 2004) and the fact that stress, caused by the transport, may make hens go from a ‘carrier’ state to a ‘shedding’ state. It is well known that stress factors such as the onset of lay, high temperatures, induced molting, final stages of the production period or transportation to the slaughterhouse can cause recurrences of *Salmonella* excretion (Line et al., 1997; Humphrey,
2006; van de Giessen et al., 2006; Golden et al., 2008). The results do suggest that individual cloaca swabs taken at the farm, which was seen as a potential user-friendly way to obtain some information about the within-flock prevalence in an infected flock, do not seem to be appropriate to detect a low prevalence of infection. This is in accordance with what has been found in the studies by Bichler et al. (1996) and Van Immerseel et al. (2004) where cloacal swabs were taken from experimentally infected chickens.

Our data also suggest that on farms which are ‘apparently Salmonella-free’, a relatively large proportion of the hens may still carry the pathogen without shedding. Therefore, the numbers of infected flocks based on the official monitoring programs are most likely an underestimation of the true number of flocks in which Salmonella is still present. The actual public health risk of the flocks, carrying low levels of Salmonella that are only detectable using intensified sampling procedures is unclear. Indeed, one can argue that the risk of egg contamination in these flocks probably is very low.

Defining a flock as positive based on the finding of at least one positive sample in whatever bacteriological sampling methodology used is a very crude way of categorizing flocks and does not take into account any estimation of the infection pressure in the flock. The results of this study do suggest that, even in those flocks where Salmonella was found, the infection pressure at the time of sampling was probably low. This is based on the observation that in the conventional sampling methodology no positive samples were found (no indication of active shedding) and only a limited number of shedders and carriers were found after submitting the birds to transport stress and intensive sampling. This low infection pressure can probably be attributed to the combination of vaccination and hygienic measures. The observed results are in accordance with the results of several studies concerning the effect of vaccination. Indeed it has been stated that vaccination, with the vaccines currently used in Belgium, can reduce but not totally prevent the faecal shedding and systemic spread of Salmonella Enteritidis (Van Immerseel et al., 2005; Gantois et al., 2006). This raises the question whether the sampling methodology as it is currently used in the Salmonella monitoring programs of several EU member states is accurate enough to detect Salmonella in low prevalence flocks. Although postmortem examination of hens is labour-intensive and expensive, the results of this study suggest that it is the best method for the detection of Salmonella in low prevalence flocks (P < 0.05). It also permits to estimate the within-flock prevalence and as a consequence, the risk of infection of humans through the consumption of infected eggs since there is a good agreement between the level of caecal carriage and the
prevalence of infected or contaminated eggs (Henzler et al., 1994, 1998; Schlosser et al., 1995; Mallinson et al., 2000).

However, in the current study the possible infection of the hens during transport also needs to be taken into account. Despite the relatively short transportation time and the hygienic precautions taken, it cannot be completely ruled out that the laying hens were infected during the transport from the farms to the Faculty since for broilers it has been described that birds got infected with Salmonella during transport due to insufficiently cleaned and disinfected transport crates and vehicles (Rigby et al., 1980; Rigby et al., 1982; Heyndrickx et al., 2002).

In conclusion, it is clear that depending on the sampling procedure different estimates of the between- and within-flock prevalence of Salmonella can be obtained. Analysis of faecal samples clearly under-estimates the actual prevalence of Salmonella in laying hen flocks. The results of this study can be a stimulus to pay further attention to the control and prevention of Salmonella Enteritidis in commercial laying hen flocks.

**ACKNOWLEDGEMENTS**

This research was funded by the EU FP6, under the contract 065547 (Safehouse project). The authors thank Sofie Haerens for technical assistance.
REFERENCES


Chapter 3


CHAPTER 4

THE AGE OF PRODUCTION SYSTEM AND PREVIOUS SALMONELLA INFECTIONS ON FARM ARE RISK FACTORS FOR SALMONELLA INFECTIONS IN LAYING HEN FLOCKS


Adapted from Poultry Science (2010) 89:1315-1319
ABSTRACT

An explorative field study was carried out to determine risk factors for Salmonella infections in commercial laying hen flocks. For this purpose twenty-nine laying hen flocks, including farms using conventional and alternative housing systems, were intensively sampled both on-farm and after transport as described in chapter 3. An on-farm questionnaire was used to collect information on general management practices and specific characteristics of the sampled flock such as flock size, age of the hens and age of the infrastructure. Salmonella was detected in laying hens from 6 of the 29 sampled farms. Using multivariate logistic regression with the Salmonella status of the flock as an outcome variable a previous Salmonella contamination on the farm and the age of the production system were identified as risk factors for Salmonella infections in laying hens (P < 0.05).

Key words: risk factor – Salmonella - laying hen
INTRODUCTION

In the EU, *Salmonella* is still the second most important cause of food-borne infections (EFSA, 2007). Although a decrease in human *Salmonella* Enteritidis infections has recently been noted in a number of EU member states (Cogan and Humphrey, 2003; Collard *et al*., 2007), contaminated eggs remain one of the most important sources of *Salmonella* Enteritidis infections for humans (Delmas *et al*., 2006; EFSA, 2007). In order to reduce *Salmonella* and other zoonotic agents of public health significance in farm animals, the EU member states have to apply Regulation EC No 2160/2003 (Anon., 2003) into their national legislation, which implies that EU member states have to invest in prevention, detection and control of *Salmonella* infections in laying hens.

Only a few epidemiological studies have been carried out to investigate risk factors for *Salmonella* infections in laying hen farms. The main risk factors that have been identified in these studies are (1) large flock sizes (Mollenhorst *et al*., 2005; Namata *et al*., 2008) (2) the age of the sampled hens, where a higher age implies a higher risk for *Salmonella* (Castellan *et al*., 2004; Namata *et al*., 2008) and (3) the housing system, where conventional battery cages showed a higher risk for *Salmonella* compared to alternative housing systems (EFSA, 2007).

However, it can be questioned whether the sampling methodologies that were used in these studies (i.e. pooled faeces and dust samples (EFSA, 2007; Namata *et al*., 2008) or serology (Mollenhorst *et al*., 2005)) are suitable for the detection of low *Salmonella* infection levels, especially when these samplings are performed in flocks which have been vaccinated against *Salmonella*. Moreover, in the EFSA baseline study it is clearly stated that the results may have been confounded by farm size, flock size and other variables.

The aim of this paper is to describe the presence of *Salmonella* on 29 laying hen farms using an extensive sampling protocol and to determine which management and farm characteristics influence the *Salmonella* status of the flock.

MATERIALS AND METHODS

Selection of the Farms

Farms were selected using the National Identification & Registration database. There are 220 laying hen farms in Belgium with a capacity of 1000 hens or more. In the target population, the distribution of the farms is as follows: 60 % conventional battery cages (n = 132) and 40 % non-cage systems. Of these non-cage housing systems 40 % are floor-raised farms (n = 37), 40 % are free-range (n = 33) and 20 % are organic systems (n = 18). To assure
that the potential effect of the housing type on the *Salmonella* prevalence could be evaluated, a stratified selection of the housing types was performed in order to keep the proportion of conventional battery cages / non-cage systems approximately 1 / 4. Only farms with more than 1000 laying hens that were in the last month of the production cycle were selected. Owners of farms fulfilling these selection criteria were contacted by telephone. Participation was voluntary.

*Sample Types and Analysis of the Samples*

In total 29 laying hen farms were sampled (8 conventional battery cage flocks, 10 floor-raised flocks, 8 free-range flocks and 3 organic flocks). Only one flock per farm was sampled. All of the sampled flocks were vaccinated against *Salmonella* with an attenuated vaccine. The following samples were collected on-farm: 5 pooled faeces samples, 1 mixed dust sample and cloacal swabs of 40 randomly selected hens. Subsequently 100 randomly selected hens per farm were transported to the Faculty of Veterinary Medicine. Of each hen a cloacal swab was taken after transport. After euthanasia both caeca of each hen were removed and pooled for further analysis. Detailed information on the bacteriological analysis of the samples is described in Van Hoorebeke *et al*. (2009). Summarized, all samples were analyzed using a modification of the ISO 6579:2002 method. Pre-enrichment was done by incubation of the samples in buffered peptone water (Oxoid, Basingstoke) during 18 ± 2 h at 37 ± 1 °C. Of the pre-enrichment solution 3 droplets were inoculated onto a modified semi-solid Rappaport-Vassiliadis (MSRV) (Difco; Becton Dickinson) agar plate and incubation was done for 2 x 24 ± 2 h at 42 ± 1°C. Suspect white culture from the border of the growth zone was plated on Brilliant Green Agar (BGA; Oxoid) and Xylose Lysine Deoxycholate agar (XLD; Oxoid), followed by incubation for 24 ± 2 h at 37 ± 1°C. Presumed *Salmonella* colonies on BGA and XLD were biochemically confirmed using urea agar, triple sugar iron agar and lysine-decarboxylase broth. All sampled flocks were screened negative for *Salmonella* by the official sampling protocols in accordance with EU regulation N° 2160/2003.

*Questionnaire Design*

The questionnaire was filled in during an on-farm interview at the same day of sample collection. Questions related to general farm and flock characteristics (e.g. flock size, breed, age of the hens, medical treatments...) and biosecurity measures. Special attention was paid to the housing system in which the sampled flock was housed (Table 1). The on-farm interview took on average 25 minutes to complete.
Table 1: Summary of the main items included in the questionnaire to identify risk factors for Salmonella in 29 laying farms (total number of questions = 92)

<table>
<thead>
<tr>
<th>Farm characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total capacity of the farm</td>
</tr>
<tr>
<td>Number of poultry houses</td>
</tr>
<tr>
<td>Other animal / poultry productions</td>
</tr>
<tr>
<td>Control of pests</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>House characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total capacity of the house</td>
</tr>
<tr>
<td>Size</td>
</tr>
<tr>
<td>Age of building and production system</td>
</tr>
<tr>
<td>Number of flocks present</td>
</tr>
<tr>
<td>Feeding / drinking / manure disposal systems</td>
</tr>
<tr>
<td>Access to out-door run</td>
</tr>
<tr>
<td>Nest boxes &amp; egg collecting systems</td>
</tr>
<tr>
<td>Cleaning &amp; disinfection status before repopulation</td>
</tr>
<tr>
<td>Biosecurity measures (footbath, clothing, all in/ all out)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sampled flock characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of hens</td>
</tr>
<tr>
<td>Age of the hens</td>
</tr>
<tr>
<td>Breed of the hens</td>
</tr>
<tr>
<td>Medical treatments</td>
</tr>
<tr>
<td><em>Salmonella</em> vaccination status</td>
</tr>
<tr>
<td>Cumulative mortality in the flock since onset</td>
</tr>
</tbody>
</table>

Data Processing and Analysis

Information from the questionnaires was coded and put in a database (Excel, Microsoft Cooperation). Data were analyzed using SPSS 16.0 for Windows (SPSS Inc., Chicago, IL). The potential relationship between risk factors and *Salmonella* status of the sampled farm was evaluated by means of a multivariate logistic regression model with the *Salmonella* status of the sampled flock as a binary outcome variable. For this a flock was defined infected if at least one of the collected samples was positive for *Salmonella*. Before entering the variables into the multivariate model a univariate evaluation of each potential risk factor was performed. All factors with a P-value < 0.20 were taken into account for the multivariate model which was constructed in a stepwise backward manner. In the final multivariate logistic regression model only factors with P < 0.05 were retained. All 2-way
interactions between significant main effects were tested. Odds ratios (OR), including 95% confidence intervals (CI), are reported for all significant variables.

**RESULTS**

A description of the main characteristics of the sampled farms is presented in Table 2. The mean age of the hens at the moment of sampling was 75.72 weeks (min 68 weeks - max 82 weeks). No significant differences in age of the hens could be observed in the different housing systems. The age of the production system was defined as the number of years that the current infrastructure is in use. For conventional battery cages this was the number of years since the current cages were installed. For alternative housing systems this was the number of years since the current equipment (nest boxes, perches, slats…) was installed in the sampled house. For this characteristic a remarkable difference was seen between the housing systems (Table 2). The age of the production system was significantly higher in conventional battery cage and organic farms compared to floor-raised and free-range farms ($P = 0.01$). With regard to the length of the interval between depopulation and repopulation, a significant longer interval could be observed in the alternative housing systems than in the conventional battery cages ($P < 0.05$).

**Table 2:** Description of the main characteristics of the 29 sampled farms

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>95 % CI of the mean</th>
<th>S.D.</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number of hens on the farm</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Battery</td>
<td>30375</td>
<td>9887.3-50862.7</td>
<td>24506.2</td>
<td>9000</td>
<td>72000</td>
</tr>
<tr>
<td>Floor-raised</td>
<td>23400</td>
<td>18487.7-28312.4</td>
<td>6867.0</td>
<td>19000</td>
<td>38000</td>
</tr>
<tr>
<td>Free-range</td>
<td>23500</td>
<td>13290.2-33709.8</td>
<td>12212.4</td>
<td>4000</td>
<td>40000</td>
</tr>
<tr>
<td>Organic</td>
<td>5500</td>
<td>1021.7-9978.3</td>
<td>1802.8</td>
<td>3500</td>
<td>7000</td>
</tr>
<tr>
<td><strong>Mean age of the production system (years)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Battery</td>
<td>14.50</td>
<td>10.50-18.50</td>
<td>4.78</td>
<td>8.00</td>
<td>22.00</td>
</tr>
<tr>
<td>Floor-raised</td>
<td>5.60</td>
<td>3.60-7.60</td>
<td>2.80</td>
<td>1.00</td>
<td>10.00</td>
</tr>
<tr>
<td>Free-range</td>
<td>9.12</td>
<td>3.57-14.68</td>
<td>6.64</td>
<td>2.00</td>
<td>19.00</td>
</tr>
<tr>
<td>Organic</td>
<td>16.33</td>
<td>2.65-30.01</td>
<td>5.51</td>
<td>10.00</td>
<td>20.00</td>
</tr>
<tr>
<td><strong>Interval depop-repop (days)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Battery</td>
<td>20.12</td>
<td>12.19-28.06</td>
<td>9.49</td>
<td>14.00</td>
<td>42.00</td>
</tr>
<tr>
<td>Floor-raised</td>
<td>30.50</td>
<td>19.96-41.04</td>
<td>14.73</td>
<td>14.00</td>
<td>70.00</td>
</tr>
<tr>
<td>Free-range</td>
<td>31.25</td>
<td>22.22-40.28</td>
<td>10.81</td>
<td>21.00</td>
<td>56.00</td>
</tr>
<tr>
<td>Organic</td>
<td>35.00</td>
<td>4.88-65.12</td>
<td>12.12</td>
<td>21.00</td>
<td>42.00</td>
</tr>
</tbody>
</table>

*this is the number of days between the end of the previous production cycle and the restocking of the house with new laying hens (all farms all-in/ all-out)
None of the 29 farms were found positive in any of the samples taken on-farm. However, *Salmonella* could be detected in hens from 6 out of the 29 laying hen farms both in the caeca and in the cloacal swabs taken after transport. A detailed overview of the results of the bacteriological analysis of the 6 positive farms is presented in Table 3. The factors associated with detection of *Salmonella* in the univariate analysis are listed in Table 4. The housing system as such was not significantly associated with the *Salmonella* infection ($P = 0.83$) since the same ratio of positive farms was found in each category of housing systems.

**Table 3: Results of bacteriological analysis of samples from laying hens of 29 flocks, after transport**

<table>
<thead>
<tr>
<th>Farm</th>
<th>Housing type</th>
<th>Cloacal swabs</th>
<th>Caeca</th>
<th>Serotype-phage type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Battery</td>
<td>3/100</td>
<td>6/100</td>
<td>Enteritidis, PT 1</td>
</tr>
<tr>
<td>2</td>
<td>Battery</td>
<td>1/100</td>
<td>14/100</td>
<td>Enteritidis, PT 12</td>
</tr>
<tr>
<td>3</td>
<td>Floor-raised</td>
<td>3/100</td>
<td>10/100</td>
<td>Enteritidis, PT 21</td>
</tr>
<tr>
<td>4</td>
<td>Free-range</td>
<td>4/100</td>
<td>7/100</td>
<td>Enteritidis, PT 1 and PT 35</td>
</tr>
<tr>
<td>5</td>
<td>Free-range</td>
<td>2/100</td>
<td>5/100</td>
<td>Enteritidis, PT 11</td>
</tr>
<tr>
<td>6</td>
<td>Organic</td>
<td>2/100</td>
<td>8/100</td>
<td>Typhimurium var. Copenhagen, PT 208</td>
</tr>
</tbody>
</table>

In the final multivariate logistic regression model (Table 5) the age of the production system ($P = 0.04$) and a previous *Salmonella* contamination on the farm ($P = 0.03$) turned out to be risk factors for a *Salmonella*-infection in laying hen flocks.

**Table 4: Results of univariable analysis for the identification of risk factors for *Salmonella* infection on 29 laying hen farms. Only factors with a $P$-value < 0.2 are listed.**

<table>
<thead>
<tr>
<th>Continuous variables</th>
<th>N</th>
<th>OR</th>
<th>95% CI for OR</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age of the production system</td>
<td>29</td>
<td>1.29</td>
<td>1.05-1.57</td>
<td>0.02</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Categorical variable</th>
<th>Salm pos/n</th>
<th>OR</th>
<th>95% CI for OR</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Previous <em>Salmonella</em>-contamination</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No (ref)</td>
<td>2/24</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Yes</td>
<td>4/5</td>
<td>44.00</td>
<td>3.18-608.16</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Other animal production on the farm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No (ref)</td>
<td>1/20</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Yes</td>
<td>5/9</td>
<td>23.75</td>
<td>2.15-262.47</td>
<td>0.01</td>
</tr>
<tr>
<td>Sanitary transition zone present</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>3/5</td>
<td>10.50</td>
<td>1.21-91.03</td>
<td>0.03</td>
</tr>
<tr>
<td>Yes (ref)</td>
<td>3/24</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Type of ventilation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Natural</td>
<td>2/3</td>
<td>11.00</td>
<td>0.80-152.04</td>
<td>0.07</td>
</tr>
<tr>
<td>Mechanical (ref)</td>
<td>4/26</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Control of rodents</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>By farmer (ref)</td>
<td>3/22</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>By farmer and specialized company</td>
<td>¼</td>
<td>2.11</td>
<td>0.16-27.58</td>
<td>0.56</td>
</tr>
<tr>
<td>By specialized company</td>
<td>2/3</td>
<td>12.67</td>
<td>0.86-186.91</td>
<td>0.06</td>
</tr>
</tbody>
</table>
Table 5: Results of multivariable analysis for the identification of risk factors for *Salmonella* infection on 29 laying hen farms (*P*-value < 0.05)

<table>
<thead>
<tr>
<th>Variable</th>
<th>n</th>
<th>OR</th>
<th>95% CI for OR</th>
<th><em>P</em>-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age of the production system</td>
<td>29</td>
<td>1.35</td>
<td>1.01-1.81</td>
<td>0.04</td>
</tr>
<tr>
<td>Previous <em>Salmonella</em> contamination</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No (Ref.)</td>
<td>24</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Yes</td>
<td>5</td>
<td>77.64</td>
<td>1.68-3596.30</td>
<td>0.03</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The number of sampled flocks in this study is relatively small which limits its power in comparison to the European baseline study on the prevalence of *Salmonella* in egg laying flocks. Also there is the fact that the numbers of farms of the different housing types sampled in this study do not exactly reflect the distribution of farms in the target population. This might explain why in contrast to what has been found in the EU baseline study (EFSA, 2007; Namata *et al*., 2008), the housing of the hens in conventional battery cages could not be identified as a risk factor for *Salmonella* in this dataset. It should be stressed that if the same sampling methodology as in the EU baseline study (i.e. 5 pooled faeces samples and 2 dust samples) was used no positive flock would have been detected. This indicates that the infection level in the positive flocks was very low. These low level infections were, at least in the subset of sampled flocks, present in equal proportions in the different housing systems. This could suggest that for these low infection levels the housing system has no influence, although more studies using extensive sampling should be performed to confirm this.

The fact that a previous *Salmonella* infection in the same house or in other houses on the farms could be identified as a risk factor is in accordance with Huneau-Salaün *et al*. (2009). Carrique-Mas *et al*. (2009) stated that the presence of *Salmonella* (Enteritidis) in the farm environment plays an important role in the presence of *Salmonella* in a laying hen flock. In all housing systems, poor standards of cleaning of the hens’ houses could be observed in many of the sampled flocks, especially in the sanitary transition zones and the anterooms of the laying hens’ houses. Similar poor results in biosecurity on laying hen farms have been reported by Davies and Breslin (2003) and Wales *et al*. (2006). It has indeed been stated that many *Salmonella* infections originate from the farm environment (van de Giessen *et al*., 1994; Davies and Breslin, 2003). Moreover, the level of environmental contamination increases
significantly during a production cycle (Wales et al., 2007). *Salmonella* can be detected both on the floor as on the equipment at different locations in and around laying hen houses such as underneath cages, the interior of the nest boxes, in laying house anterooms and egg storage areas (Davies and Breslin, 2001; Davies and Breslin, 2003). In this study, cleaning and disinfection during and between 2 production cycles of the above mentioned spots was marginal or non existent on many of the sampled farms.

Furthermore the infection of a flock with *Salmonella* through vectors such as rodents, flies and beetles (Davies and Breslin, 2001; Carrique-Mass et al., 2009) and the reported capacity of some *Salmonella* strains isolated in poultry to develop a biofilm could contribute to the survival of *Salmonella* in the environment of poultry houses (Marin et al., 2009).

Independent of the housing system, a higher age of the production system increased the risk of presence of *Salmonella* on the farm. The significant longer interval between depopulation and repopulation (*P*-value < 0.05) in alternative housing systems compared to conventional battery cages does not seem to have a protective influence on the prevalence of *Salmonella*. This is in accordance with Davies and Wray (1996) who described survival of *Salmonella* in empty poultry houses for a period of 12 months.

A previous *Salmonella* contamination on the farm and the age of the production system were found to be the main risk factors for low-level *Salmonella* infections occurring in laying hen flocks, irrespective of the housing system in which the hens are housed. This indicates that in flocks which are vaccinated against *Salmonella*, persistent biosecurity measures are necessary in all types of housing systems in order to prevent the recurrent contamination or new infections of laying hen flocks in subsequent production cycles.

**ACKNOWLEDGEMENTS**

This research was funded by the EU FP6, under contract 035547 (Safehouse project).
REFERENCES


Determination of the within- and between-flock prevalence and identification of risk factors for *Salmonella* shedding in laying hen flocks


*Adapted from Preventive Veterinary Medicine (2010) 94: 94-100*
ABSTRACT

Salmonella outbreaks in humans are often linked with the consumption of contaminated eggs. Therefore a profound knowledge of the actual prevalence of Salmonella spp. in laying hens and the factors that influence the presence and persistence of Salmonella on a farm is of utmost importance. The housing of laying hens in conventional battery cages will be forbidden in the European Union (EU) from 2012 onwards. There is an urgent need to evaluate whether this move to alternative housing systems will influence the prevalence of Salmonella in laying hens. Therefore, a cross-sectional study was performed in 5 European countries (Belgium, Germany, Greece, Italy and Switzerland) to determine the between and within flock prevalence of hens shedding Salmonella and to investigate whether there is an effect of the housing type on the shedding of Salmonella. In total 292 laying hen farms were sampled in the month prior to depopulation. The on-farm questionnaire described in chapter 4 was used to collect information on general management practices and specific characteristics of the sampled flock. Twenty-nine flocks were found positive for at least 1 Salmonella-serotype. In these flocks the within flock prevalence of shedding hens, determined by individual sampling of 40 hens, varied between 0 % and 27.50 %. A wide variety of serotypes was isolated with Salmonella Enteritidis as the most common. Housing in conventional battery cages, the absence of dry cleaning in between production rounds and sampling in winter turned out to be risk factors for the shedding of Salmonella Enteritidis or Typhimurium (P < 0.05).

Key words: Salmonella – laying hens – prevalence – risk factors – housing
INTRODUCTION

Already since the late 1960’s there was a growing public awareness concerning farm animal welfare which generally resulted in a consumer’s aversion to eggs produced by laying hens housed in cages (Appleby, 2003). This finally led to a ban of the housing of laying hens in conventional battery cages in the EU, from January 1st 2012 onwards (Council Directive 1999/74/EC). From this date on, the housing of laying hens in the EU will be restricted to enriched cages and alternative systems. According to the legislation laying hens housed in enriched cages must have at least 750 cm² of floor space per hen, a nest, perches and litter. These enriched cages exist in a wide variety of group sizes (EFSA, 2005). The so-called alternative systems are non-cage systems consisting of an indoor area either or not combined with outdoor facilities (EFSA, 2005; LayWel, 2006). The outdoor run may be covered (‘wintergarden’) or uncovered (‘free-range’). Two main categories can be distinguished: single level systems where the ground floor is fully or partially covered with litter and aviaries with a ground floor area plus one or more platforms (EFSA, 2005; LayWel, 2006). The above mentioned ban on conventional battery cages aims to improve the welfare of laying hens (Wall et al., 2004). Yet it has also initiated the question whether there aren’t any adverse consequences of this decision on the spread and/or persistence of infectious diseases in a flock. This fear is based on the opinion that one of the big advantages of conventional battery cages is that, because hens are separated from their faeces, the risk for disease transmission through faeces can be minimized (Duncan, 2000). As an example, in Switzerland an increase in the incidence of bacterial infections in chicks and laying hens could be seen in a 12 year period after the Swiss ban of conventional battery cages (Kaufmann-Bart and Hoop, 2009). This leads to the question whether the same effect is to be expected for zoonotic pathogens such as Salmonella or Campylobacter. After all Salmonella is worldwide still an important cause of human disease (EFSA, 2009). In Europe, Salmonella Enteritidis and Salmonella Typhimurium are the most commonly isolated serotypes in human cases of salmonellosis (WHO, 2006; EFSA, 2007) and contaminated eggs still remain the most important source of infection with Salmonella Enteritidis for humans (Crespo et al., 2005; De Jong and Ekdahl, 2006; Delmas et al., 2006).

The EU ban on conventional battery cages combined with high EU Salmonella prevalence data, indicated by the results of the European Food Safety Authority (EFSA) baseline study on the prevalence of Salmonella in European laying hen flocks (EFSA, 2007) urged the European Commission to call for a specific targeted research project to analyze and
control egg contamination by *Salmonella* after the move of laying hens to enriched cages and alternative housing systems (the Safehouse-project). One of the main tasks was a cross-sectional field study carried out in 5 European countries (Belgium, Germany, Greece, Italy and Switzerland).

The aim of this paper was to determine the between and within flock prevalence of *Salmonella* spp. in laying hen flocks housed in conventional battery cage and alternative non-cage housing systems and to identify risk factors for the shedding of *Salmonella* on laying hen farms.

**MATERIAL AND METHODS**

*Selection of the sampled farms*

Flocks were selected based upon a list of contact addresses of registered laying hen farms provided by the official Identification & Registration authorities. Only farms with a farm size of >1000 hens were selected. Also the housing system was an important selection criterion. The composition of the subset of sampled farms that was aimed at was 1/5 conventional battery cage farms and 4/5 non-cage housing systems. Within these non-cage housing systems an equal presence of aviaries, floor-raised, free-range and organic farms was strived at.

The farmers were contacted by telephone. The purpose of the study was explained and 100% anonymity was guaranteed (except for Germany), the foreseen date of depopulation was marked to make sure that the farm could be sampled in the month prior to depopulation. The aim was to sample in total 340 farms. Since not all of the contacted farmers volunteered to participate and because of logistic reasons, this number was not entirely reached. In the final study 292 flocks from 292 different farms were sampled in Belgium (n = 69), Germany (n = 84), Greece (n = 10), Italy (n = 30) and Switzerland (n = 99), comprising conventional battery cage flocks and four types of non-battery cage housing systems (Table 1). On farms with more than one house, only one house was randomly selected to be sampled. When several flocks were present in a given house, only one flock was selected at random.

*Moment of sampling*

Participating laying hen farms were sampled one month prior to depopulation. The samplings were performed during a 19-month period from January 2007 to August 2008.
Table 1: Numbers and types of laying hen farms that have been sampled

<table>
<thead>
<tr>
<th>Country</th>
<th>Conventional battery cage</th>
<th>Floor raised</th>
<th>Free range</th>
<th>Organic</th>
<th>Aviary</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belgium</td>
<td>18</td>
<td>21</td>
<td>21</td>
<td>8</td>
<td>1</td>
<td>69</td>
</tr>
<tr>
<td>Germany</td>
<td>26</td>
<td>20</td>
<td>24</td>
<td>14</td>
<td>-</td>
<td>84</td>
</tr>
<tr>
<td>Italy</td>
<td>12</td>
<td>10</td>
<td>8</td>
<td>-</td>
<td>-</td>
<td>30</td>
</tr>
<tr>
<td>Greece</td>
<td>3</td>
<td>7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>Switzerland</td>
<td>-</td>
<td>16</td>
<td>33</td>
<td>14</td>
<td>36</td>
<td>99</td>
</tr>
<tr>
<td>Total</td>
<td>59</td>
<td>74</td>
<td>86</td>
<td>36</td>
<td>37</td>
<td>292</td>
</tr>
</tbody>
</table>

Number and sample type taken on-farm

On-farm sampling consisted of 5 pooled faeces samples, 40 cloaca swabs of 40 laying hens and 1 sample of 200 red mites. Depending on the housing system, the pooled faeces samples were taken as follows:

- Conventional battery cage flocks and aviaries: 5 samples of mixed fresh faeces were taken from dropping belts and scrapers. The farmer was asked to operate the manure belts of each stack. In this way fresh piles of faeces could be picked up from each level of manure belts and of cages both at the right and the left side of the stack and along the entire length of stack. Each pooled faeces sample consisted of approximately 250 g.

- Floor-raised, free-range and organic farms: for each of the 5 pooled faeces samples 60 piles of fresh faeces were collected from the floor and the slats. Each pooled faeces sample consisted of approximately 250 g.

The pooled faeces samples were placed in a sterile recipient. Gloves were changed in between collection of each pooled faeces sample. The red mites were collected wherever in the house they were available. Using a disinfected brush they were swept into a sterile recipient.

The cloaca swabs were taken from hens that could be caught without causing too much agitation. Care was taken that hens were selected evenly throughout the house. Forty hens in the flock were selected and from each hen a cloaca swab was taken by inserting a cotton-tipped swab approximately 5 cm into the cloaca, taking care to avoid contact with the surrounding feathers and skin. Afterwards, the swabs were placed in tubes containing Ames medium. The sample size of 40 swabs was calculated in function of an expected within-flock prevalence of 10 %, an accepted error of 10 %, a confidence level of 95 % and a flock size of 10000 (WinEpiscope 2.0). All samples were placed in an appropriate leak-proof bag and outer
container and transported to the lab under ambient conditions and were incubated for bacteriological analysis (see further). Bacteriological analysis started on the day of sampling. If not, samples were stored at 4°C for no more than 24 hours.

**Bacteriological analysis of samples**

All samples were analyzed using ISO 6579:2002_Amd1:2007, as recommended by the Community Reference Laboratory for *Salmonella* in Bilthoven, The Netherlands for the detection of *Salmonella* in animal and environmental samples from primary production. From each pooled faeces sample, 25 g was added to 225 ml of buffered peptone water (BPW) (Oxoid, Basingstoke, Hampshire, UK). Each cloacal swab was placed in 9 ml of BPW. Pooled faeces samples were mixed in a stomacher bag for 1 minute. All samples were incubated for 18 ± 2 h at 37 ± 1°C. Next, 3 droplets of the pre-enrichment culture were inoculated onto a modified semi-solid Rappaport-Vassiliadis (MSRV) (Difco; Becton Dickinson) agar plate containing 0.01 g l⁻¹ novobiocine and incubated for 2 x 24 ± 3 h at 41.5 ± 1°C. Suspect white culture from the border of the growth zone was plated on Brilliant Green Agar (BGA; Oxoid) and Xylose Lysine Deoxycholate agar (XLD; Oxoid), followed by incubation for 24 ± 3 h at 37 ± 1°C. Presumed *Salmonella* colonies on BGA and XLD were biochemically confirmed using ureum agar, triple sugar iron agar and lysine-decarboxylase broth. Serotyping of *Salmonella*-isolates according to the Kauffmann-White scheme was performed in the national Reference Labs for *Salmonella* in each participating country.

**Questionnaire design**

The questionnaire was filled in during an on-farm interview on the same occasion of the collection of the samples. Questions related to general farm and flock characteristics (e.g. flock size, breed, age of the hens, medical treatments…) and biosecurity measures. Special attention was paid to the system in which the sampled flock was housed. A summary of the main items included in the questionnaire is given in Table 2. The same questionnaire was used in all participating countries. Before use the questionnaire was tested on 2 laying hen farms, one with conventional battery cages and one with a free-range production system to check whether the questions were relevant to the aim of this study.
Data processing and analysis

Information from the questionnaires was coded and put in a database (Excel, Microsoft Cooperation). Data were analyzed using SPSS 16.0 for Windows (SPSS Inc., Chicago, IL) and MLwiN (MLwiN version 2.0, Centre for Multilevel Modelling, Institute of Education, London, UK). One-way analysis of variance was used to examine differences in farm and flock size and age of the infrastructure between the different housing systems. For conventional battery cages this was the number of years since the current cages were installed. For the other housing systems this was the number of years since the current equipment (nest boxes, perches, slats...) was installed in the sampled house.

Table 2: Summary of the main items included in the questionnaire to identify risk factors for Salmonella in laying hen farms (for continuous variables: Median; for categorical variables: proportions) (total number of questions = 92)

<table>
<thead>
<tr>
<th>All flocks</th>
<th>Salm.* positive flocks</th>
<th>Salm.* negative flocks</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Farm characteristics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total number of hens</td>
<td>9700.0</td>
<td>57500.0</td>
</tr>
<tr>
<td>Number of poultry houses</td>
<td>1.0</td>
<td>1.5</td>
</tr>
<tr>
<td>Other animal production present (% of the farms)</td>
<td>48.3</td>
<td>22.7</td>
</tr>
<tr>
<td>Control of pests (% of the farms)</td>
<td>91.0</td>
<td>90.1</td>
</tr>
<tr>
<td><strong>House and management characteristics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age of building (in years)</td>
<td>21.0</td>
<td>38.0</td>
</tr>
<tr>
<td>Age of infrastructure</td>
<td>9.0</td>
<td>21.0</td>
</tr>
<tr>
<td>Number of flocks present</td>
<td>1.0</td>
<td>1.5</td>
</tr>
<tr>
<td>Access to out-door run (% of the farms)</td>
<td>63.4</td>
<td>22.3</td>
</tr>
<tr>
<td>Number of egg collections per day</td>
<td>2.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Dry cleaning status (% of the flocks)</td>
<td>82.2</td>
<td>31.8</td>
</tr>
<tr>
<td><strong>Sampled flock characteristics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of hens in the flock</td>
<td>4820</td>
<td>22000.0</td>
</tr>
<tr>
<td>Cumulative mortality in the flock since onset (in %)</td>
<td>8.00</td>
<td>7.00</td>
</tr>
<tr>
<td>Age of the hens (in weeks)</td>
<td>71.0</td>
<td>73.0</td>
</tr>
<tr>
<td>Salmonella vaccination status (% of the flocks)</td>
<td>28.1</td>
<td>13.6</td>
</tr>
<tr>
<td>Medical treatment (% of the flocks)</td>
<td>9.2</td>
<td>18.2</td>
</tr>
</tbody>
</table>

*Salmonella Enteritidis and/or Typhimurium

The within flock prevalence of excretion for each flock positive for Salmonella Enteritidis or Typhimurium was calculated based on the 40 individual cloacal swabs. The 95% confidence interval (CI) was calculated as the prevalence ± 1.96 SD/√n with SD being the standard deviation and n the sample size. For positive flocks with 0 positive cloaca swabs (only positive in pooled faeces) the maximum possible prevalence that could be missed due to
coincidence was calculated using following formula: \( D = \left( 1 - (1 - CL)^{1/n} \right) \left( N - \frac{(n-1)}{2} \right) \) (WinEpiscope 2.0). With \( D \) = maximum number of positive animals missed due to coincidence, \( CL \) = the confidence level, \( N \) = population size and \( n \) = sample size.

The relationship between risk factors and \textit{Salmonella} Enteritidis or Typhimurium status of the sampled farm was evaluated by means of a multilevel logistic regression model with the \textit{Salmonella} Enteritidis/Typhimurium status of the sampled flock as a binary outcome variable. For this a flock was defined as being infected if at least one of the collected samples was positive for \textit{Salmonella} Enteritidis or Typhimurium. The decision to take this outcome variable is based on the fact that these 2 serovars are the 2 most common isolated serovars in case of human salmonellosis (EFSA, 2009). To check the potential effect of the housing system, the following categories were used: conventional battery cages, indoor production (which means aviaries and floor-raised systems), free-range systems and free-range organic systems.

In a first step, all potential risk factors were tested univariably and only variables with a \( P \)-value < 0.2 were selected. The shape of the relationship with the outcome variable was assessed for all continuous variables by plotting the log odds of the outcome versus the continuous variable (Parkin et al., 2005). If there was a non-linear relationship, the continuous variable was categorized.

Next, a multivariable logistic regression model was built, including all variables with a univariable \( P \)-value < 0.20. Correlations between the selected independent variables (Pearson’s and Spearman’s \( \rho \) correlations) were determined. If 2 variables were highly correlated (this means with an \( r^2 \)-value above 0.60), only the variable with the smallest \( P \)-value was included in the final multivariable model.

The distribution of the housing types within the different countries could possibly influence the risk factor analysis, because of clustering or non-independence of data. To address this, the analyses were performed using MLwiN. Hereby, ‘country’ was taken into account as a random effect. To evaluate the presence of confounding, a Mantel-Haensel analysis was used. Confounding was considered potentially significant when changes in the \( OR \) of more than 20 \% could be observed (Dohoo et al., 2003).

In the final multilevel logistic regression model, all two-way interactions between significant variables were evaluated (with the significance level set at \( P < 0.05 \)). Odds ratios (OR), including 95 \% confidence intervals (CI), are reported for all significant variables.
RESULTS

A total of 292 commercial laying hen farms have been sampled in Belgium, Germany, Greece, Italy and Switzerland. A detailed overview of the numbers and types of farms is presented in Table 1. The participation rate was more than 90 % in Belgium, Greece, Italy and Switzerland and 70 % in Germany. A description of the main characteristics of the sampled farms is presented in Table 3. The mean age of the sampled hens was 72.1 weeks, ranging from 45 to 121 weeks. Because of practical reasons 1 Greek flock was sampled halfway the production cycle, at 38 weeks of age. The size of the selected flocks varied between 480 and 96000 hens. The number of laying hens on conventional battery cage farms was significantly higher than the number of hens on farms with a non-cage housing system ($P < 0.05$). The age of the infrastructure in conventional battery cage systems was significantly higher than on floor-raised, free-range and organic farms ($P < 0.05$). No significant difference in the number of houses on the farm could be seen between the different housing systems, neither was there a significant difference in the number and type of sampled houses between the seasons.

<table>
<thead>
<tr>
<th>Table 3: Main characteristics of the 292 sampled farms</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Amount of hens on the farm</strong></td>
</tr>
<tr>
<td>Battery</td>
</tr>
<tr>
<td>Aviary</td>
</tr>
<tr>
<td>Floor-raised</td>
</tr>
<tr>
<td>Free-range</td>
</tr>
<tr>
<td>Organic</td>
</tr>
<tr>
<td><strong>Amount of hens in the flock</strong></td>
</tr>
<tr>
<td>Battery</td>
</tr>
<tr>
<td>Aviary</td>
</tr>
<tr>
<td>Floor-raised</td>
</tr>
<tr>
<td>Free-range</td>
</tr>
<tr>
<td>Organic</td>
</tr>
<tr>
<td><strong>Age of the production system (years)</strong></td>
</tr>
<tr>
<td>Battery</td>
</tr>
<tr>
<td>Aviary</td>
</tr>
<tr>
<td>Floor-raised</td>
</tr>
<tr>
<td>Free-range</td>
</tr>
<tr>
<td>Organic</td>
</tr>
<tr>
<td><strong>Age hens (weeks)</strong></td>
</tr>
<tr>
<td>Battery</td>
</tr>
<tr>
<td>Aviary</td>
</tr>
<tr>
<td>Floor-raised</td>
</tr>
<tr>
<td>Free-range</td>
</tr>
<tr>
<td>Organic</td>
</tr>
</tbody>
</table>
Of the 292 sampled farms, 29 were found positive for *Salmonella* in at least one of the samples taken. The between flock prevalences differed significantly ($P < 0.05$) between the countries: 1.43% (0.00 – 3.73%) (Belgium), 20.00% (11.68 – 28.72%) (Germany), 20.00% (0.00 – 44.58%) (Greece) and 30.00% (13.81 – 46.19%) (Italy). In none of the 99 sampled Swiss farms *Salmonella* could be found: 0.00% (0.00 – 3.70%). When looking only at *Salmonella* Enteritidis or Typhimurium, 22 flocks were found positive with respective between flock prevalences of 1.43% (0.00 – 3.73%) (Belgium), 20.00% (11.68 – 28.72%) (Germany), 0.00% (0.00 – 2.40%) (Greece) and 13.30% (1.32 – 25.34%) (Italy).

Only 7 of the 29 positive farms were found positive both in the pooled faeces samples and in the cloacal swabs. Twenty flocks were only positive in the pooled faeces samples and 2 flocks were positive in one or more cloacal swabs but not in the pooled faeces samples. The number of positive pooled faeces samples ranged from 1 to 5. In the 9 flocks where positive cloacal swabs were found the within-flock prevalence varied between 2.50% and 27.50%. In the 20 flocks with positive pooled faeces but no positive cloacal swabs the maximum prevalence of hens shedding *Salmonella* that could have been missed due to coincidence was 7.33%. A detailed overview of the results of the bacteriological analysis with the within flock prevalences is presented in Table 4. The number of positive swabs was not significantly associated with the housing type, nor was the number of positive pooled faeces samples. On 214 of the 292 sampled farms red mites could be collected. None of the red mites samples was found positive for *Salmonella*. 
Table 4: Detailed overview of the 29 laying hen flocks found positive for *Salmonella*

<table>
<thead>
<tr>
<th>Country</th>
<th>Housing type</th>
<th>N positive / Sample type</th>
<th>Within flock prevalence (95% CI)</th>
<th>Serotype and phage type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belgium</td>
<td>Conv. Batt.</td>
<td>5/5 faeces, 0/40 swabs</td>
<td>0% (0.00 – 7.21%)</td>
<td><em>S. Enteritidis</em> PT 4a</td>
</tr>
<tr>
<td>Germany</td>
<td>Conv. Batt.</td>
<td>5/5 faeces, 0/40 swabs</td>
<td>0% (0.00 – 7.21%)</td>
<td><em>S. Enteritidis</em> PT 21c, RDNC</td>
</tr>
<tr>
<td>Germany</td>
<td>Conv. Batt.</td>
<td>3/5 faeces, 0/40 swabs</td>
<td>0% (0.00 – 7.21%)</td>
<td><em>S. Enteritidis</em> PT 4</td>
</tr>
<tr>
<td>Germany</td>
<td>Conv. Batt.</td>
<td>1/5 faeces, 0/40 swabs</td>
<td>0% (0.00 – 7.21%)</td>
<td><em>S. Enteritidis</em> PT 4</td>
</tr>
<tr>
<td>Germany</td>
<td>Conv. Batt.</td>
<td>1/5 faeces, 2/40 swabs</td>
<td>5.00% (0.00 – 11.65%)</td>
<td><em>S. Enteritidis</em> PT 8</td>
</tr>
<tr>
<td>Germany</td>
<td>Conv. Batt.</td>
<td>1/5 faeces, 0/40 swabs</td>
<td>0% (0.00 – 7.21%)</td>
<td><em>S. Enteritidis</em> PT 4</td>
</tr>
<tr>
<td>Germany</td>
<td>Conv. Batt.</td>
<td>1/5 faeces, 0/40 swabs</td>
<td>0% (0.00 – 7.21%)</td>
<td><em>S. Enteritidis</em> PT 4</td>
</tr>
<tr>
<td>Germany</td>
<td>Conv. Batt.</td>
<td>1/5 faeces, 0/40 swabs</td>
<td>0% (0.00 – 7.21%)</td>
<td><em>S. Enteritidis</em> PT 4</td>
</tr>
<tr>
<td>Germany</td>
<td>Conv. Batt.</td>
<td>1/5 faeces, 0/40 swabs</td>
<td>0% (0.00 – 7.21%)</td>
<td><em>S. Enteritidis</em> PT 8</td>
</tr>
<tr>
<td>Germany</td>
<td>Floor-raised</td>
<td>3/5 faeces, 4/40 swabs</td>
<td>10.00% (0.73 – 19.27%)</td>
<td><em>S. Enteritidis</em> PT 4</td>
</tr>
<tr>
<td>Germany</td>
<td>Floor-raised</td>
<td>3/5 faeces, 1/40 swabs</td>
<td>2.50% (0.00 – 7.33%)</td>
<td><em>S. Enteritidis</em> PT 8</td>
</tr>
<tr>
<td>Germany</td>
<td>Floor-raised</td>
<td>3/5 faeces, 0/40 swabs</td>
<td>0% (0.00 – 7.21%)</td>
<td><em>S. Enteritidis</em> PT 8, <em>S. subsp. I</em></td>
</tr>
<tr>
<td>Germany</td>
<td>Free-range</td>
<td>5/5 faeces, 0/40 swabs</td>
<td>27.5% (13.94 – 41.06%)</td>
<td><em>S. Enteritidis</em> PT 4</td>
</tr>
<tr>
<td>Germany</td>
<td>Free-range</td>
<td>1/5 faeces, 0/40 swabs</td>
<td>0% (0.00 – 7.18%)</td>
<td><em>S. Enteritidis</em> PT 4</td>
</tr>
<tr>
<td>Germany</td>
<td>Free-range</td>
<td>1/5 faeces, 0/40 swabs</td>
<td>0% (0.00 – 7.16%)</td>
<td><em>S. Enteritidis</em> PT 4</td>
</tr>
<tr>
<td>Greece</td>
<td>Floor-raised</td>
<td>5/5 faeces, 0/40 swabs</td>
<td>0% (0.00 – 7.21%)</td>
<td><em>S. Heidelberg</em></td>
</tr>
<tr>
<td>Greece</td>
<td>Floor-raised</td>
<td>5/5 faeces, 0/40 swabs</td>
<td>0% (0.00 – 7.18%)</td>
<td><em>S. Heidelberg</em></td>
</tr>
<tr>
<td>Italy</td>
<td>Conv. Batt.</td>
<td>2/5 faeces, 0/40 swabs</td>
<td>0% (0.00 – 7.21%)</td>
<td>*S. Napoli, <em>enterica</em> subsp. <em>ent.</em></td>
</tr>
<tr>
<td>Italy</td>
<td>Conv. Batt.</td>
<td>3/5 faeces, 0/40 swabs</td>
<td>0% (0.00 – 7.21%)</td>
<td><em>S. Virchow</em></td>
</tr>
<tr>
<td>Italy</td>
<td>Conv. Batt.</td>
<td>4/5 faeces, 0/40 swabs</td>
<td>0% (0.00 – 7.21%)</td>
<td><em>S. Enteritidis</em> PT 27, NT</td>
</tr>
<tr>
<td>Italy</td>
<td>Conv. Batt.</td>
<td>0/5 faeces, 2/40 swabs</td>
<td>5.00% (0.00 – 11.75%)</td>
<td><em>S. Enteritidis, S. Bredeny</em></td>
</tr>
<tr>
<td>Italy</td>
<td>Conv. Batt.</td>
<td>0/5 faeces, 1/40 swabs</td>
<td>2.50% (0.00 – 7.34%)</td>
<td><em>S. Typhimurium</em> NT</td>
</tr>
<tr>
<td>Italy</td>
<td>Conv. Batt.</td>
<td>1/5 faeces, 0/40 swabs</td>
<td>0% (0.00 – 7.21%)</td>
<td><em>S. enterica subsp. enterica</em> NT</td>
</tr>
<tr>
<td>Italy</td>
<td>Floor-raised</td>
<td>3/5 faeces, 1/40 swabs</td>
<td>2.50% (0.00 – 7.33%)</td>
<td>*S. Bareilly, <em>S. Thompson</em></td>
</tr>
<tr>
<td>Italy</td>
<td>Free-range</td>
<td>2/5 faeces, 0/40 swabs</td>
<td>0% (0.00 – 7.21%)</td>
<td><em>S. Kottbus</em></td>
</tr>
<tr>
<td>Italy</td>
<td>Free-range</td>
<td>3/5 faeces, 1/40 swabs</td>
<td>2.50% (0.00 – 7.33%)</td>
<td><em>S. Enteritidis</em> PT 14, NT</td>
</tr>
</tbody>
</table>
For the identification of risk factors only *Salmonella* Enteritidis and Typhimurium and the following housing types were taken into account: conventional battery cages, indoor production systems, free-range and free-range organic systems. The factors associated with detection of *Salmonella* in the univariable analysis are listed in Table 5. In the final multivariable logistic regression model (Table 5) the absence of dry cleaning (*P* < 0.01), the housing type (*P* < 0.01) and the season of sampling (*P* = 0.01) turned out to be risk factors for the shedding of *Salmonella* Enteritidis/Typhimurium in laying hen flocks.

**Table 5**: Results of the univariable and multivariate analysis for the identification of risk factors for *Salmonella* Enteritidis or Typhimurium infection on 292 European laying hen farms.

<table>
<thead>
<tr>
<th>Continuous variables</th>
<th>Univariable analysis</th>
<th>Multivariable analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age of the infrastructure in years</td>
<td>1.07</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Number of flocks in the sampled house</td>
<td>1.39</td>
<td>0.04</td>
</tr>
<tr>
<td>Number of egg collections per day</td>
<td>0.19</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Categorical variable</th>
<th>n</th>
<th>OR</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry cleaning</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>52</td>
<td>13.49</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Yes (ref)</td>
<td>240</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Vaccination status against <em>Salmonella</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>210</td>
<td>2.62</td>
<td>0.13</td>
</tr>
<tr>
<td>Yes (ref)</td>
<td>82</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Type of housing</td>
<td></td>
<td></td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Conventional battery (ref)</td>
<td>59</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Indoor production</td>
<td>11</td>
<td>0.09</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Free-range</td>
<td>86</td>
<td>0.16</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Organic</td>
<td>36</td>
<td>0.09</td>
<td>0.02</td>
</tr>
<tr>
<td>Season of sampling</td>
<td></td>
<td></td>
<td>0.02</td>
</tr>
<tr>
<td>Winter (ref)</td>
<td>49</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Spring</td>
<td>80</td>
<td>0.30</td>
<td>0.04</td>
</tr>
<tr>
<td>Summer</td>
<td>97</td>
<td>0.09</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Autumn</td>
<td>66</td>
<td>0.44</td>
<td>0.15</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The housing of laying hens in conventional battery cages turned out to be a significant risk factor for the shedding of *Salmonella* Enteritidis and/or Typhimurium. This is in accordance with the results of the baseline study on the prevalence of *Salmonella* in laying hen flocks, both at the EU level (EFSA 2007) and at the level of individual member states (Methner et al., 2006; Namata et al., 2008; Huneau-Salaün et al., 2009). The reasons why a
higher prevalence of *Salmonella* is found in cage housed laying hen flocks is likely to be a combination of factors. First of all flock size may have an effect (Mollenhorst et al., 2005; EFSA, 2007; Carrique-Mas et al., 2008; Huneau-Salaün et al., 2009). As also demonstrated in this study, in cage farms flock sizes are in general larger in comparison to non-cage farms. Second, the number of years that the current infrastructure is in use could also play an important role. In this study the age of conventional battery cages was significantly higher than that of the floor-raised, free-range and organic systems. In the univariable analysis the age of the infrastructure was identified as a significant risk factor, as was the age of the building. However, because there was a too strong correlation between these 2 variables, only the first one was considered. The effect of the age of the infrastructure may be explained by the fact that the older the infrastructure, the more difficult it gets to achieve sufficient standards of cleaning due to the wear of the materials, both of the production system and of the building itself, especially when it is taken into account that the level of environmental contamination increases significantly during a production cycle (Wales et al., 2007). The importance of such latent environmental sources of infections should not be underestimated since Davies and Wray (1996) described the survival of *Salmonella* in empty poultry houses for a period of 12 months. This implies the risk of ‘transfer’ of an infection between successive production cycles.

The multivariate model shows that “age of the infrastructure” does not remain significant when combined with production type. This implies that this factor is not sufficient to explain the risk conventional housing systems pose to *Salmonella* Enteritidis infections, and thus that other factors related to the housing system are involved. One of these other factors might be the fact that it is more difficult to thoroughly clean and disinfect cage systems and therefore remainders from previous infections may be more difficult to eliminate (Davies and Breslin, 2003; EFSA, 2007).

Another proof of the importance of cleaning is given by the fact that absence of dry cleaning in between production rounds was found to be a significant risk factor for the shedding of *Salmonella*. Dry cleaning is the mechanical removal of organic material (manure, dust, feed spills…) before the wet cleaning (using water) is carried out. Only 82.2 % of the sampled farms carried out dry cleaning of the laying hen houses where for wet cleaning and disinfection this was 93.2 % and 92.5 % respectively. The value of dry cleaning in between production rounds is 2-fold: on one hand it is very useful to remove organic matter that can harbor *Salmonella*. On the other hand it contributes to a more efficient disinfection since the
presence of considerable amounts of organic protective matter such as manure, dust and spilled feed has an adverse effect on the efficacy of disinfection (Davies and Breslin, 2003; Gradel et al., 2004).

In contrast to other studies (Bouwknegt et al., 2004; Mollenhorst et al., 2005; Namata et al., 2008) but in accordance with Wales et al. (2007), a seasonable effect could be observed. The odds to detect *Salmonella* were significantly higher in flocks that were sampled in winter compared to flocks that were sampled in the other seasons of the year. This could be explained by the fact that in housing systems with an outdoor run the hens are kept inside due to wet and cold weather conditions. A high density of animals is a well-known risk factor for *Salmonella* (EFSA, 2007; Huneau-Salaün et al., 2009). Furthermore, the air quality in laying hen houses seems to be lower in winter (Ellen et al., 2000; Nimmermark et al., 2009). This can cause stress in the hens, leading them from a *Salmonella*-carrying state to a *Salmonella*-shedding state.

Because of reasons of multicollinearity, the protective influence of vaccination against *Salmonella* could not be statistically confirmed. This finding will be further elaborated in the general discussion section of this thesis.

The estimates of the within flock prevalence based on the cloacal swabs were usually relatively low indicating that in general only a small percentage of birds in the positive flocks were shedding the bacterium. It needs to be stressed that the estimates obtained are an indication of the number of birds shedding *Salmonella*, and not necessarily an accurate indication of the number of birds infected with *Salmonella*. It is likely that in a substantial proportion of the birds sampled negative still some low level *Salmonella* infection may be present. In that case the negative sample is due to the fact that the birds were not shedding the bacteria at the moment of sampling or the used sampling technique was not sensitive enough to detect the limited shedding. The sometimes large difference between the prevalence of infected and shedding birds has recently been clearly demonstrated by Van Hoorebeke et al. (2009). This also holds for the between flock prevalence.

Compared to the results of Belgium [27.7 % (22.1 – 33.9 %)], Germany [24.2 % (21.2 – 27.5 %)], Greece [25.7 % (20.5 – 31.6 %)] and Italy [7.9 % (5.9 – 10.5 %)] in the EFSA baseline study on the between herd prevalence of *Salmonella* Enteritidis and/or Typhimurium on laying hen holdings (EFSA, 2007), only in Belgium and Greece the prevalence detected in our study, was lower. However, these findings should be interpreted with care because the
farms that were contacted to be sampled were selected on the base of the housing type and thus not random. In addition, the sampling design of this study is different from the one of the EFSA baseline study.

CONCLUSION

The results of this study illustrate that, despite the fact that in non-cage housing systems the chance of oro-faecal transmission of *Salmonella* is much higher than in conventional battery cage systems, no higher prevalence of *Salmonella* could be observed in flocks housed in these alternative systems. Several management and biosecurity measures such as strict cleaning and disinfection practices have been identified as protective factors to minimize the introduction and persistence of *Salmonella* on laying hen farms. In future, a close follow up of the evolution in time, both of the prevalence of *Salmonella* spp. in laying hen flocks housed in different housing systems and in the diversity of serovars isolated and their significance for public health, will be necessary.

ACKNOWLEDGMENTS

This research was funded by the EU FP6, under the contract 035547 (Safehouse project). The authors thank all farmers for their permission to sample their farm and all lab technicians at the different institutions for their help during the bacteriological analyses.
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Chapter 6

THE EFFECT OF THE HOUSING SYSTEM ON ANTIMICROBIAL RESISTANCE IN INDICATOR BACTERIA IN LAYING HENS


Submitted in Epidemiology and Infection (2010)
ABSTRACT

The aim of this study was to determine the potential association between housing type and multi-drug resistance in *Escherichia coli* and *Enterococcus faecalis* isolates recovered from 283 laying hen flocks. In each flock, a cloacal swab from four hens was collected and 1102 *E. coli* and 792 *E. faecalis* isolates were recovered. Broth microdilution was used to test susceptibility to antimicrobials including multiple drug resistance (MDR). A generalized estimating equations logistic regression model was used to identify factors influencing MDR. Country and housing type interacted differently with the MDR levels of both species. In the *E. coli* model, the housing in a floor raised system was associated with an increased risk of MDR in comparison to the conventional battery system. In the *E. faecalis* model, the MDR levels were lower in free-range systems than in conventional battery cages. In Belgium, ceftiofur-resistant *E. coli* isolates were more abundantly present than in the other countries.

**Key words:** antimicrobial resistance – *E. coli* – *E. faecalis* – laying hen
INTRODUCTION

The therapeutic, prophylactic and metaphylactic use of antimicrobials is common practice in modern food animal husbandry (Berge et al., 2003; Aarestrup, 2004; EFSA, 2008). Concerns have grown that this widespread use of antibiotic drugs may lead to an increase in antimicrobial resistance in numerous bacteria potentially affecting public health (EFSA, 2009; Jordan et al., 2009). Standardized and continuous surveillance programs are necessary to monitor the occurrence and persistence of antimicrobial resistance in food animals (Aarestrup, 2004; Wallmann, 2006; WHO, 2008). Indicator bacteria are generally used to monitor antimicrobial resistance since they can be commonly found in healthy animals, giving an indication of the level of resistance in a particular population. In addition, they acquire antimicrobial resistance faster than other commonly found bacteria (Aarestrup, 2004; WHO, 2007; Miranda et al., 2008). Commensal Escherichia coli and Enterococcus faecalis are internationally used as respective Gram-negative and Gram-positive indicator bacteria for monitoring antimicrobial resistance because of their common presence in the animal intestinal tract (Wray and Gnanou, 2000; Sørum and Sunde, 2001; de Jong et al., 2009). Surveillance of antimicrobial resistance is performed in several countries (Aarestrup, 2004), yet these surveillance programs have generally been focused on cattle, pig and broiler production. Programs in laying hens are still scarce. Therefore, there is a need to monitor antimicrobial resistance development in laying hens (EFSA, 2008).

The Council Directive 1999/74/EC states that for welfare reasons as of January 2012 onwards conventional battery cages will be forbidden in the European Union (EU) (Wall et al., 2004). Only enriched cages and non-cage housing systems will be allowed. Non-cage housing systems consist of an indoor area that may or may not be combined with covered (‘wintergarden’) or uncovered (‘free-range’) outdoor facilities (EFSA, 2005; LayWel, 2006). The non-cage systems can be categorized into 2 groups: single level systems with a ground floor area which is fully or partially covered with litter and aviaries, consisting of a ground floor area plus one or more platforms (EFSA, 2005; LayWel, 2006). Free-range organic flocks have the same structure as a free-range system but there are some additional requirements concerning maximum flock size, beak trimming and the origin of the feed. Moreover, the application of antimicrobials in these organic flocks is strictly restricted to the therapeutic usage. Presently it is not clear whether the different housing and management systems for laying hens will influence the occurrence of antimicrobial resistance. Recent reports indicate that both in poultry and other animal species the move from conventional
indoor production towards free-range and organic production exerted a beneficial effect on the levels of antimicrobial resistance in zoonotic and indicator bacteria (Avrain et al., 2003; Thakur and Gebreyes, 2005; Ray et al., 2006, Schwaiger et al., 2008; 2010, Young et al., 2009). On the other hand it has been shown that this move to non-cage housing systems resulted in an increased incidence of particularly bacterial diseases (Fossum et al., 2009; Kaufmann-Bart and Hoop, 2009), which could potentially lead to increased antibiotic usage. However, epidemiological data on the prevalence of antimicrobial resistance in the above mentioned indicator bacteria in laying hens in different housing systems is still limited. It is therefore necessary to investigate the impact of these new laying hen housing systems on the prevalence of antimicrobial resistance and to study antimicrobial resistance development in laying hens (EFSA, 2008).

The aim of this study is to investigate the prevalence of antimicrobial resistance in E. coli and E. faecalis isolates recovered from 283 laying flocks in four European countries and to evaluate the potential association between housing systems and observed multi-drug resistance.

**MATERIAL AND METHODS**

*Farm selection*

Flocks were selected from registry list of registered laying hen farms provided by the official identification and registration authorities of the participating countries (Belgium, Germany, Italy and Switzerland). The farm size (>1000 laying hens) and the housing type were the only 2 selection criteria. The distribution of sampled farms aimed at was 20 % conventional battery cage systems and 80 % (alternative) non-cage housing systems. Within the group of alternative housing systems an equal number of aviaries, floor-raised, free-range and free-range organic farms was targeted. The farmers were contacted by telephone and the purpose of the study was explained. The foreseen date of depopulation was marked to make sure that the farm could be sampled in the month prior to depopulation.

*Sampling of the laying hen farms*

The cloacal swabs were taken from randomly selected hens in each of the flocks as carefully as possible to avoid unnecessary stress. Four hens in the flock were evenly selected throughout the house and from each hen a cloacal swab was taken by inserting a sterile cotton-tipped swab approximately 5 cm into the cloaca, taking care to avoid contact with the
surrounding feathers and skin. The swabs were directly placed in tubes containing Ames medium.

A questionnaire was completed during an on-farm interview at the same occasion as collection of the samples took place. The questions were related to general farm and flock characteristics such as flock size, breed, age of the hens and biosecurity measures. Special attention was paid to the housing system of the sampled flock and the antimicrobial treatments the hens had received during the current production cycle. The same questionnaire was used in all participating countries. In each of the participating countries, there was one person who collected the samples and did the interview with the farmer.

Bacteriological examination of the samples

For the isolation of E. coli the swabs were plated on MacConkey plates (Oxoid®, France) and incubated aerobically for 24 h at 37°C. From each primary plate, one colony was picked and plated again on a MacConkey agar plate. Suspected colonies were confirmed as E. coli by positive glucose/lactose fermentation, gas production and absence of H₂S production on Kligler Iron Agar (Oxoid) and absence of aesculin hydrolysis (Bile aesculin agar; Oxoid). Enterococcus was isolated on Slanetz & Bartley agar (Oxoid). After incubation for 48 h at 42 ± 1°C, one suspected Enterococcus faecalis colony per sampled animal was purified and verified by using Rapid ID 32 STREP strips (BioMérieux, France). In Switzerland, the following equivalent methods were used: for the isolation of E. Coli, the cloacal swabs were plated on MacConkey Agar (Oxoid) and incubated for 24 h at 37°C under aerobic conditions. Strains which were lactose-positive were sub-cultured on Brolacin Agar (Merck®, Darmstadt, Germany) and incubated for 24 h at 37°C under aerobic conditions. Confirmation of the strains as E. coli was carried out using Rapid 20 E (BioMérieux). E. Faecalis were isolated from the cloacal swabs on Enterococcus Agar (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and incubated for 48 h at 37°C under aerobic conditions. On Columbia 5% Sheep Blood Agar (Oxoid) strains were sub-cultured and incubated for 24 h at 37°C under anaerobic conditions. Confirmation of the strains as E. faecalis was carried out using Rapid ID 32 STREP strips (BioMérieux).

Antimicrobial susceptibility testing

For both indicator bacteria, susceptibility was tested by means of broth microdilution using custom made Sensititre® plates (Trek Diagnostics Systems Ltd., UK). The antimicrobials tested and their ranges are listed in Tables 1 and 2 for E. coli and E. faecalis.
respectively. The results were read visually after 24 h of incubation at 37°C and the Minimum Inhibitory Concentration (MIC) was defined as the lowest concentration of the antimicrobial that completely inhibited visible growth. *Escherichia coli* ATCC 25922 and *Enterococcus faecalis* ATCC 29212 were used for quality control. The EUCAST (European Committee on Antimicrobial Susceptibility Testing) epidemiological breakpoints for MIC determination were used, since epidemiological cut-off values are considered more useful than clinical break points to achieve optimum sensitivity for detection of acquired resistance (Aarestrup et al., 2007; EFSA, 2008). When these EUCAST breakpoints were not available (which was the case for apramycin, sulfamethoxazole and kanamycin), the break points mentioned in the reports of Danish Integrated Antimicrobial Resistance Monitoring and Research Program were used (Danmap, 2007).
Table 1: Minimum Inhibitory Concentration distribution (%) for all *Escherichia coli* isolates (vertical black line indicates cut-off value)

| Compound                  | 0.015 | 0.03 | 0.06 | 0.12 | 0.25 | 0.5  | 1    | 2    | 4    | 8    | 16   | 32   | 64   | 128  | 256  | 512  | 1024 | 2048 | 10.4 | 4.7  | 4.6  | 15.8 | 2.5  | 1.8  | 1.1  | 0.3  | 5.5  | 22.8 | 0.9  | 8.7  | 1.4  | 0.1  | 9.4  | 10.7 | 2.8  | 17.2 |
|---------------------------|-------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|
| ciprofloxacin             | 89.6  | 0.5  | 1.3  | 5.6  | 1.1  | 1.7  | 0.2  | 0.0  |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| cefpodoxime               | 9.5   | 56.1 | 26.5 | 3.1  | 0.3  | 4.4  |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| ceftriaxone               | 94.6  | 0.8  | 0.4  | 0.4  | 3.8  |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| ampicillin                | 5.2   | 32.0 | 43.2 | 3.6  | 0.0  | 15.8 |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| gentamicin                | 94.5  | 3.0  | 0.9  | 0.3  | 0.7  | 0.6  |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| amoxi/clav. acid          | 20.3  | 55.5 | 22.3 | 1.1  | 0.7  |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| chloramphenicol           | 2.0   | 25.3 | 67.3 | 4.3  | 0.4  | 0.7  |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| florfenicol               | 3.8   | 42.6 | 49.3 | 4.0  | 0.3  | 0.0  |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| neomycin                  | 90.6  | 3.4  | 0.5  | 0.4  | 5.1  |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| tetracycline              | 73.8  | 2.9  | 0.5  | 0.0  | 22.8 |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| colistin                  | 0.0   | 0.9  | 0.0  |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| streptomycin              | 66.9  | 20.5 | 3.4  | 1.8  | 6.9  |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| apramycin                 | 83.4  | 13.3 | 1.9  | 1.4  |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| cefalothin                | 24.6  | 50.1 | 18.1 | 7.1  |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| trimethoprim              | 90.2  | 0.5  | 0.5  | 8.9  |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| nalidixic acid            | 87.4  | 1.5  | 0.4  | 0.4  | 10.3 |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| spectinomycin             | 68.8  | 22.5 | 5.9  | 1.5  | 1.3  |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| sulfamethoxazole          | 81.1  | 0.8  | 0.2  | 17.0 |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
Statistical data analysis

The Pearson’s $\chi^2$-test was used to determine significant differences between categorical variables ($P < 0.05$), such as the presence of other animal production on the farm and the treatment status of the flock. An isolate was defined as multiple drug resistant (MDR) if it exhibited resistance to 2 or more antimicrobials. For both indicator bacteria, MDR was used as an outcome variable in the statistical analysis. For *E. coli*, ceftiofur-resistance was also used as an outcome variable. The exploratory factors, consisting of country, housing type, presence of other farm animals (pigs, cattle, sheep…) on farm, the presence of hens in the flock originating from different rearing sites and antimicrobial treatment of the flock were tested for inclusion in the models. To identify influential factors a stepwise forward selection process was used for the variable selection in a population average logistic regression model with a $p$-value $\leq 0.2$ for entry, and with a $p$-value $\leq 0.10$ for retention in model. The factors that were significant in this model were introduced into a model using generalized estimating equations (GEE) to control for clustering of samples within a farm using an independent correlation matrix. Interaction effects were tested for variables retained in the GEE model. Odds ratios and 95% confidence intervals were calculated for the parameters that were retained in the GEE model using the criteria of $p$-value $\leq 0.05$ for retention in the GEE model. The statistical software package SAS (SAS for Windows, version 9.1, SAS Institute Inc, Cary, NC, USA) was used for data analysis.

Antimicrobial resistance patterns were described using cluster analysis. Binary cluster analysis was performed using the Jaccard matching coefficient and the centroid method to obtain discrete clusters with no intra-cluster variability. Due to the large number of antimicrobial resistance clusters, the descriptive and stratified analysis was limited to the 15 most common resistance patterns describing more than 80% of the isolate data set.
Table 2: Minimum Inhibitory Concentration distribution (%) for all *Enterococcus faecalis* isolates (vertical black line indicates cut-off value)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Distribution (%) of MIC's (µg/ml)</th>
<th>Total % resistance</th>
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<td>0.015</td>
<td>0.03</td>
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<td>daptomycin</td>
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<td>0.4</td>
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<td>ciprofloxacin</td>
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<td>chloramphenicol</td>
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<td>streptomycin</td>
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<td>1.6</td>
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</table>
**Results**

In total, 1102 *E. coli* isolates and 792 *E. faecalis* isolates were collected from 283 laying hen flocks (69 Belgian, 85 German, 30 Italian and 99 Swiss flocks). The participation rate was more than 90% in Belgium, Italy and Switzerland and 70% in Germany. A detailed description of the number of isolates per housing type and per country is presented in Table 3.

Twenty-four of the 283 sampled flocks were treated with antimicrobials according to the declaration of the farmers. The number of treated flocks differed significantly between countries (*P* < 0.05) but not between housing types. In Italy none of the sampled flocks were treated with antimicrobials, for Belgium this was 3 of the 69 sampled flocks, for Switzerland 5 of the 99 flocks and for Germany 16 of the 85 sampled flocks. Colistin was the most frequent treatment (16 flocks), followed by amoxicillin (5 flocks), neomycin (2 flocks) and enrofloxacin (1 flock). The antimicrobial treatments per flock and per housing type are presented in Table 4. There was a significant difference between countries in the number of laying hen farms where also other production animals were kept (*P* < 0.05). In Italy, only 16.7% of the sampled laying hen farms managed other animal production on the same site, whereas in Germany, Belgium and Switzerland this was 38.5%, 43.8% and 75.5% respectively.
<table>
<thead>
<tr>
<th></th>
<th>Belgium</th>
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<th>Italy</th>
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<th>Switzerland</th>
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<td>No. of flocks</td>
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<td>No. of isolates</td>
<td>No. of flocks</td>
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<td>-</td>
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<td>98</td>
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<td>64</td>
<td>21</td>
<td>92</td>
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<td>65</td>
<td>-</td>
<td>-</td>
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<td>32</td>
<td>14</td>
<td>58</td>
<td>-</td>
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<td>Total</td>
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<td><strong>256</strong></td>
<td><strong>85</strong></td>
<td><strong>356</strong></td>
<td><strong>30</strong></td>
<td><strong>119</strong></td>
<td><strong>99</strong></td>
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<tr>
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<td>E. faecalis</td>
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<td><strong>236</strong></td>
<td><strong>85</strong></td>
<td><strong>323</strong></td>
<td><strong>30</strong></td>
<td><strong>95</strong></td>
<td><strong>99</strong></td>
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</table>
The distribution of the MIC’s (in %) of each antimicrobial is described in Table 1 for *E. coli* and Table 2 for *E. faecalis*. The isolates were grouped according to their multi-resistance patterns, resulting in 120 and 94 antimicrobial resistance (AR)-clusters for *E. coli* and *E. faecalis* respectively. Due to space limitations, only the top 15 clusters for each bacterial species are shown (complete descriptions of patterns can be obtained from corresponding author upon request).

*Escherichia coli*

The majority of the isolates (55.0 %) were susceptible to all 18 antimicrobials, 16.9 % were resistant to 1 antimicrobial and the remaining 28.1 % were multi-resistant. Only to tetracycline (22.8 %), sulfamethoxazole (17.2 %), ampicillin (15.8 %), ciprofloxacin (12.3 %) and nalidixic acid (10.7 %), more than 10 % of all isolates showed resistance. The 15 most common resistance phenotypes of AR-clusters are described in Table 5. These clusters included 81.8 % of the isolates.

**Table 4**: Antimicrobial treatments in 292 European laying hen flocks

<table>
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<tr>
<th>Country</th>
<th>Housing system</th>
<th>Antimicrobial agent</th>
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<tbody>
<tr>
<td>Belgium</td>
<td>Conventional battery cage</td>
<td>colistin</td>
</tr>
<tr>
<td>Belgium</td>
<td>Floor-raised</td>
<td>colistin</td>
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<td>Free-range</td>
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<td>Germany</td>
<td>Conventional battery cage</td>
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<td>colistin</td>
</tr>
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<td>Germany</td>
<td>Conventional battery cage</td>
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<tr>
<td>Switzerland</td>
<td>Floor-raised</td>
<td>enrofloxacin</td>
</tr>
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</table>
Table 5: The 15 most common antimicrobial resistance clusters in *E. coli* isolated from European laying hens

<table>
<thead>
<tr>
<th>AR-cluster (^1)</th>
<th>No. of isolates</th>
<th>%</th>
<th>AR-Freq (^2)</th>
<th>AMC</th>
<th>AMP</th>
<th>APR</th>
<th>CEF</th>
<th>CEP</th>
<th>CHL</th>
<th>CIP</th>
<th>COL</th>
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<th>NEO</th>
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</tbody>
</table>

| Sum \(^3\)         | 900            | 81.8 |


\(^1\) AR-cluster = Antimicrobial Resistance cluster describing the pattern of resistance of the isolates

\(^2\) AR-Freq = Antimicrobial Resistance frequency: the number of antimicrobials to which the *E. coli* were classified as resistant (R)

\(^3\) The number and percentage of isolates described in this table, representing 81.8 % of the 1102 total isolates included in this study
The housing of hens in floor-raised systems, compared to conventional battery cages ($P = 0.02$) and the country ($P = 0.03$) turned out to be risk factors for higher levels of MDR in the final GEE logistic regression model (Table 6). Other factors such as other animal production on the farm, the presence of hens originating from different rearing farms in the sampled flock and antimicrobial treatment of the flock were not retained in the final model.

Table 6: Results of the GEE logistic regression analysis for the identification of risk factors for the presence of multiple drug resistance in *Escherichia coli* from European laying hens.

<table>
<thead>
<tr>
<th>Categorical variable</th>
<th>N of isolates</th>
<th>OR</th>
<th>95 % CI</th>
<th>$P$-value</th>
</tr>
</thead>
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<td><strong>Type of housing</strong></td>
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</tr>
<tr>
<td>Conventional battery (ref)</td>
<td>217</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Aviary</td>
<td>142</td>
<td>0.87</td>
<td>0.90-2.26</td>
<td>0.77</td>
</tr>
<tr>
<td>Floor-raised</td>
<td>284</td>
<td>2.12</td>
<td>1.13-3.97</td>
<td>0.02</td>
</tr>
<tr>
<td>Free-range</td>
<td>309</td>
<td>0.84</td>
<td>0.42-1.68</td>
<td>0.62</td>
</tr>
<tr>
<td>Free-range organic</td>
<td>150</td>
<td>1.02</td>
<td>0.45-2.33</td>
<td>0.96</td>
</tr>
<tr>
<td><strong>Country</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Belgium (ref)</td>
<td>256</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Germany</td>
<td>356</td>
<td>0.54</td>
<td>0.31-0.93</td>
<td>0.03</td>
</tr>
<tr>
<td>Italy</td>
<td>119</td>
<td>0.73</td>
<td>0.38-1.42</td>
<td>0.36</td>
</tr>
<tr>
<td>Switzerland</td>
<td>371</td>
<td>0.86</td>
<td>0.48-1.60</td>
<td>0.67</td>
</tr>
</tbody>
</table>

When looking at factors affecting ceftiofur resistance in *E. coli* (Table 7), Belgium was more likely to have ceftiofur-resistant isolates than the three other countries. Ceftiofur-resistance varied from 0.0 % (Switzerland), over 2.5 and 4.5 % (Italy and Germany), to 12.1 % in Belgium. No significant potential association between the other tested risk factors (housing system, other animal production on the same farm and hens originating from different rearing farms) was observed.

Table 7: Results of the GEE logistic regression analysis for the identification of risk factors for the presence of ceftiofur resistance in *Escherichia coli* from European laying hens.

<table>
<thead>
<tr>
<th>Categorical variable</th>
<th>N of isolates</th>
<th>OR</th>
<th>95 % CI</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Country</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Belgium (ref)</td>
<td>256</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Germany</td>
<td>356</td>
<td>0.72</td>
<td>0.42-1.24</td>
<td>0.24</td>
</tr>
<tr>
<td>Italy</td>
<td>119</td>
<td>0.62</td>
<td>0.29-1.32</td>
<td>0.21</td>
</tr>
<tr>
<td>Switzerland</td>
<td>371</td>
<td>0.40</td>
<td>0.21-0.76</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>
**Enterococcus faecalis**

The majority of *E. faecalis* isolates were multiresistant (51.1%), 34.5% were resistant to 1 antimicrobial and only 14.4% of all isolates were pan-susceptible. Resistance to tetracycline (68.6%) was most common, followed by erythromycin (35.5%), tigecyclin (26.1%), streptomycin (23.5%) and kanamycin (16.4%). The 15 most common resistance phenotypes of AR-clusters representing 81.2% of the dataset are described in Table 9.

The results of the GEE logistic regression model, showing factors associated with MDR in *E. faecalis* are presented in Table 8. The isolates from laying hens housed in free-range systems were more likely to have lower levels of MDR (*P* = 0.03) compared to conventional battery cage systems. Isolates from Belgian hens had lower levels of resistance than hens in Germany and Italy. Similar to the observations in *E. coli*, other factors such as other animal production on the farm, antimicrobial treatment of the flock and the presence of hens originating from different rearing plants in the flock did not significantly interact with the levels of MDR.

**Table 8:** Results of the GEE logistic regression analysis for the identification of risk factors for the presence of multiple drug resistance in *Enterococcus faecalis* from European laying hens.

<table>
<thead>
<tr>
<th>Categorical variable</th>
<th>N of isolates</th>
<th>OR</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Type of housing</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conventional battery (ref)</td>
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<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Aviary</td>
<td>50</td>
<td>1.13</td>
<td>0.39-3.28</td>
<td>0.83</td>
</tr>
<tr>
<td>Floor-raised</td>
<td>207</td>
<td>0.95</td>
<td>0.52-1.72</td>
<td>0.85</td>
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<tr>
<td>Free-range</td>
<td>214</td>
<td>0.51</td>
<td>0.27-0.94</td>
<td>0.03</td>
</tr>
<tr>
<td>Free-range organic</td>
<td>121</td>
<td>0.57</td>
<td>0.28-1.18</td>
<td>0.13</td>
</tr>
<tr>
<td><strong>Country</strong></td>
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<tr>
<td>Belgium (ref)</td>
<td>236</td>
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<td>-</td>
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</tr>
<tr>
<td>Germany</td>
<td>323</td>
<td>2.67</td>
<td>1.57-4.55</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Italy</td>
<td>95</td>
<td>13.07</td>
<td>5.69-30.00</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Switzerland</td>
<td>149</td>
<td>1.86</td>
<td>0.91-3.79</td>
<td>0.09</td>
</tr>
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</table>
Table 9: The 15 most common antimicrobial resistance clusters in *E. faecalis* isolated from European laying hens

<table>
<thead>
<tr>
<th>AR-cluster</th>
<th>No. of isolates</th>
<th>%</th>
<th>AR-Freq²</th>
<th>AMP</th>
<th>AVI</th>
<th>CHL</th>
<th>CIP</th>
<th>DAP</th>
<th>ERY</th>
<th>FLO</th>
<th>GEN</th>
<th>KAN</th>
<th>LIN</th>
<th>SAL</th>
<th>STR</th>
<th>TET</th>
<th>TIG</th>
<th>VAN</th>
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<tbody>
<tr>
<td>1</td>
<td>138</td>
<td>15.1</td>
<td>1</td>
<td>.</td>
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<tr>
<td>2</td>
<td>116</td>
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<td>0</td>
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<td>3</td>
<td>89</td>
<td>11.1</td>
<td>1</td>
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<td>R</td>
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<td>4</td>
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<td>6</td>
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<td>R</td>
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<td>8</td>
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<td>3.5</td>
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<tr>
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<td>11</td>
<td>13</td>
<td>2.1</td>
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<td>1.6</td>
<td>3</td>
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<td>13</td>
<td>7</td>
<td>0.9</td>
<td>3</td>
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<td>R</td>
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<td>.</td>
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<td>R</td>
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<tr>
<td>14</td>
<td>7</td>
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<td>2</td>
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<td>.</td>
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<td>.</td>
<td>.</td>
<td>.</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
</tbody>
</table>

Sum³ | 664 | 81.2


¹AR-cluster = Antimicrobial Resistance cluster describing the pattern of resistance of the isolates

²AR-Freq = Antimicrobial Resistance frequency: the number of antimicrobials to which the *E. faecalis* were classified as resistant (R)

³The number and percentage of isolates described in this table, representing 81.2 % of the 803 total isolates included in this study
DISCUSSION

In this study, the alternative non-cage production systems for laying hens did not show consistent results with regard to MDR in *E. coli* and *E. faecalis*. The levels of MDR in *E. faecalis* were lower in free-range laying hens than in the conventional battery cage system, whereas increased levels of MDR were seen in *E. coli* in floor-raised hens. This is different from the studies of Schwaiger et al. (2008; 2010) who found significantly lower levels of antimicrobial resistance in *E. coli* and faecal enterococci in organic laying hens compared to laying hens housed in conventional battery cages.

There are several possible explanations for the ambiguous association between the non-cage housing types on the level of MDR. A first important factor is the exposure to antimicrobials. In the current study, apart from the free-range organic flocks, the reported antimicrobial use in laying hens in the alternative systems was not significantly lower than in conventional battery cages. Maybe the higher incidence of bacterial diseases in laying hens housed in alternative non-cage systems that has been described in several studies (Fossum et al., 2009; Kaufmann-Bart and Hoop, 2009) may have resulted in a more frequent use of antimicrobials. If this would be the case, it raises the question whether, besides the advantages on the animal-welfare level, in the future there won’t be any adverse consequences for public health on the level of the spread and persistence of antimicrobial resistance in laying hens. Secondly, in non-cage systems the chance of oro-faecal transmission of bacteria is much higher than in conventional battery cages, both between hens and between the animals and the environment. This could also be of importance since, apart from antimicrobial usage, other factors such as the localization and size of the microbial population (van den Bogaard and Stobberingh, 1999), immunity and contact intensity of the host (Walson et al., 2001) play a role in antimicrobial resistance development. Finally, the fact that many of the sampled farms with non-cage production systems made the move to the new production systems only a few years before the onset of the study and that these new production systems are often located in the same house where previously the battery cages were present, might also explain the observed results. It has been described that the decrease in antimicrobial resistance after changes in the production system or in the antimicrobial usage policy in food producing animals is very slow and that resistance against certain antimicrobials can be detected even long after direct selection pressure by using antimicrobials came to an end (Dunlop et al., 1998; De Gelder et al., 2004).
The difference in effect of the housing system on MDR in \textit{E. coli} and \textit{E. faecalis} might be the result of different biological characteristics of both bacterial species. \textit{E. coli} is typical an inhabitant of the intestinal tract and is to a considerable extent present in the hens’ faeces (Huang et al., 2009). The animals have frequent oro-faecal contacts in non-cage indoor production systems, resulting in an intense exchange of \textit{E. coli} between hens. This high contact intensity could cause the higher levels of MDR in laying hens in floor-raised systems. Since enterococci are widely distributed in the soil and the environment (Aarestrup, 2002), the access to a pasture may exert some kind of diluting effect on the intestinal enterococcal population, leading to lower levels of MDR in free-range laying hens. However, although these differences between the housing types are statistically significant, it is not yet clear whether they have some biological relevance and therefore further study is necessary to confirm and clarify these findings.

Although differences in methodology of sampling and analysis between different studies have to be taken into account, the results of this study illustrate that, in general, the levels of antimicrobial resistance in indicator bacteria in laying hens are relatively low compared to broilers, pigs and –to a lesser extent– cattle (Bywater et al., 2004; de Jong et al., 2009; Persoons et al., 2009). This is in accordance with previous studies in laying hens (Schwaiger et al., 2008; 2010; Kojima et al., 2009). The overall limited antimicrobial usage in egg-producing laying hens, as shown in the results, could play a role in this observation since it is generally accepted that the use of antimicrobials is one of the major risk factors for the development and spread of antimicrobial resistance (Wegener et al., 1999; van den Bogaard et al., 2001; Johnsen et al., 2009). A reason for the lower levels of resistance seen in the layers compared to broilers may be that the layers in this study were sampled on average at 74 weeks of age, compared to broilers that are usually 6 weeks at the time of slaughter. It has been described indeed for several animal species that antimicrobial resistance levels decrease with increasing age (Langlois et al., 1988; Butaye et al., 1998). From this point of view, it would be very interesting to carry out longitudinal studies to see the interaction between antimicrobial resistance and the age of the hens and to monitor the use of antimicrobials during the rearing of the pullets.

The fact that the samples were analyzed in different labs in different countries may have slightly influenced the results, despite the equivalent methodology used in all 4 participating countries. However, the observed differences in MDR levels between countries, probably also result from regional differences in animal husbandry and antimicrobial usage.
and from the fact that the distribution of the housing system of the sampled farms was not the same in each country. A remarkable finding in this respect is the ceftiofur resistance in *E. coli* in Belgium. Whereas in Germany, Italy and Switzerland only very low levels of ceftiofur resistance were found, in Belgium this resistance was 12.1% in *E. coli*. Consequentially the odds to have resistance against ceftiofur were higher in Belgium compared to the other countries, although only the difference with Switzerland was significant. This study result coincides with recent findings of high levels of ceftiofur resistance in broilers in Belgium (Persoons et al., 2009). It has recently been stated by several authors that one of the reasons for the increasing levels of ceftiofur resistance may be the worldwide and systematic use of ceftiofur in breeding eggs and one-day-old chicks in the hatcheries (Collignon and Aarestrup, 2007; CMAJ, 2009; Webster, 2009). In Belgium the use of ceftiofur in poultry has not been licensed for a decade (Bertrand et al., 2006) but might be continued off-label (Persoons et al., 2009). This is an additional argument to include laying hens in antimicrobial resistance monitoring programs. Also monitoring of the use of antimicrobials during the rearing of the pullets needs attention since it has been described for pigs and poultry that the younger animals exhibit a higher prevalence of resistance than the full-grown animals (Langlois et al., 1988; Butaye et al., 1998). To our knowledge, data on antimicrobial use and antimicrobial resistance in pullets are extremely scarce.

Another hypothesis for the increased ceftiofur resistance might be the production on the same farm of other animal species, in which the use of ceftiofur is still registered. This could result in horizontal transmission of resistance genes between bacterial populations of different animal species (Kruse and Sorum, 1994; Lorenz and Wackernaegel, 1994). However, the number of mixed-production farms in Belgium did not significantly differ from the situation in Germany and was even less than in Switzerland. Possibly the high density of farms in the northern part of Belgium, where 90% of all animal production is situated, may enhance contact between the bacterial populations of different ecological systems, for example surface water, leading to an efficient horizontal spread of antimicrobial resistance. For humans, it has been demonstrated that a higher population density enhances the development and spread of antimicrobial resistance (Garau et al., 1999; Bruinsma et al., 2003). Further eco-epidemiological studies are needed to elucidate this possibility.
CONCLUSION

The results of this study suggest that the levels of antimicrobial resistance in indicator bacteria such as *E. coli* and *E. faecalis* in laying hen flocks are relatively low. The differences observed between both indicator bacteria with respect to the potential association between the housing system and MDR suggest that it is important to not focus on a sole bacterial species when trying to assess risk factors for antimicrobial resistance. It is crucial to conscientiously monitor the prevalence and evolution in time of antimicrobial resistance in laying hens, both during the rearing period and the production cycle, to be able to detect early changes in antimicrobial resistance and to minimize the spread of resistant bacteria to humans. Therefore it is recommended to carry out detailed epidemiological studies in the field and under experimental conditions on a broader spectrum of indicator bacteria.

ACKNOWLEDGEMENTS

This research was funded by the EU FP6, under the contract 035547 (Safehouse project). The authors thank all farmers for their permission to sample their farm and all lab technicians at the different institutions for their skillful help during the bacteriological analyses.
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CHAPTER 7

GENERAL DISCUSSION
The scope of this thesis was to determine if, and to what extent, the move from battery cages to non-cage housing systems influences the prevalence of *Salmonella* and antimicrobial resistance in laying hens and to identify risk factors for the presence of *Salmonella* in laying hen flocks in different housing systems.

1. **Sampling of Laying Hen Flocks: Detecting the Shedding or the Carriage of *Salmonella*?**

   It has been described that a vaccinated flock is not guaranteed *Salmonella*-free, a finding which is corroborated by the results presented in chapter 3. In this study, 19 vaccinated laying hen flocks were extensively sampled at the end of the production cycle, both on-farm (5 pooled faeces samples, 40 cloacal swabs and 1 mixed dust sample) and after transport (cloacal swabs of 100 hens, caeca of 100 hens). All of the sampled farms were found negative for *Salmonella* according to the official monitoring program.

   The data of chapter 3 suggest that on farms which are ‘apparently *Salmonella*-free’, a relatively large proportion of the flocks may still carry the pathogen without shedding. Therefore, the numbers of infected flocks based on the official monitoring programs are most likely an underestimation of the true number of flocks in which *Salmonella* is still present.

   The actual public health risk presented by these positive flocks is very difficult to assess. The fact that *Salmonella* is still present in apparently negative flocks implies the risk of a renewed shedding of the pathogen, especially at moments of stress, leading to egg contamination. Shedding of the pathogen can cause surface contamination of the eggs, following oviposition (Gantois et al., 2009). Moreover, the pathogen can easily penetrate through cracked egg shells, leading to internal egg contamination (Braden, 2006).

   However, based on the observation that using the conventional sampling methodology no positive samples were found (no indication of active shedding) and only a limited number of shedders and carriers were found after submitting the birds to transport stress and intensive sampling, one can argue that the risk of egg contamination in these vaccinated flocks is probably very low because of the low infection pressure. It has been described that the lower the degree of environmental contamination, the lower the numbers of contaminated eggs produced by an infected flock (Henzler et al., 1998; Chemaly et al., 2009).

   Moreover, these results indicate that the sampling methodology as it is currently used in the *Salmonella* monitoring programs of several EU member states might not be accurate
enough to detect *Salmonella* in flocks where the bacterium is only shed in very small amounts, or not shed at all. It can be expected that in the forthcoming years these low-level *Salmonella* infections will more regularly occur because of measures such as mandatory vaccination and a more thorough sensibilization of the farmers.

As described in the general introduction of this thesis, the current monitoring programs are based on the collection of pooled faeces samples or overshoes. However, even the intensified on-farm sampling, combining bacteriological analysis of 40 cloaca swabs, 1 mixed dust sample and 5 pooled faeces samples, did not result in the detection of *Salmonella*. The absence of *Salmonella* in pooled faeces and cloacal swabs in these flocks can be attributed to several factors. The first one is the very low degree of shedding at the moment of sampling. This can be the result of the vaccination against *Salmonella* or it can simply be the result of the intermittent excretion of *Salmonella* by infected animals (Van Immerseel et al., 2004). Another factor possibly interfering with the detection of *Salmonella* in faeces is the acidification of the drinking water. A wide variety of products for *Salmonella* control are available, claiming a decrease in shedding of the bacterium in the treated laying hens (Van Immerseel et al., 2002). At the same time, it raises the question whether the inhibited shedding caused by the use of these additives does not lead to failure to detect infected flocks. In the study described in chapter 4, 75% of the sampled flocks and 5 out of 6 positive flocks were supplied with acids in the drinking water. All this can also be an explanation why the individual cloacal swabs taken on-farm did not seem to be appropriate to detect *Salmonella* in the flocks, which is in accordance with the results of Bichler et al. (1996) and Van Immerseel et al. (2004). Analyzing cloacal swabs of laying hens can give an indication of the number of shedding birds but it is not indicative of the number of hens carrying the pathogen.

The mixed dust sampling yielded no positive results although the collection and analysis has often been described as a suitable sample type to detect *Salmonella* in a laying hen house (Davies and Wray, 1996; Kwon et al., 2000; Davies and Breslin, 2003; Gast et al., 2004). Again, the low level of excretion in the sampled flocks could have played a role. Arnold et al. (2009) demonstrated that the sensitivity of dust samples increases when the within-flock prevalence of shedding birds, and thus the infection pressure, increases. Other factors that are thought to influence the efficiency of dust sampling are the stage of infection and the housing type.

The results of this thesis indicate that the post-mortem examination of hens (caecal and/or ovary/oviduct samples) is the most accurate test to determine the infection status of a
laying hen flock. This way of sampling also allows estimating the number of infected hens within a flock. However, the fact that it is an expensive and time-consuming technique makes it impossible to use it in routine monitoring programs. To circumvent this, a possible solution could be the post-mortem examination of culled hens in the slaughterhouse. In this way, data on the between- and within-flock prevalence could be obtained without any healthy hen killed during the production round. The sampling of culled hens in the slaughterhouse also has some disadvantages. First there is the risk of cross-contamination, not only between hens of the same flock but also between hens of different flocks or farms, both during transport and processing. This would lead to an over-estimation of the prevalence of Salmonella. Therefore, strict cleaning and disinfection measures are necessary. Also the risk of infection of laying hens during transport needs to be taken into account. Another major drawback of sampling culled hens is that the information on the Salmonella status of the flock is only obtained after the eggs are sold or processed. In this way, the information forming the base on which the public health risk presented by such flock is assessed, comes too late. This is one of the main differences between the control of Salmonella in laying hens and other food producing animals: in broilers and pigs the level of Salmonella contamination can be heavily influenced by the different processing steps (e.g. transport to the slaughterhouse, hygiene along the slaughter line, storage after processing...), whereas in the egg industry the prevalence of Salmonella contaminated eggs changes only very little between the time the eggs are laid and the consumption of the eggs. Therefore, Salmonella control in laying hens should be focused to an even larger extent at the level of the primary production, i.e. the laying hen farms.

In this respect, the combination of the on-farm sampling as it is currently performed and the testing of culled hens in the slaughterhouse would give the most complete information on the infection status of a flock. The shedding of Salmonella could be monitored using the on-farm sampling, the presence of hens carrying Salmonella could be detected in the slaughterhouse. Any positive results in the caecal or ovarian samples would imply that extra measures, for instance cleaning and disinfection in accordance with official guidebooks, must be taken before the house can be restocked with new pullets.

2. PREVALENCE OF SALMONELLA IN EUROPEAN LAYING HEN FLOCKS

In the cross-sectional field study carried out during this thesis (chapter 5), 29 of the 292 sampled flocks were found positive for Salmonella in at least one of the samples taken. The between flock prevalences differed significantly between the countries, ranging from 0.0 %
(Switzerland) to 30.0 % (Italy). The prevalence in each country with the 95 % CI is presented in Table 1. *Salmonella* Enteritidis is still the most commonly isolated serovar: in 21 of these 29 positive flocks *S.* Enteritidis could be found.

**Table 1: Prevalence of *Salmonella* in 292 European laying hen flocks**

<table>
<thead>
<tr>
<th>Country</th>
<th>No. of flocks</th>
<th>% pos</th>
<th>CI 95 %</th>
<th>% pos</th>
<th>CI 95%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belgium</td>
<td>69</td>
<td>1.4</td>
<td>0.0-3.7</td>
<td>1.4</td>
<td>0.0-3.7</td>
</tr>
<tr>
<td>Germany</td>
<td>84</td>
<td>20.0</td>
<td>11.7-28.7</td>
<td>20.0</td>
<td>11.7-28.7</td>
</tr>
<tr>
<td>Greece</td>
<td>10</td>
<td>20.0</td>
<td>0.0-44.6</td>
<td>0.0</td>
<td>0.0-2.4</td>
</tr>
<tr>
<td>Italy</td>
<td>30</td>
<td>30.0</td>
<td>13.8-46.2</td>
<td>13.3</td>
<td>1.3-25.3</td>
</tr>
<tr>
<td>Switzerland</td>
<td>99</td>
<td>0.0</td>
<td>0.0-3.7</td>
<td>0.0</td>
<td>0.0-3.7</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>292</strong></td>
<td><strong>9.9</strong></td>
<td><strong>6.7-13.1</strong></td>
<td><strong>7.5</strong></td>
<td><strong>4.7-10.3</strong></td>
</tr>
</tbody>
</table>

When comparing these results to the results of the EFSA baseline study, only in Belgium and Greece a significantly lower prevalence of *S.* Enteritidis and/or Typhimurium could be observed. For several reasons these findings should be interpreted with care. First of all, the farms in this study were not randomly sampled but they were selected based on the housing type. This will certainly have influenced the outcome of the study. The fact that conventional battery cage farms only accounted for 25 % of the sampled farms most probably lead to an under-estimation of the prevalence. The influence of the housing system on the presence of *Salmonella* in laying hens will be discussed further on in the section about the risk factors. Secondly, participation of the contacted farmers in this study was voluntarily, which could have caused bias. The participation rates for Belgium, Greece, Italy and Switzerland were > 90 % since absolute anonymity could be guaranteed to the participating farmers; therefore a positive result could not have any adverse consequences for the farmer. For Germany the participation rate was lower (70 %) since positive results had to be reported to the authorities. Furthermore the German farmers were awaiting the decision on the housing of laying hens in the so called German type of enriched cages. This uncertainty caused some reluctance to participate in this particular study. On the other hand, the fact that also red mites were collected for further research was a big stimulus for farmers to participate since these ectoparasites are of major concern for the farmers and are perceived much more as a problem than *Salmonella* is. Finally, the sampling scheme used in this study was different from the one of the EFSA baseline study. In our study 5 pooled faeces samples and 40 cloacal swabs were
collected from each flock. These samples are suitable for detecting the excretion of *Salmonella* but not for detecting carriers of *Salmonella*. In the EFSA baseline study 5 pooled faeces samples and 2 mixed dust samples were collected. It is thought that the sampling of dust permits the detection of *Salmonella* even when no hens are shedding at the moment of sampling: the bacterium’s capacity to resist long-term desiccation (Haysom and Sharp, 2003) makes that settled dust in laying hen houses can give more information on the past *Salmonella* status of the poultry house (Arnold et al., 2009).

For Belgium, it is remarkable that the prevalence of *S. Enteritidis* and/or Typhimurium was much lower in this study compared to the EFSA baseline study (1.43 % vs. 26.2 %). It has been stated that the large-scale vaccination of commercial laying hens since 2005, together with sanitary and prevention campaigns, has been of major importance for the decline of *S. Enteritidis* (Collard et al., 2008).

From this point of view, it may seem strange that vaccination against *Salmonella* did not turn out to be a significant protective factor when all 292 flocks sampled in the cross-sectional study were taken into account. After all, the commonly used vaccines claim to reduce the faecal shedding and systemic spread of *S. Enteritidis* and to reduce the reproductive tract colonization (Van Immerseel et al., 2004; Gantois et al., 2006). The fact that the protective effect of vaccination could not be confirmed in our study is due to the different vaccination policies and *Salmonella* prevalences in the participating countries. Therefore vaccination policy and country were strongly correlated and resulted in multicollinearity. Although in Belgium the vaccination of commercial laying hens became mandatory only in 2007, vaccination already occurred in large scale since 2005 (Collard et al., 2008). In Switzerland on the other hand, vaccination of laying hens against *Salmonella* is prohibited. In Germany, Greece and Italy vaccination occurred on a voluntary basis, explaining why in these three countries respectively 1.2 %, 0 % and 40 % of the sampled flocks were vaccinated. Together with the large national differences in *S. Enteritidis* and/or Typhimurium prevalence, no well-founded conclusion on the protective effect of vaccination could be drawn on the basis of the results of our cross-sectional study.
3. **Risk factors for Salmonella infection: Influence of the age of the production system and previous Salmonella shedding**

One of the aims of this thesis was to determine risk factors for an infection with *Salmonella* in vaccinated laying hen flocks, meaning that factors were identified that could explain why in some flocks the risk was higher to find *Salmonella*, even when there were no birds shedding the bacterium. The 2 variables which turned out to be significant were a) the age of the production system and b) previous *Salmonella* shedding on the farm. The housing system as such was not significantly associated with *Salmonella* infection.

**Age of the infrastructure**

For conventional battery farms the age of the infrastructure is the number of years the current cages are in use, for non-cage systems this is the number of years the current infrastructure (nest boxes, slats, perches...) has been installed. The effect of the age of the infrastructure may be explained by the fact that the older the infrastructure becomes, the more difficult it gets to achieve sufficient standards of cleaning due to the wear of the materials, both of the production system and of the building itself, especially when it is taken into account that the level of environmental contamination increases significantly during a production cycle (Wales et al., 2007). The importance of such latent environmental sources of infections should not be underestimated since Davies and Wray (1996) described the survival of *Salmonella* in empty poultry houses for a period of 12 months, which implies the risk of ‘transfer’ of an infection between successive production cycles. This leads us to the second significant risk factor: previous *Salmonella* shedding on the farm.

**Previous Salmonella shedding**

The odds to detect *Salmonella* are higher on farms where previous *Salmonella* shedding has occurred, i.e. where *Salmonella* was detected in pooled faeces samples or bootswabs collected in the framework of the official monitoring program. This is in accordance with the studies of Gradel et al. (2004) and Carrique-Mas et al. (2009), stating that the major part of *Salmonella*-infections on laying hen farms are not newly introduced on the farm but are the result of re-introduction of the pathogen from the farm’s environment.

The importance of sanitary measures is illustrated by the fact that absence of dry cleaning in between successive production rounds was found to be a significant risk factor for the presence of *Salmonella*. Dry cleaning is the mechanical removal of organic material
(manure, dust, feed spills...) before the wet cleaning (using water) is carried out. A remarkable observation in our field study was that only 82.2% of the 292 sampled farms carried out dry cleaning of the laying hen houses, whereas for wet cleaning and disinfection this was 95.5% and 92.5% respectively, with large differences between the countries. In Germany and Italy for instance more than 1/3 of the farms did not carry out dry cleaning between production rounds. This constitutes a risk since the value of dry cleaning in between production rounds is 2-fold: on one hand it is very useful to remove organic matter that can harbour *Salmonella*. On the other hand it contributes to a more efficient disinfection since the presence of considerable amounts of organic protective matter such as manure, dust and spilled feed has an adverse effect on the efficacy of disinfection (Davies and Breslin, 2003; Gradel et al., 2004).

These findings underline once again the necessity of sensibilization and informing farmers about the importance of continuing efforts to achieve and maintain high levels of biosecurity on a laying hen farm.

4. **RISK FACTORS FOR *Salmonella* SHEDDING: INFLUENCE OF THE HOUSING SYSTEM**

Already in 2002, the results of a retrospective epidemiological study of Mølbak and Neimann suggested that eggs derived from hens housed in conventional battery cages yielded a higher risk for infection of humans with *Salmonella* Enteritidis compared to eggs derived from hens housed in non-cage housing systems. This finding was corroborated by the results of the EFSA baseline study from 2004-2005, both at the Community level (EFSA, 2007) and at the level of the individual member states (Methner et al., 2006; Namata et al., 2008; Huneau-Salaün et al., 2009). Also the results of our cross-sectional study in 292 laying hen flocks identified the housing in conventional battery cages as a risk factor.

However, the observed influence of the housing type does not necessarily mean that there is a causal relationship between the housing system and the level of *Salmonella* excretion. On the contrary, it is more likely that the housing system is strongly entangled with several other production characteristics such as the farm and the flock size, the age of the building, previous *Salmonella* infections on the farm etc. Below, a number of laying hen husbandry characteristics that may be related to both the housing system and the probability of detecting *Salmonella* are discussed.
First of all, the number of flocks on the farm and number of hens in a flock are shown to be risk factors for *Salmonella* infections in laying hens, independently of housing type (Heuvelink et al., 1999; Mollenhorst et al., 2005; EFSA, 2007; Snow et al., 2007; Carrique-Mas et al., 2008; Huneau-Salaün et al., 2009). Several studies, including our cross-sectional study, have shown that conventional battery cage farms are in general larger farms, not only with more hens per flock but also with more flocks on the farm (EFSA, 2007; Carrique-Mas et al., 2008; Van Hoorebeke et al., 2010). This could be one of the confounding factors explaining why conventional battery cage farms are more frequently positive for *Salmonella* than non-cage housing systems. The presence of multiple flocks on one farm may enhance cross-contamination from one flock to another, especially when the different flocks and laying hen houses on the farm are linked through egg conveyor belts, feed pipes, passageways etc. (Carrique-Mas et al., 2008). Furthermore, as is often the case on farms with multiple flocks, not all the hens are of the same age. Multistage production has also been identified as a risk factor for *Salmonella* in laying hens (Mollenhorst et al., 2005; Wales et al., 2007; Carrique-Mas et al., 2008; Huneau-Salaün et al., 2009).

Another factor which could play a role is the higher stocking density in conventional battery cage housing compared to non-cage housing systems. For many infectious diseases in production animals it has been demonstrated that a higher stocking density increases the prevalence of disease and the ease of spread (Dewulf et al., 2007). With reference to *Salmonella*, it has been shown in pigs that higher stocking densities increase the risk of *Salmonella* infections (Funk et al., 2001; Nollet et al., 2004). However, to our knowledge no results on this parameter are available regarding *Salmonella* infections in laying hens. Maybe this is due to the fact that this parameter was never evaluated or that it has been studied but never had been found to be significantly influential. Possibly the high density of laying hens in conventional battery cages, and as a consequence the large volume of faeces and dust increases the incidence of *Salmonella* infections in this type of housing (Davies and Breslin, 2004). High stocking densities could also indirectly interact with *Salmonella* infections because of the stress caused. Yet, literature is not unequivocal on the influence of stocking density on stress in laying hens and the relationship between stress and different housing types (Craig et al., 1986; Koelkebeck et al., 1987; Davis et al., 2000).

The difference in ease to clean and disinfect the different housing systems is also an important factor. In contrast to broiler and finishing pigs houses, which are in general relatively simple structured, laying hen houses are notoriously difficult to clean and disinfect.
because of their intrinsically complicated structures (Wales et al., 2006). In particular conventional battery cage systems are considered to be extremely hard to clean and disinfect sufficiently because of the restricted access to cage interiors, feeders, egg belts, and so forth (Davies and Breslin, 2003; Carrique-Mas et al., 2009a). It is thought that the big advantage of non-cage systems is that in between production rounds the whole infrastructure (nest boxes, slats, perches, feeding troughs...) can be dismantled and thoroughly cleaned and disinfected, in contrast to batteries of cages which are never dismantled. However, based on the results of our cross-sectional study, 21.2 % of the farmers keeping laying hens in non-cage systems do not dismantle the infrastructure. This means that cleaning and disinfection of the sampled house is carried out when the nest boxes etc. are still installed. It may be questioned whether in such conditions a sufficient cleaning and disinfection of both the house and the infrastructure is possible.

It has been suggested that conventional battery cage housing presents a more attractive environment to pests because the laying hens can interfere less with their movements since the birds are restrained to cages (Carrique-Mas et al., 2009b). Because of their role as vectors in the transfer of *Salmonella*, various pests such as rodents, flies, wild birds etc. have been extensively discussed (Guard-Petter, 2001; Davies and Breslin, 2003; Kinde et al., 2005; Carrique-Mas et al., 2009a). In our study, the focus was on another widespread pest in laying hens’ houses: the poultry red mite (*Dermanyssus gallinae*).

Besides the direct physiological harmful effects on the hens, poultry red mites also present a serious threat to health of the animals (Kilpinen, 2005): since long its role as a vector for several diseases such as chicken pox virus, Newcastle virus, *Salmonella Pullorum/Gallinarum* has been recognized (Zeman et al., 1982). The poultry red mite’s role in *Salmonella* infections in laying hens is three-fold. Firstly, mass red mite infestations can lead to immunosuppression (Kowalski and Kosol, 2009), increasing the hen’s susceptibility for infections. Secondly, the presence of large numbers of red mites causes agitation and stress. Finally, it has been reported under experimental conditions that mites could play a role in the persistence of *Salmonella* in laying hens, either by transferring the bacterium from hen to hen or by hens consuming contaminated mites leading to a persisting infection (Valiente-Moro et al., 2007; Valiente-Moro et al., 2009). Information on the role of *Dermanyssus gallinae* as an active vector for *Salmonella* under field conditions was scarce. Therefore it was decided to include the bacteriological analysis of red mite samples in the cross-sectional field study.
In total, in 214 of the 292 sampled houses red mites were collected. Concerning the prevalence of red mites, no significant differences between the different housing types and the different countries were observed. In fact, in none of the red mite samples *Salmonella* was detected, not even in the flocks found positive for *Salmonella*. Whether this means that *Dermanyssus gallinae* plays no role at all in the spread of *Salmonella* in a laying hen flock is not yet clear. Further research is needed to elucidate this matter.

Finally, there is the influence of stress. Stress has been shown to have an immunosuppressive effect in laying hens (El-Lethey et al., 2003; Humphrey, 2006), which can have negative consequences with respect to *Salmonella* shedding. There are several moments in the laying hen’s life where the bird is subjected to stress: moving from the rearing site to the egg producing plant (Hughes et al., 1989), the onset of lay (Jones and Ambali, 1987; Humphrey, 2006), in the final stages of the production period, during thermal extremes (Thaxton et al., 1974; Marshally et al., 2004) or transportation to the slaughterhouse (Beuving and Vonder, 1978). Typical for laying hen husbandry is the practice of induced moulting. The effects on S. Enteritidis infections during moult have been extensively studied: moulted hens shed more S. Enteritidis in their eggs and faeces (Holt, 2003; Golden et al., 2008), have higher levels of internal organs colonization (Holt et al., 1995) and exhibit more pathology in the intestinal tract than non-moulted hens (Holt and Porter, 1992; Holt and Porter, 1993; Macri et al., 1997). Furthermore, moulting causes the recurrence of previous S. Enteritidis infections (Holt and Porter, 1993). There are some contradictory data on the influence of the housing type on the stress levels in laying hens. Some studies suggest that laying hens have less stress in conventional battery cages (Koelkebeck et al., 1986; Craig et al., 1996) whereas other authors state that hens housed in non-cage systems experience less stress (Hansen et al., 1993; Colson et al., 2008). With regard to the housing system, the age of the hens (Singh et al., 2009) and the breed of the hens could also play a role: certain hen breeds exhibit significantly higher stress responses when raised in deep litter versus free-range systems, compared to other breeds (Campo et al., 2008). Based on the few studies exploring the stress response of hens housed in different housing systems, no consistent conclusion on the influence of the housing system can be drawn.

All this suggests that the nature of this topic is very complex and that a multi-level approach is required to tackle the problem. In a first step it is necessary to control the shedding of *Salmonella*. The housing system has an influence on this: the housing of laying hens in conventional battery cages increases the risk of shedding *Salmonella*. In a further
stage, the *Salmonella* infection on the farm, i.e. the actual presence of the pathogen in the hens or the environment, will have to be controlled. This is much more difficult to achieve because other factors than the commonly known ones (e.g. housing in battery cages, larger flocks sizes, age of the hens…) seem to play a role. Strict and maintained biosecurity measures and an optimized management will be the key to success.

5. **ANTIMICROBIAL RESISTANCE IN INDICATOR BACTERIA IN LAYING HENS**

It is often believed that the role of laying hens in the transfer of antimicrobial resistance to humans is low compared to other animal species such as broilers, pigs and cattle. This is based on the fact that antimicrobial usage is generally low in laying hens and that eggs are thought not to be very suitable vectors for transfer of resistant bacteria to the consumer (Yoshimura et al., 2000; Musgrove et al., 2006). However, the consumption of eggs and meat of culled hens could pose a threat to public health if antimicrobial resistant bacteria would be present. Moreover, differentiation between laying hens and broilers in monitoring programs for poultry allows drawing conclusions on the selective pressure of antimicrobial usage.

Recent reports indicate that both in poultry and other animal species the move from conventional indoor production towards free-range and organic production exerted a beneficial effect on the levels of antimicrobial resistance in zoonotic and indicator bacteria (Avrain et al., 2003; Thakur and Gebreyes, 2005; Ray et al., 2006, Schwaiger et al., 2008; 2010, Young et al., 2009). On the other hand it has been shown that this move to non-cage housing systems for laying hens resulted in an increased incidence of particularly bacterial diseases (Fossum et al., 2009; Kaufmann-Bart and Hoop, 2009), which could potentially lead to increased antibiotic usage. Epidemiological data on antimicrobial resistance in laying hens in different housing systems are scarce. Therefore, the levels of antimicrobial resistance in 2 indicator bacteria in laying hens were determined in chapter 6. Special attention was paid to the influence of the housing system on antimicrobial resistance.

One of the most remarkable findings is the high prevalence of ceftiofur-resistant isolates in Belgium (12 %) compared to the results of Switzerland, Italy and Germany where 0 %, 3 % and 4 % of the isolates were resistant. Although the level of ceftiofur-resistance in Belgian laying hens is much lower than the levels observed in Belgian broilers (44 %) (Persoons et al., 2010), it is necessary to sort out what are the causes of the emergence and spread of the resistance against such a critically important antimicrobial.
Although the problem of increasing Extended Spectrum Beta Lactamases-resistance is not limited to Belgium, it is striking that in our study Belgian isolates showed significant higher levels of ceftiofur-resistance. One of the reasons for the increasing levels of ceftiofur resistance may be the worldwide and systematic use of ceftiofur in breeding eggs and one-day-old chicks in the hatcheries (Collignon and Aarestrup, 2007; CMAJ, 2009; Webster, 2009). In Belgium the use of ceftiofur in poultry has not been licensed for a decade (Bertrand et al., 2006) but might be continued off-label (Persoons et al., 2010). Another hypothesis for the increased ceftiofur resistance might be the production on the same farm of other animal species such as pigs and cattle, in which the use of ceftiofur is still registered. This could result in horizontal transmission of resistance genes between different bacterial species (Kruse and Sorum, 1994; Lorenz and Wackernaegel, 1994).

Concerning the influence of the housing system, the non-cage production systems for laying hens did not show consistent results with regard to MDR in E. coli and E. faecalis. The levels of MDR in E. faecalis were lower in free-range laying hens than in the conventional battery cage system, whereas increased levels of MDR were seen in E. coli in floor-raised hens.

Although the microbiological integrity of eggs still seems to be the most urgent food safety aspect of laying hen husbandry, this study indicates that the issue of antimicrobial resistance in laying hens should not be neglected. Our results provide an interesting snapshot of the situation in laying hens. However, the limited amount of data makes it very hard to assess the size of the problem, to evaluate the evolution in time and to take corrective steps. Therefore, it would be very useful to include laying hens as a separate production entity in resistance monitoring programs. Since laying hens can be considered as a reference population with rare antimicrobial usage, continuous resistance monitoring in laying hens could serve as a useful tool for the detection of trends in the distribution of antimicrobial resistance. To maximize the impact and value of such programs, standardization of protocols and reporting in all member states is necessary (Wallmann, 2006; WHO, 2008; EFSA, 2008). This would guarantee a more focused and generalized approach of the problem.

One of the cornerstones of such control programs should be the strict registration of the amounts of antibiotics used in agriculture. Indeed, the curative, prophylactic and metaphylactic use of large amounts of antimicrobials is common practice in modern husbandry of several food animal species (Aarestrup, 2004; EFSA, 2008) and concerns have
grown that this usage raised antimicrobial resistance in animals and humans (Wegener et al., 1999; Aarestrup, 2005; Acar and Moulin, 2006; Aminov and Mackie, 2007). Registration of the amounts and types of antibiotics used and the epidemiological analysis of the relationship between antimicrobial usage and the emergence and spread of resistance would allow to further evaluate this link between antimicrobial usage and the prevalence of antimicrobial resistance.

Even though it certainly has played a huge role in the development and spread of antimicrobial resistance in animals and humans, the debate should not be narrowed down to a yes-or-no argument on the –abundant– use of antibiotics in food animal production. There is little doubt that the whole issue of antimicrobial resistance is more complex than that and that factors such as the use of antibiotics in humans (Cizman et al., 2001), the spread of resistance from companion animals to humans (Canton and Coque, 2006; Skurnik et al., 2006) and the presence of resistant flora in diverse eco-systems such as surface water (Acar and Moulin, 2006; Moore et al., 2006; Servais and Passerat, 2009) also contribute to the complexity of the problem. Further epidemiological and microbiological research is needed to better understand the spread and mechanisms of antimicrobial resistance.

6. **Specific targets for future research**

This thesis has provided some useful information on the presence of *Salmonella* and antimicrobial resistance on laying hen farms and the associated risk factors, with special emphasis on the influence of the housing system. However, changing legislation and increasingly critical consumers’ demands will require continuous efforts and adaptations of the laying hen sector. Scientific research can be very useful in this process, identifying factors to fine tune and optimize the management of the farms and to provide guidelines for policy makers.

Until now, the focus of control programs in laying hens has been on *S. Enteritidis* and *S. Typhimurium*. When in the foreseeable future a zero-prevalence of all *Salmonella*-serovars may be required, additional or different measures will have to be taken. In order to increase the efficiency of such measures, more epidemiological data on the prevalence of these serovars and the risk factors associated with their presence must be available.

The strict monitoring of the prevalence of *Salmonella* in different housing systems must continue. Further studies must be carried out to determine which sampling protocol is
sensitive enough to provide accurate estimates of the prevalence in vaccinated flocks with low levels of infection.

Although the antimicrobial resistance issue seems less outspoken in laying hens compared to broilers, pigs and cattle, it would be useful to include laying hens in antimicrobial resistance monitoring programs in order to assess the size of the problem and to monitor the evolution in time. Special attention should be paid to the prevalence and spread of β-lactam resistance in laying hens.

7. CONCLUDING REMARKS

Based on the results of the studies carried out during the current thesis, it is most unlikely that the move from conventional battery cages towards non-cage housing systems will increase the prevalence of *Salmonella* in laying hen flocks. However, it would be very naïve to state that the ban on battery cages will be the answer to the well-known egg-related *Salmonella* problem in public health. The apparent advantage of non-cage systems is most probably influenced and biased by other factors such as the age of the infrastructure, flock size and sanitary conditions.

Our results from the extensively sampled flocks clearly indicate that in a considerable proportion of these vaccinated flocks *Salmonella* is still present in the hens, regardless of the housing type. These findings suggest that if the decision would be taken to stop the mandatory vaccination of commercial laying hens, the prevalence of *Salmonella* could rapidly increase again, also in the non-cage housing systems.

Furthermore, there is the issue of the *Salmonella* serovars different from Enteritidis and Typhimurium. The presence of such serovars does not seem to be influenced by the housing type and vaccination strategy, suggesting that the control of these serovars will be completely dependent on biosecurity and management strategies. This raises the question whether the commonly accepted measures to control *Salmonella* will be adequate enough to counter all serovars of *Salmonella*.

Concerning the antimicrobial resistance in laying hens, no consistent conclusion on the influence of the housing system could be drawn for *Escherichia coli* and *Enterococcus faecalis*. Compared to battery cages, the levels of multiple drug resistance were higher for *E. coli* in floor-raised systems and lower for *E. faecalis* in free-range systems. Although these
differences are statistically significant, it is not yet clear whether they have some biological relevance.
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The housing of laying hens in conventional battery cages will be forbidden in the EU from 1 January 2012 onwards. Council Directive 1999/74/EC states that from then onwards the housing must be restricted to enriched cages and non-cage housing systems. This ban on conventional battery cages aims to improve the welfare of laying hens. Although the influences of these alternatives for conventional battery cages on laying hen welfare, productivity and user-friendliness have been extensively evaluated and discussed, it has also initiated the question whether there are any adverse consequences of this decision on the spread and/or persistence of infectious diseases in a flock. For instance, the intensive and frequent hen-to-hen contacts and the presence of litter in non-cage systems jeopardize the biosecurity and increase the risk for specific diseases to develop and spread. This has led to the question whether the same effect is to be expected for zoonotic pathogens.

One of these zoonotic agents is Salmonella, which is worldwide still an important cause of human disease. In Europe, Salmonella Enteritidis and Salmonella Typhimurium are the most commonly isolated serovars in human cases of salmonellosis and contaminated eggs still remain the most important source of infection with Salmonella Enteritidis for humans.

Apart from the microbiological integrity of the eggs, the emergence and spread to humans of antimicrobial resistance through the consumption of food from animal origin plays a very important role (CHAPTER 1). The aims of this thesis were to determine the influence of laying hen housing systems on the prevalence of Salmonella and antimicrobial resistance in laying hens (CHAPTER 2).

In CHAPTER 3, the results of a study to determine whether the current sampling protocols (i.e. a limited number of pooled faeces and/or dust samples) are sufficiently sensitive to detect expected low within-flock prevalences of Salmonella are presented. In total, 19 flocks were sampled. All the sampled flocks were found negative for Salmonella based on the results from the official Salmonella control program. Using an extended on-farm sampling methodology (5 pooled faeces samples, 40 cloacal swabs of 40 hens and 1 mixed dust sample), Salmonella could not be detected in any of the flocks. After transportation of the hens to the laboratory and subsequent analysis of cloacal swabs and caecal contents, Salmonella Enteritidis was detected in laying hens from 5 out of 19 farms. The observed within-flock prevalence, based on the cloacal swabs, ranged from 1 – 14%. The differences between the samples taken on-farm and after transport may be explained by the intermittent excretion of Salmonella by infected animals and the fact that stress, caused by the transport, may make hens go from a ‘carrier’ state to a ‘shedding’ state. These results indicate that depending on
the sampling procedure different estimates of the between- and within-flock prevalence of Salmonella can be obtained. Although postmortem examination of hens is labour-intensive and expensive, the results of this study suggest that it is the best method for the detection of Salmonella in low prevalence flocks.

Risk factors for Salmonella infections in laying hen flocks were identified in CHAPTER 4. For this purpose 29 laying hen flocks, including farms using conventional and alternative housing systems, were intensively sampled both on-farm and after transport as described in chapter 3. An on-farm questionnaire was used to collect information on general farm and flock characteristics (e.g. flock size, breed, age of the hens, medical treatments...) and biosecurity measures. Salmonella was detected in laying hens from 6 of the 29 sampled farms. A previous Salmonella contamination on the farm and the age of the production system (the number of years that the current infrastructure is in use) were identified as risk factors for Salmonella infections in laying hens. The results of this study indicate that, irrespective of the housing system in which the hens are housed, persistent biosecurity measures are necessary in order to prevent the recurrent contamination or new infections of laying hen flocks in subsequent production cycles.

In the next study (CHAPTER 5), the aims were to determine the between- and within-flock prevalence of Salmonella spp. in laying hen flocks housed in conventional battery cage and alternative non-cage housing systems and to identify risk factors for the shedding of Salmonella on laying hen farms. Therefore, a cross-sectional study was carried out in 5 European countries (Belgium, Germany, Greece, Italy and Switzerland). In total 292 laying hen flocks were sampled in the month prior to depopulation. The on-farm questionnaire described in chapter 4 was used to collect information on general management practices and specific characteristics of the sampled flock. Twenty-nine flocks were found positive for at least 1 Salmonella-serotype. In these flocks the within flock prevalence of shedding hens, determined by individual sampling of 40 hens, varied between 0 % and 27.50 %. A wide variety of serotypes was isolated with Salmonella Enteritidis as the most common. Housing in conventional battery cages, the absence of dry cleaning in between production rounds and sampling in winter turned out to be risk factors for the shedding of Salmonella Enteritidis or Typhimurium. The results of this study illustrate that, despite the fact that in non-cage housing systems the chance of oro-faecal transmission of Salmonella is much higher than in conventional battery cage systems, no higher prevalence of Salmonella could be observed in flocks housed in these alternative systems. Several management and biosecurity measures
such as strict cleaning and disinfection practices have been identified as protective factors to minimize the introduction and persistence of *Salmonella* on laying hen farms.

In a last study (CHAPTER 6), the influence of the housing type on antimicrobial resistance in *Escherichia coli* and *Enterococcus faecalis* from laying hens was determined. In total, 1102 *E. coli* and 792 *E. faecalis* isolates were tested from 4 European countries (Belgium, Germany, Italy and Switzerland). The isolates were described by multiple drug resistance (MDR) patterns. Country and housing type displayed different influences on the MDR levels of both species. In *E. coli*, the housing in a floor-raised system resulted in an increased risk of MDR in comparison to the conventional battery system. In *E. faecalis* the MDR levels were lower in free-range systems than in conventional battery cages. In Belgium, ceftiofur-resistant *E. coli* isolates were more abundantly present than in the other countries. The results of this study suggest that the levels of antimicrobial resistance in indicator bacteria such as *E. coli* and *E. faecalis* in laying hen flocks are relatively low compared to the levels observed in broilers and pigs. The differences observed between both indicator bacteria with respect to the influence of the housing system suggest that it is important to not focus on a sole bacterial species when trying to assess risk factors for antimicrobial resistance. It is crucial to conscientiously monitor the prevalence and evolution in time of antimicrobial resistance in laying hens, both during the rearing period and the production cycle, to be able to detect early changes in antimicrobial resistance and to minimize the spread of resistant bacteria to humans.

Based on the results of the studies carried out during the current thesis, it is most unlikely that the move from conventional battery cages towards non-cage housing systems will increase the prevalence of *Salmonella* in laying hen flocks. However, the apparent advantage of non-cage systems is most probably influenced and biased by other factors such as the age of the infrastructure, flock size and sanitary conditions. The results from the extensively sampled flocks clearly indicate that in a considerable proportion of these vaccinated flocks *Salmonella* is still present in the hens, regardless of the housing type, suggesting that stopping of vaccination against *Salmonella* of commercial laying hens could rapidly lead to an increase of the prevalence. Concerning the antimicrobial resistance in laying hens, no consistent conclusion on the influence of the housing system could be drawn for *Escherichia coli* and *Enterococcus faecalis* (CHAPTER 7).
SAMENVATTING

Eén van deze pathogenen is Salmonella, wereldwijd nog steeds een belangrijke ziekteverwekker bij de mens. In Europa zijn Salmonella Enteritidis en Typhimurium de vaakst geïsoleerde serotypes bij gevallen van humane salmonellosis. Voor de mens blijven besmette eieren de belangrijkste bron van infectie met Salmonella Enteritidis.

Naast de microbiologische integriteit van de eieren is ook de verspreiding van antibioticumresistentie naar de mens door de consumptie van voedsel van dierlijke oorsprong een heel belangrijk facet van de voedseelveiligheid (Hoofdstuk 1). De doelstellingen van deze thesis waren de effecten van huisvestingssystemen op de prevalentie van Salmonella en antibioticumresistentie in leghennen na te gaan (Hoofdstuk 2).

In Hoofdstuk 3 worden de resultaten beschreven van een studie waarin werd nagegaan of de huidige bemonsteringsprotocols (i.e. een beperkt aantal mengmeststalen al dan niet gecombineerd met een stofstaal) accuraat genoeg zijn om op toomniveau lage Salmonella-prevalenties te kunnen detecteren. In totaal werden 19 tomen bemonsterd. Al deze tomen waren op basis van het officiële controleprogramma Salmonella-negatief bevonden. Ook na een intensieve bemonstering (i.e. 5 mengmeststalen, 40 cloacaswabs van 40 hennen en 1 stofstaal) op het bedrijf kon in geen enkel van de bemonsterde tomen Salmonella teruggevonden worden. Na transport van 100 hennen naar het labo en daaropvolgende analyse van cloacaswabs en caecale inhoud werd Salmonella Enteritidis teruggevonden in leghennen van 5 tomen. Op basis van de cloacaswabs varieerde de prevalentie binnen een toom van 1 tot 14 %. Het verschillende resultaat van de staalnames op het bedrijf zelf en na transport kan verklaard worden door de intermitterende excretie van Salmonella door geïnfecteerde dieren.
en het feit dat de stress die veroorzaakt wordt door het transport de dieren van een ‘drager status’ naar een ‘excreterende status’ doet overgaan. Deze resultaten tonen aan dat afhankelijk van de gebruikte bemonsteringsschema’s verschillende schattingen van de Salmonella-prevalentie worden bekomen. De resultaten van deze studie wijzen er op dat post mortem onderzoek van leghennen de beste methode is om Salmonella te detecteren, hoewel het een arbeidsintensieve en bijgevolg dure methode is.

Risicofactoren voor Salmonella-infecties werden geïdentificeerd in Hoofdstuk 4. Negenentwintig tomen, gehuisvest in batterijkooien en niet-kooisystemen werden intensief bemonsterd zoals beschreven in Hoofdstuk 3. Tijdens het bedrijfsbezoek werd een vragenlijst ingevuld om informatie te bekomen over bedrijf- en toomeigenschappen (bv. de toomgrootte, het ras, de leeftijd van de hennen, behandelingen van de toom,...) en de bioveiligheidsmaatregelen. Salmonella werd teruggevonden in hennen uit 6 van de 29 bemonsterde tomen. Uit de risicofactoren-analyse bleek dat een voorgaande Salmonella-besmetting op het bedrijf en de leeftijd van het productiesysteem (i.e. het aantal jaren dat de huidige installaties in dienst zijn) een significante invloed hebben op de kans op Salmonella-besmetting in leghennen. De resultaten van deze studie tonen aan dat, ongeacht het huisvestingssysteem, strikte en aanhoudende bioveiligheidsmaatregelen nodig zijn om nieuwe infecties of heropflakkeren van bestaande infecties te voorkomen tijdens opeenvolgende productierondes.

In een volgende studie (Hoofdstuk 5) werd de prevalentie van Salmonellauitscheiding nagegaan op leghennenbedrijven met verschillende huisvestingssystemen en werden risicofactoren voor de uitscheiding van Salmonella geïdentificeerd. Er werd in 5 Europese landen een cross-sectionele veldstudie uitgevoerd: België, Duitsland, Griekenland, Italië en Zwitserland. In totaal werden 292 leghennentomen bemonsterd de maand voorafgaand aan depopulatie. De vragenlijst die beschreven werd in Hoofdstuk 4 werd gebruikt om informatie te verzamelen over het algemene management op het bedrijf en specifieke eigenschappen van de bemonsterde toom. In 29 van de bemonsterde tomen werd tenminste 1 Salmonella-serotype gevonden. Gebaseerd op de cloacaswabs van 40 hennen varieerde de prevalentie van Salmonella-uitscheidende hennen tussen 0 en 27.5 %. Er werden verschillende Salmonella-serotypes geïsoleerd hoewel S. Enteritidis toch de meest voorkomende was. De volgende risicofactoren voor de uitscheiding van Salmonella Enteritidis/Typhimurium werden geïdentificeerd: het houden van leghennen in batterijkooien, het ontbreken van droge reiniging tussen twee productierondes in en het bemonsteren tijdens
de winterperiode. Er werd dus geen hogere *Salmonella*-prevalentie gevonden in tomen die niet in batterijkooien gehouden werden, niettegenstaande in niet-kooisystemen de kans op de oro-faecale overdracht van *Salmonella* tussen de dieren veel groter is. Verscheidene managements- en bioveiligheidsmaatregelen zoals een strikte reiniging en disinfectie werden geïdentificeerd als factoren die de insleep en verspreiding van *Salmonella* op een leghennenbedrijf kunnen helpen minimaliseren.

In een laatste studie (HOOFSKSTUK 6) werd de invloed van het huisvestingssysteem op het voorkomen van antibioticumresistentie in *Escherichia coli* en *Enterococcus faecalis* onderzocht. In totaal werden 1102 *E. coli* en 792 *E. faecalis* isolaten van leghennen verzameld in 4 Europese landen (België, Duitsland, Italië en Zwitserland). De isolaten werden beschreven aan de hand van multiple drug resistance (MDR) patronen. Uit de risicofactoren analyse bleek dat land en huisvestingstype een invloed uitoefenen op de MDR in beide bacterie species. In vergelijking met batterijkooien vergrootte het huisvesten van hennen in scharrelsystemen het risico op hoge MDR niveaus in *E. coli*. Voor *E. faecalis* waren de geobserveerde MDR niveaus lager in hennen gehuisvest in vrije uitloop-systemen vergeleken met hennen in batterijkooien. In België werden significant meer ceftiofur-resistente *E. coli* isolaten gevonden dan in de andere landen. De resultaten van deze studie tonen aan dat de niveaus van antibioticumresistentie in indicatorbacteriën in leghennen relatief laag zijn in vergelijking met de situatie bij andere diersoorten zoals braadkippen en varkens. De verschillende invloed van de huisvesting op de graad van antibioticumresistentie in beide indicatorbacteriën duidt erop dat het belangrijk is om niet op één enkele bacteriesoort te focussen wanneer men risicofactoren voor antibioticumresistentie wil identificeren. Het is noodzakelijk om zowel tijdens de opfokperiode als tijdens de productiecyclus de prevalentie en de evolutie in de tijd van antibioticumresistentie in leghennen zorgvuldig te monitoren om in staat te zijn veranderingen snel te detecteren en om indien nodig in te grijpen teneinde de verspreiding van resistentie naar de mens te minimaliseren.

Gebaseerd op de resultaten beschreven in deze thesis kan geconcludeerd worden dat het hoogst onwaarschijnlijk is dat het huisvesten van leghennen in niet-kooisystemen de prevalentie van *Salmonella* zal doen toenemen. Desondanks moet men er zich toch bewust van zijn dat dit schijnbare voordeel van de niet-kooisystemen meer dan waarschijnlijk beïnvloed en vertekend wordt door andere factoren zoals de leeftijd van de infrastructuur, de toomgrootte en hygiënestatus. De resultaten van de intensief bemonsterde tomen tonen duidelijk aan dat in een aanzienlijk deel van de gevaccineerde tomen *Salmonella* nog steeds
aanwezig is, ongeacht het huisvestingstype. Dit wijst er op dat het afschaffen van de vaccinatie van leghennen snel zou kunnen leiden tot een stijging van de prevalentie. Wat de antibioticumresistentie betreft kon geen éénduidige conclusie getrokken worden voor *Escherichia coli* en *Enterococcus faecalis* wat betreft de invloed van het huisvestingsysteem. *(HOOFDSTUK 7).*
CURRICULUM VITAE

In oktober 2006 trad hij in dienst van de Vakgroep Voortplanting, Verloskunde en Bedrijfsdiergeneeskunde als doctoraatsbursaal met een driejarig mandaat gefinancierd door de Europese Unie. Hij verrichtte een onderzoeksstudie over de prevalentie van *Salmonella* en antibioticumresistentie bij leghennen in verschillende huisvestingssystemen. Vanaf 1 oktober 2009 was hij aan de zelfde vakgroep werkzaam op een éénjarig FOD-project over *Salmonella Gallinarum*.

Gedurende de 4 jaren aan de vakgroep was hij ook actief in de bedrijfsbegeleiding Varken en stond hij mee in voor de opleiding van de optievakkers. Hij nam eveneens deel aan de nacht- en weekenddiensten van de kliniek verloskunde Rund.

Sebastiaan Van Hoorebeke is auteur of mede-auteur van meerdere wetenschappelijke publicaties in internationale tijdschriften en was spreker op verschillende nationale en internationale congressen.
DANKWOORD
DANKWOORD

Weggemoffeld in de cloaca van dit boekje vinden jullie dit dankwoord.

Elke gelijkenis met bestaande personen berust louter op de werkelijkheid.

“Noblesse obligé” en daarom begin ik graag met mijn beide promotoren: Prof. Dr. Dewulf en Prof. Dr. Van Immerseel. Jeroen, hartelijk dank voor je blijvende geloof in dit onderzoek, ook als ik een andere richting uitstuurde. Het was af en toe eens stevig zweten als een nieuwe deadline me bijna bij de lurven had maar vandaag ben ik er helemaal van overtuigd dat het dubbel en dik de moeite waard is geweest, bedankt. Filip, je heel deskundige en immer flegmatieke input waren van onmisbare waarde in de voortgang van dit onderzoek. Het is echt een luxe om zulk een autoriteit als promotor te hebben, of het nu over *Salmonella* of *Clostridium* gaat!

Ook een hartelijke dank aan Prof. Dr. Ducatelle. Vanaf het prille begin was u heel nauw betrokken bij het Safehouse-project. Dank voor uw enthousiaste, niet aflatende interesse in mijn onderzoek.

Prof. Dr. Haesebrouck, bedankt dat ik gebruik mocht maken van uw labo’s en de snelle en accurate verbeteringen van mijn manuscripten.

Ten slotte ook nog mijn welgemeende dank aan Prof. Dr. Pasmans (Frank) voor de ijver waarmee je mijn wetenschappelijke kattebelletjes las en corrigeerde.

Ik wil ook nogmaals van harte de leden van de begeleidings- en examencommissie bedanken voor de tijd en moeite die ze staken in het nalezen en corrigeren van mijn thesis: Dr. Lieve Herman, Prof. Dr. Berge, Dr. Hilde Van Meirhaeghe, Dr. Yves Van der Stede, Prof. Dr. De Zutter en Prof. Dr. Ducatelle.

Jantina, jij en ik waren de ‘jonge wolven’ van het Belgische Safehouse-luik. Bedankt voor alle hulp bij de vele euthanasies en labo-analyses. Veel succes en plezier in je nieuwe job!

Ook een woord van dank voor de mensen van Degudap voor het aanleveren van een heleboel leghenbedrijven die wilden deelnemen aan ons onderzoek.

Het bemonsteren van al die bedrijven is één ding, bij aankomst in het labo begint het echte werk. BPW bereiden, media maken, platen gieten, overenten… Daarom mijn zeer grote dank aan Sophie Callens, Sofie Haerens en Lonneke Verbeek voor het vele werk in het labo.

To all the partners of the Safehouse-project: thank you very much for the nice cooperation. It was a wonderful experience to work with people from so many different countries and institutions. The yearly meetings always were very productive and enjoyable occurrences.

Johan Z (Proefbedrijf Pluimveehouderij): hartelijk dank voor uw interesse in mijn onderzoek en de toelating om ook op uw bedrijf stalen te mogen nemen.

Marc V, je volgde van in het begin met veel belangstelling de voortgang van mijn onderzoek, het was dan ook een aangename verrassing toen we plots ‘facultaire’ collega’s werden.
Na 3 jaar Safehouse kwam Salmopoul, hetgeen me opnieuw de kans gaf om enkele fijne mensen te ontmoeten. Hilde VM, je rechtstreeks aanpak was heel verfrissend en motiverend. Ik hoop dat we in de toekomst nog zullen kunnen samenwerken. Mieke G., veel succes met je bestrijding van Java en consorten! Isabelle DW, Koen DR en Marc H. (ILVO): ik ben jullie heel dankbaar voor alle knowhow en de uitvoering van de typeringen. Johan VE (Galluvet): hartelijke dank voor het aanleveren van de te bemonsteren bedrijven en relevante praktijkkennis allerhande.

Koen P, heel hartelijk dank voor het kunstwerk dat de kaft van dit boekje siert!

Hoewel ik officieel tot de DI08 behoorde, speelde veel van de actie zich ook af op de vakgroep Bacterio, Patho en Pluimveeziekten. En hoewel ik een koekoejong was, heb ik absoluut nooit te klagen gehad over de ontvangst, zelfs niet als ik het ganse gebouw murw sloeg met een bombardement van vloei- en op lichaamstemperatuur gebrachte kippenmest. Daarom een hele grote dankjewel aan iedereen voor alle uitleg, de leuke babbels en de prettige ‘buitenschoolse activiteiten’. Echte mannen doen aan sport, dat weet iedereen. Daarom wil ik uitdrukkelijk de Verbancksjes danken, de voetbalploeg waar een goed resultaat als een leuk extraatje wordt beschouwd. Jurgen, Gunter, Patrick V, Jo DW, Ward, Alexander, Serge, Koen en Baptist: hartelijk dank voor de zeer fijne sportieve en para-sportieve uitspattingen op en naast het veld. Ook dank aan de moedige collega’s die de sportieve strijd met de studenten aangingen: Bart P., Peter DS, Hans D, Miguel (always a pleasure!), Stefanie S, Tamara L, Johnny V, Marc G. en Ruben VG.

Prof. Dr. de Kruif, dank voor uw interesse in mijn onderzoek, zowel wat de wetenschappelijke voortgang als de meer praktijkgerichte benadering ervan betreft.

De vakgroep Voortplanting, Verloskunde en Bedrijfsdiergeneeskunde: een rovershol waarnaar het altijd prettig terugkeren was na één of andere wetenschappelijk verantwoorde veroveringstocht. Het echte hoofdkwartier is zonder de minste twijfel ‘den 48’. Stefaan, een heel hartelijke dankjewel voor alles wat je hebt gedaan voor mij, in de soms woelige omstandigheden. Of het nu over epidemiologie ging, of over gecompliceerde keizersnedes of over allerhande andere chaotische en intense toestanden: je hulp is van onschatbaar belang geweest. Ik wens je het allerbeste toe in alles wat je wilt en doet. Davy, we hadden dat prima naar ons zin, zo op den 48. Merci voor alle leute, alle hulp (met de computer en alle mogelijke praktische calamiteiten) en alle gesprekken. Veel succes ook nog met je (post)doc! Ge weet het: “ge moogt nooit zeggen dat ge iets nie lust als ge’t nog nie geproefd hebt”. En dan Sarah. Ons gemeenschappelijke wetenschappelijke carrière heeft maar een tweetal jaar geduurd maar het was een fijne tijd (“Avez-vous une sasse hygiénic?”). Ik memde over het buitenland en werk nu in Deinze, jij memde over het buitenland en werkt nu tussen de glooiende heuvels van Rwanda… Ik vind het chique dat jullie die stap durfden zetten, heel veel succes gewenst! My dear Alfonso, I like you so much that you’re mentioned twice in this dankwoord. We shared a lot of memorable moments,
both in the context of Science and in ‘other contexts’. You’re a fantastic guy. De volgende in de rij is Loes, die een stevige portie Hollandse bravoure toevoegde aan den 48. Je heerlijke no-nonsense stijl was een hele verrijking en je bent het levende bewijs dat er vrouwen bestaan die kunnen meepraatsen over epidemiologie én over voetbal (weliswaar over de verkeerde ploeg maar goed). Bénédicte, ik heb nooit het geluk gehad om samen met jou den 48 te bevolken maar na het voorbije jaar (en Nantes) ben ik er heel erg van overtuigd dat je er helemaal thuis hoort (“Parlez-vous français?”).

Naast het E-team had ik het genoegen om deel uit te maken van een andere sub-unit, zijnde Bedrijfsdiergeneeskunde Varken. Prof. Dr. Maes, Dominiek, hartelijk bedankt voor de fijne samenwerking en het vertrouwen gedurende de voorbije 4 jaren. Caroline, bedankt voor al je input ivm bedrijfsproblemen en het bijhorende gebabbel. Querido Alfonso i Rubén, fue un gran placer de conocer vos. The both of you are really een verrijking for the pig unit, I wish you all the best with your research. Espero que nos volveremos a encontrar pronto, para tomar unas cervezas con vosotros. Emily, Ellen, Josine, Liesbet, Iris V. en An C: bedankt voor de vlotte samenwerking én de wetenschappelijke input gedurende de journal clubs!

Bart M. en Tom M., jullie zijn diegenen die me als student en als beginnend bursaaltje de kneepjes van de bedrijfsdiergeneeskunde hebben bijgebracht, bedankt hiervoor.

Philip V., hartelijk dank voor de interesse in mijn onderzoek, de verhelderende gesprekken en de nuttige feedback bij bedrijfsproblemen.

Tamara VDS, ik heb de varkens nu wel verlaten maar ik weet zeker dat we elkaar zeker nog zullen ontmoeten.

En uiteraard moet ik ook de varkenshouders bedanken voor de aangename samenwerking. Een zeer dikke merci aan Christian en Isabelle B., Herman en Dina L., Jim, Geert en Lydia (Agrivet) en Kristof D., Hans, Bart, Jan en Jos (ILVO). De bedrijfsbezoeken op het ILVO brachten me ook in contact met Sam M. en Marijke A. Sam, bedankt voor je interesse in mijn onderzoek en je kijk op het onderzoek. Marijke, het allerbeste met jouw verder onderzoek en carrière.

Nadine, we hebben alle twee reeds de faculteit verlaten maar het is altijd fijn om je terug te zien. Bedankt voor de zeer fijne tijd. Ria en Els, Marnik en Véro, Willy, Dirk en Wilfried: wat zou het zijn zonder jullie? Een supermerci voor alle praktische hulp in de apotheek, de kliniek en de stallen, het transport van de varkens, de ontlilbare babbels en verfrissinkjes! Steven, dankjewel voor het geduld dat je had met mijn ‘digibeet-zijn’. En voor de gesprekjes natuurlijk. Sandra en Leïla, dankjewel voor het regelen en controleren van alle mogelijke en onmogelijke (financiële) administratie. Nicole, je hebt soms het genoegen (?) gehad om me ’s morgens uit mijn slaap te halen. Heel erg bedankt voor al je werk om de vakgroep en de rest van de faculteit netjes te houden, al zal ik wel altijd een sloddervos blijven.
Geen inspanning zonder ontspanning. Gelukkig hadden we daar koffiepauzes, middagpauzes, terrasjes en een ski-reis voor. Aan mijn prachtcollega’s Muriel (“There’s a crack in everything…”), Leen, Josine, Iris, Isabel, Petra, Vanessa, Eline, Hilde en Lars: bedankt voor alle leuke gesprekken en de gezelligheid! Voor diegenen die nog bezig zijn aan hun onderzoek: succes.

Ik heb een bloedhekel aan paarden maar dat neemt niet weg dat ik de collega’s van Paard heel erg op prijs stel. Te beginnen met Jan, Maarten en Catharina. Eerst en vooral dankjewel voor alle nachten dat jullie tijdens die allereerste maanden ons, de beginnende assistenten, de keizersneden aanleerden. Daarnaast -en vooral- ook een hele grote merci voor alle leute. Ik wens jullie nog heel veel succes met het voltooien van jullie onderzoek! Katrien, daarmee zit het grote doctoraatsavontuur er bijna op. Wie had dat ook alweer 4 jaar terug kunnen bedenken? Nog heel veel succes met alle toekomstige avonturen! (nooit vergeten: surfen en paardrijden is voor snobs). Kim, jij ook nog veel succes met je residency. Je was een supercollega.

Mocht Vlaanderen ooit ingelijfd worden bij Nederland dan is het beter om goed voorbereid te zijn. Daarom heb ik altijd met veel plezier samengewerkt met mijn Hollandse collega’s Iris, Cyrillus en Wendy. Iris, het is dan toch geen Varken geworden voor mij (maar kalkoen is ook niet slecht!). Je bent heel hard bedankt voor alle leuke momenten op de faculteit en daarbuiten. Veel succes met je verdere carrière! Cyrillus en Wendy, onze samenwerking op de faculteit is van korte duur geweest maar daarom niet minder aangenaam. Allebei veel succes gewenst in jullie nieuwe job. Ik neem aan dat we elkaar regelmatig zullen blijven tegenkomen, hetgeen fijn is.

Jef en Marcel, de buitenpraktijk Rund heb ik nooit ‘gedaan’ maar dat neemt niet weg dat ik steeds met veel plezier naar jullie verhalen over het Echte Werk (vroeger en nu) kon luisteren. Ook de andere collega’s van Rund (Stefaan, Iris, Miel, Sofie, Karlien) wil ik bedanken om te komen depanneren als een sectio op de kliniek een tikje te gecompliceerd bleek. Tot slot ook nog dank aan de overige collega’s van de vakgroep voor de prettige samenwerking.

Ik ben natuurlijk niet de eerste die de vakgroep verlaat. Enkele fantastische vrouwen zijn me voorgegaan: Mirjan, Jo B (nog steeds met voorsprong mijn favoriete bio-ingenieur), Nele G, Nele E, Emilie VH en Leen M. Bedankt voor alle leuke momenten op en buiten de faculteit. Het is altijd fijn om jullie terug te zien. Ook fantastisch doch veel minder vrouwelijk zijn de éminences grises die de faculteit verlieten net toen ik er toekwam: Jo L, Boudewijn C, Geert H en Steven V. Jullie waren op vele manieren een voorbeeld voor velen van de jonkies.

Niettegenstaande ik met heel veel plezier terugkijk op mijn periode aan de faculteit, ben ik toch heel tevreden dat ik de stap heb gezet naar Quartes (Versele-Laga). Laat er geen twijfel over bestaan: kalkoenen zijn de nobelste aller hoenders! Daarom gaat mijn dank in de eerste plaats uit naar Eric H, Patrick Z en Ann V om me wegwijze te maken in ‘all things turkey’. Daarnaast ook woorden van dank
voor P. Galle, Eveline DW en de overige collega’s “in de villa”. Tot slot nog een hartelijke groet aan K. De Praetere en Philippe DL van Volys Star.

Mijn ouders wil ik bedanken voor de prachtige jeugd die ik mocht beleven, een gegeven dat ik steeds meer naar waarde ben gaan schatten. Ook toen alles anders werd kon ik steeds op jullie blijven rekenen.


Liesbeth (ofte Pr*t voor de vrienden), ook voor jou een grote dankjewel. Nu het doctoraat gedaan is maak ik je plezier mijn belofte waar: dat wordt dus een dagje Boudewijnpark! En ik weet zeker dat het ‘begeleid wonen-project’ zal slagen. Ik ben er trots op dat ik je broer ben.

Paps en Alice, ook jullie heel hartelijk bedankt voor alle leuke en gezellige momenten en de onvoorwaardelijke steun. Paps, met betrekking tot het trauma van Biochemie (2de kan): bij deze beloof ik je plechtig dat ik je nooit meer vraag om een cursus te onderlijnen exact 14 u vóór het examen.

Virginie, Thierry, Bernard, Yannick, Marjorie, Tom, Fanny, Geoff, Claire, Sébastien, Sarah en Fleur: we liepen elkaar toevallig tegen het lijf in de “Nieuw samengestelde gezinnen”-statistieken en het mag gezegd: ik ben heel blij dat ik jullie heb leren kennen. We hebben al bijzonder veel pret gemaakt en dat zal in de toekomst niet anders zijn.

Het klassieke beeld van Dé Dierenarts wankelt en daar hebben de volgende zeer fijnbesnaarde personen veel mee te maken: Hans en Philippe, jullie zijn schitterende vrienden. Ongetallebare uren hebben we reeds zitten zeuren, schateren of grienen, een cola of pilsje in onze knuistjes gekneld en dat mag voor mijn part nog zeer lang blijven duren. Bedankt om er steeds te zijn als er ‘even top-overleg moet gepleegd worden’. Steven C, jij hebt niet kunnen weerstaan aan de lokroep van het Grote Geld maar het is je vergeven. Je bent één van de meest onkreukbare mensen die ik ken en dat apprecieer ik heel erg. Daarnaast denk ik je plezier aan onze bier- en frietensessies en de bijhorende gesprekken terug. Diedrich, het is een waar genoegen om nog steeds contact te houden met jou. Waar is de tijd dat we als schriele kuikens zaten te piepen dat “het nog allemaal moest beginnen”? Frederik VH, je bent voor de eigen praktijk aan het gaan. Respect daarvoor! Ik heb enkele schitterende casussen, dus zeer graag kortelings een wetenschappelijk symposium organiseren, wat denkt u?

Aldwin, Godfried, Hendrik, Jonas, Pieter C. en Pieter W: het mag heel duidelijk zijn waarvoor ik jullie bedank: voor de jarenlange vriendschap, de heerlijke tijd in de Ottergemsesteenweg en de Friedrich Froëbelstraat en de ongetallebare momenten met een lach en een traan. De posse is uitgebreid met vrouwen en kinderen maar ons vermogen tot volslagen debiliteit blijft godzijdank onaangetast. Jullie zijn flinke knappen!
Toen het penthouse in de Friedrich Froëbelstraat was uitgebrand moest er een nieuw onderkomen gevonden worden. Dan maar de Stropstraat. En ook daar heb ik mij vré goed gehad. Miel, ik bedacht zonet dat we melkander zowaar bijna 10 jaar kennen, een periode waarin het aantal waanzinnige toestanden en interessante feestjes moeilijk te tellen is. Het zijn alleszins prachtige herinneringen. Tuur, plots stonden we met onze hele inboedel bij jou op kot. En we hebben er een fantastisch jaar van gemaakt! Ik vind het chique hoe jij je weg zoekt binnen de machtige praktijkwereld, mijn onvoorwaardelijke steun heb je alvast. En tot slot… ons Cumrolientje, het enige echte Orakel van Leke. We zijn nu verschillende jaren verder en ook jij bent intussen aan de weg aan het timmeren, veel succes! Wie weet komt er dra een reünie van de Stropstraat?

Maarten H, toen ik plots ‘dakloos’ was bood jij de bovenverdieping in Balegem aan. Ik besef dat het niet mijn meest constructieve periode was maar bij deze wil ik je uitdrukkelijk bedanken voor je gastvrijheid en alle goeie zorgen. Ik hoop dat je in de job terechtkomt die je wilt, je verdient het dubbel en dik.

Vincent, we plegen van tijd tot tijd nog eens te mijmeren over die tijd dat we als hevig puberende rebellen het mooie weer maakten op ons aller Don Boscocollege. We’ve come a long way… En het stemt me bijzonder gelukkig dat we nog steeds het mooie weer kunnen blijven maken (“Als zelfs Fidel Castro en de paus…”). Lynn, de tijd van de zebraabrilt ligt ook alweer ver achter jou. Ik ben erg blij dat we elkaar hebben leren kennen. Ik wens jullie samen het allerbeste toe. Aan de kleine Jeff: tot dra!

Ik kus m’n beide handjes want ik ken nog enkele schitterende mensen die ik heel erg apprecieer! Daarom een dikke merci aan Pieter M, Debby, Frederief, Mandy (Phil Collins!), Fleur M, Wouter B., Filip, Lin, Wimpel, Valerie en Leen S.

David en Virginia, Jeanne en Jaak: hartelijk dank voor de gastvrijheid, de gezelligheid en de lekkere etentjes.

En om te eindigen…

Venessa, mijn liefste Pluim, niemand is zijn of haar plaatsje in dit dankwoord zo hard waard als jij. Bedankt voor alles, ik vind je fantastisch (en ik heb nog gelijk ook!). Bedankt voor wie je bent en wat je betekent voor mij. Het heden met jou is al super, en de toekomst ziet er zelfs nog beter uit. Je weet: ik ben een Handige Harry én een computerwizzard dus dat droomhuis en de layout van jouw boekje komen voor elkaar!

Hoe graag ik je zie? Superveel graag!!
“And in the end
the love you take
is equal
to the love you make”
(The Beatles)
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