# *Salmonella* epidemiology in broiler flocks

by

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| | |
|---|---|
| **API** | Identification system for Enterobacteriaceae and other Gram-negative rods |
| **BES** | Boletín epidemiológico semanal |
| **BPW** | Buffered Peptone Water |
| **º C** | Celsius degrees |
| **C&D** | Cleaning and disinfection |
| **cfu** | Colony-forming units |
| **CI** | Confidence interval |
| **CITA** | Centro de Tecnología Animal |
| **cm** | Centimeter |
| **DNA** | Deoxiribonucleic acid |
| **EC** | European Commission |
| **EFSA** | European Food Safety Authority |
| **e.g** | For example |
| **ELISA** | Enzyme-linked immunosorbent antibody assay |
| **EU** | European Union |
| **g** | Gram |
| **h** | Hours |
| **ISO** | International Organization for Standardization |
| **IVIA** | Instituto Valenciano de Investigaciones Agrarias |
| **LB** | Lauria-Bertoni |
| **M** | Molar |
| **MARM** | Ministry of Environment and Rural and Marine Affairs |
| **min** | Minute |
| **mL** | Millilitre |
| **MS** | Member States |
| **MSRV** | Modified Semisolid Rappaport Vassiliadis |
| **n** | Number of observations |
| ***P*** | probability value |
| **PCR** | Polymerase chain reaction |
| **PFF** | Previous flock faeces |
| **PT** | Phage type |
| **PVC** | Polyvinyl chloride |
| **REA** | Real Escuela de Avicultura |
| ***S*** | *Salmonella* |
| **SE** | Standard error |
| **USA** | United States of America |
| **UV** | Ultraviolet |
| **vol/vol** | Volume to volume |
| ***vs*** | versus |
| **W** | Water |
| **WHO** | World Health Organization |
| **XLD** | Xylose-Lysine-Desoxycholate |
| **XLT4** | Xylose-Lysine-Tergitol-4 |

Eggs and poultry meat are a common source of human salmonellosis. Contamination of poultry or poultry meat may occur throughout the production chain. Nevertheless, in Spain non contaminated broiler meat may be sold for human consumption from 2011. Before *Salmonella* prevalence in broiler flocks (EFSA, 2007) and the Community objectives for *Salmonella* control in poultry farms were established (EC, 2007) non official dates were available regarding the *Salmonella* situation in broiler production in Spain. In this context, in 2005 the Regional Ministry of Environment and Rural and Marine Affairs of the Valencia Region invested in the study of the main sources of *Salmonella* contamination in broiler flocks reared in the Region of Valencia and the study of *Salmonella* epidemiology in field conditions. These studies were intended to anticipate the National Control Programme for *Salmonella* in broiler production with the aim of applying proper measures to comply with the future Community objectives. This study started in October 2005 and finished in October 2007. Over a two year period, 65 flocks from different farms were intensively sampled during the rearing stage. Broiler farms were distributed throughout the Valencia Region.

The objectives of the first experiment were to determine the main sources of *Salmonella* contamination in broiler production, assess the risk factors for *Salmonella* contamination in broiler flocks at the end of the rearing period, and determine the serotypes involved in broiler production systems in the Valencia Region. Each house was visited 11 times during the rearing period. First, when the previous flock was taken to the slaughterhouse, samples of dust, surfaces and faeces were collected; after cleaning and disinfection, samples of dust and surfaces were also taken. On the first day of rearing, samples of water, bedding, farmers' boots, meconiums, delivery-box liners and feed were collected. During rearing, 6 visits took place and samples of feed were taken. On slaughter day, samples of dust, surfaces, water, feed and faeces were also collected. Finally, two days after slaughter, carriers (rodents, flies and beetles) were trapped. All samples were analysed in accordance to ISO 6579:2002 (Annex D) and positive samples were serotyped by Kauffman-White-Le-Minor technique. Our results showed that all different types of samples collected were contaminated with *Salmonella* (ranged between 1.5 % and 38.6 %), except some feed samples collected from the truck. The most contaminated samples related with poultry production were: delivery-box liners, faeces samples, dust samples, farmers' boots and feed from feeders.

Nevertheless, the most important risk factors for *Salmonella* contamination of the flocks at the end of the rearing period were contaminated feed from feeders, *Salmonella* status of the house after cleaning and disinfection and *Salmonella* status of day-old chick flocks. Twenty-one different serotypes were isolated from the samples analysed. The most prevalent were in decreasing order: *S.* Enteritidis (52.9 %), *S.* Hadar (17.8 %), *S.* Virchow (8.9 %) and *S.* Ohio (5.4 %). The study suggested that there are many sources for *Salmonella* contamination and persistence in broiler production. Therefore, the whole production chain has to be controlled to eradicate the bacteria from the primary production.

The objectives of the second experiment were to assess *Salmonella* detection from faeces samples during rearing, and assess the influence of live transport to the slaughterhouse on *Salmonella* detection. During this study, 65 flocks were sampled at weekly intervals from first day of rearing until slaughter. Samples of faeces were taken from the litter using five pairs of cellulose sock swabs attached to boots and applied over the length of the house (EC, 2005). To assess *Salmonella* detection rates before and after live transport to the slaughterhouse, faeces samples were collected. Before loading, faeces were taken with five pairs of cellulose sock swabs as described above (EC, 2005). After transport, two pooled faeces samples were taken directly from the truck (200-300 g each; EC, 2005). All samples were analysed in accordance with ISO 6579:2002 (Annex D). Results showed that regardless of whether broiler flocks arrived at the farm already shedding the bacteria in faeces, or were infected during rearing, both groups described the same detection pattern, with the highest detection in faeces at 14th day of rearing (50.5 % and 34.5 %, respectively). Moreover, *S.* Enteritidis was the most prevalent serotype isolated during rearing (66.7 %), followed by *S.* Virchow (13.7 %), *S.* Hadar (9.4 %) and *S.* Ohio (2.8 %). On the other hand, before loading and after transport to the slaughterhouse, 15.4 % and 41.2 % of faeces samples collected were determined positive, respectively. In addition, a change in the serotype pattern was also observed. *S.* Enteritidis remains the most prevalent serotype isolated (54.5 %). *S.* Hadar doubled the excretion rates (39.3 %), and *S.* Virchow and *S.* Ohio were not isolated after transport.

Finally, the objectives of the third experiment were to determine the biofilm development capacity of the strains isolated from poultry risk factors. Then,

glutaraldehyde (50 % vol/vol), formaldehyde (37 % vol/vol) and hydroxide peroxide (35 % vol/vol) were applied to evaluate their capacity to remove *Salmonella*, biofilm and non-biofilm strains, isolated from each risk factor in an artificial contamination test in field conditions. Samples of faeces, dust, surfaces, meconiums, delivery-box liners, water tank, water dispensers, litter, vectors (rodents, flies and beetles) and surfaces of the slaughter trucks were taken throughout the rearing period. All samples were analysed in accordance with ISO 6579:2002 (Annex D). To evaluate biofilm development, a screening method based on the fluorescence of *Salmonella* colonies on calcofluor agar plates was used. In the artificial contamination test, the chemical solutions were prepared at a concentration of 1.0 % and applied at exact times (1, 15 or 60 min). Our results showed that irrespective of the origin of different *Salmonella* strains, around 50 % of the different serotypes were able to produce biofilm. Finally, the use of glutaraldehyde, formaldehyde and peroxygen at a concentration of 1.0 % in field conditions are inadequate for *Salmonella* elimination irrespective of the serotype, biofilm development capacity and disinfectant contact time.

Due to the above considerations, there are many sources for *Salmonella* persistence in poultry houses and the whole production chain has to be monitored to eradicate the bacteria from the primary production. Nevertheless, *Salmonella* surveillance and control programmes do not have to stop at farm level. The control should be considered until the end of the line, the processing plant and markets. Considering the importance of transport on *Salmonella* spreading, C&D is an important challenge in *Salmonella* control and eradication from poultry production, although more studies are necessary to find the correct concentrations and application of disinfectants in field conditions and compare this effect with biofilm or non-biofilm strains.

# CHAPTER 1. INTRODUCTION ON *SALMONELLA* SPP

# 1.1. General aspects of *Salmonella*

## 1.1.1. Historical context of *Salmonella*

*Salmonella* have been the subject of many studies throughout the world since 1885, when Salmon and Smith (1886), first isolated the organism from swine in association with "swine plague". Thomas Willis can be regarded as the pioneer in typhoid fever studies. Until his classic description in 1659 and its translation into English in 1684, little had been done to separate this disease from similar diseases (Adelantado *et al.*, 2008). In 1826, Trousseau attempted to clarify the picture of typhoid mainly from the pathological angle, and to separate true typhoid from all the other gastro-intestinal infections. He described the classic inflammation of the Peyer glands and gave detailed description of post-mortem appearances (Kelterborn, 1967). In 1829, Pierre Louis gave another classic picture of typhoid and described post-mortem findings in detail, especially the enlargement and ulceration of the Peyer patches. He was also the first to use the word "Typhoid". However, he did not clearly differentiate between typhoid and typhus, which were often confused. The first to differentiate between the typhoid and typhus fevers was Gerhard in 1837 (Adelantado *et al.*, 2008). William Budd, 20 years before the bacterial origin of infectious diseases had been discovered, stated that typhoid fever was not spread by stench, was an alimentary disease in which the infective material in faeces contaminated water, milk, and the hands of those who attended the sick (Moorehead, 2002). In 1888, when 58 persons became ill in Frankenhausen, Germany, the outbreak of gastro-enteritis was associated with consumption of beef, and *Bacterium enteritidis* was isolated from that outbreak. A. Gärtner isolated a bacterium from both red meat and the spleen of one of the patients who had died and he named the organism *Bacillus enteritidis* (Kelterborn, 1967). Castellani and Chalmers (1919) renamed the organism as *Salmonella enteritidis*. The first published table based on the terminology introduced by White (1929) and modified by Kauffmann, contained 20 serotypes. Nowadays, more than 2500 serologically distinct *Salmonella* serotypes have been recognized and this list increases every year (WHO, 2005). Almost all of the 2500 *Salmonella* serovars are believed to be able to cause illness in humans. The epidemiology of human disease is dominated by only a

few serovars. In the late 1970s, *S.* Typhimurium was the most common and *S.* Agona before this (Harbour *et al.*, 1977). Many of these peaks of infection have been associated with a particular food or animal vehicle, such as *S.* Enteritidis with poultry.

### 1.1.2. General characteristics

The *Salmonella* genus is classified in the family Enterobacteriaceae, whose members are non-encapsulated, gram-negative bacilli. With the exception of *S.* Gallinarum and *S.* Pullorum, all salmonellas are motile, as they have peritrichous flagella (Van de Giessen, 1996). The organism is 0.7-1.5 μm wide and 2.0-5.0 μm long.

*Salmonella*s are facultative anaerobic bacteria and usually utilize citrate as a sole carbon source (Van de Giessen, 1996). The organism usually produces hydrogen sulphide gas on triple-sugar iron agar; usually decarboxylate lysine and ornithine reduce nitrates to nitrites, but are urease- and indole-negative (Cotrubo *et al.*, 2004).

*Salmonella* growth rate is dependent on several factors including temperature, pH, water activity and nutrients, and reflects the interactions between these factors (Van de Giessen, 1996). As a generalization, *Salmonella* grow at temperatures between 10 and 49 ºC, with an optimum of approximately 37 ºC (Bowmer, 1965). At temperatures between 0 and 5 ºC the organisms remain viable even though there is no growth (Bowmer, 1965). However, there is a marked reduction in the number of *Salmonella*s during freezing and long-term frozen storage, but not all are destroyed (D'Aoust, 1991). *Salmonella* are killed when exposed to temperatures of 55 ºC for one hour or 60 ºC for 15 to 20 minutes (Bowmer, 1965). The cooking of food will destroy *Salmonella*s if the internal temperature of the food reaches 74 to 77 ºC. *Salmonella*s in animal feeds and feed ingredients are killed by a process where the feed is heated to boiling point in steam-jacketed agitating cookers (110 ºC for 2.5 to 3.6 hours, Meara, 1973; Prost, 1967). Nevertheless, household cooking procedures used for eggs and egg containing foods are frequently insufficient to ensure a safe meal (Coetzer and Tustin, 2004).

The optimal pH for *Salmonella* growth lies between 6.5 and 7.5, with possibilities for growth in media with a pH range of 4.5 to 9.0, lower or higher pH values cause them to die (Baird-Parker, 1991).

*Salmonella* grow at water activities above 0.93 (Baird-Parker, 1991). The organism is resistant to drying and can survive for prolonged periods of storage at ambient temperature (D'Aoust, 1989) and in faecal material, in slurry or on pasture (Wray, 1975). *Salmonella* is sensitive to gamma irradiation (Clavero *et al.*, 1994) and organic acids (Smulders, 1987).

### 1.1.3. Nomenclature

Since Kauffmann in 1929 introduced the method for antigenic analysis of the *Salmonella* group, more than 2500 serovars have been identified (Popoff *et al.*, 2001; WHO, 2005). The names of the serovars (such as Typhimurium or Enteritidis) should be used without italicization or underlining, and with the first letter capitalized, e.g. *S. enterica* subsp. *enterica* ser. Typhimurium. As it is tedious and impractical to adopt this long, formal nomenclature for everyday use, it is common to refer to serovars as, for example, *Salmonella* Typhimurium, or as serovar Typhimurium (Le Minor and Popoff, 1987).

The classification of *Salmonella* has been controversial for many years. According to the latest nomenclature which reflects recent advances in *Salmonella* taxonomy (Le Minor and Popoff., 1987; Reeves *et al.*, 1989), the genus *Salmonella* consists of two species: *S. enterica*, which is divided into six subspecies (*S. enterica* subsp. *enterica*, *S. enterica* subsp. *salamae*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp. *houtenae* and *S. enterica* subsp. *indica*) and *S.* bongori. In veterinary literature, a distinction is usually made between infections caused by the (i) two host-adapted serovars of *S.* Pullorum (pullorum disease) and *S.* Gallinarum (fowl typhoid), (ii) the Arizonae subspecies of *Salmonella* (arizonosis) and

(iii) the remainder of the *Salmonella* (salmonellosis or paratyphoid infection). Generally, the serotypes of zoonotic importance are non-host-adapted serovars, but there are exceptions, e.g. *S.* Cholerasuis (adapted to pigs) or *S.* Dublin (adapted to cattle and sheep) which can cause severe disease in humans (Gradel, 2004).

### 1.1.4. Detection, identification, typing and phage typing

#### *1.1.4.1. Bacteriological isolation and identification*

Bacteriological isolation and subsequent confirmation by an appropriate biochemical and serological test are the traditional methods used in salmonellosis diagnostic (Mousing *et al.*, 1997). The bacteriological isolation of *Salmonella* requires four stages:

***Pre-enrichment in non-selective liquid medium.*** *Salmonella*s have simple nutrient requirements. However, samples that may contain only low numbers of the bacteria, such as food and environmental samples usually include a pre-enrichment step, e.g. Buffered Peptone Water (Aho, 1992; Van de Giessen, 1996). Pre-enrichment of samples with an abundant microbial flora, such as faecal samples from animals, was controversial for years, given the risk of false positives (Aho, 1992). Nevertheless, these days pre-enrichment in non-selective liquid medium is a common practice in isolation of the bacteria (Davies et al 2000).

***Enrichment.*** This is a critical point in bacteria isolation, because the selective medium is involved in competitive flora elimination and permits *Salmonella* proliferation. The enrichment in selective medium demanded by ISO 6579:2002 (Annex D) is the Modified Semi-Solid Rappaport Vassiliadis (MSRV). This medium allows the motile *Salmonella*s to spread through the medium plate. This medium favours *Salmonella* differentiation from other non-motile bacteria. Nevertheless, other media are frequently used for *Salmonella* enrichment, such as Tetrathionate broth and Selenite broth (Waltma, 2000).

***Plating-out and identification.*** These media are characterized by their "selectivity" and "differentiation". Selectivity means the addition of an inhibitory substance that prevents the growth of different Enterobacteria. Differentiation means the addition of a substance that allows us to characterize *Salmonella* against other bacteria (Mallison *et al.*, 2000). The main characters used in *Salmonella* identification are the production of sulphydric acid and the inability to ferment glucose.

***Confirmation.*** Colonies of presumptive *Salmonella* are subcultured then plated-out and their identity is confirmed by means of an appropriate biochemical and serological test (ISO 6579:2002). The most common biochemical test used to confirm presumptive *Salmonella*s is the API-20. This test consists of 20 microtubes containing dehydrated substrates. Bacterial suspension is inoculated and reconstitutes the media. During incubation, metabolism produces colour changes that are either spontaneous or revealed by the addition of reagents. The identification is obtained by referring to the Analytical Profile Index.

### *1.1.4.2. Immunoenzymatic methods applied in bacteriological diagnostic*

The capture ELISA is a common method used in bacteria diagnostics. Whereas traditional isolation methods need from 3 to 7 days to diagnose the bacteria, ELISA can detect the microorganism in 1 day. Nevertheless, in highly contaminated samples such as faeces, the test is not appropriate (Fedorka-Cray *et al.*, 1994). Several authors suggested that the main disadvantage of this method is requiring $10^4$-$10^5$ cfu/ mL to detect the microorganism (Lambiri *et al.*, 1990; Van Poucke, 1990).

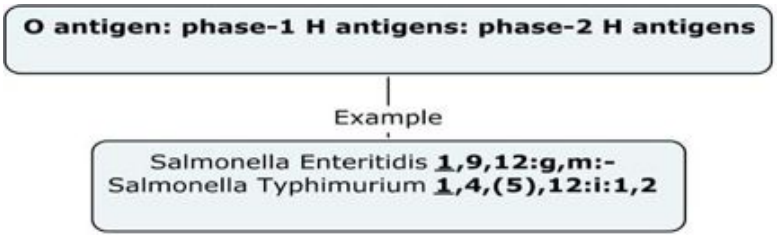
### *1.1.4.3. Typing*

The members of the genus *Salmonella* are typed into serovars which are differentiated from each other by the combinations of their somatic (O) and flagella (H) antigens and, to a lesser extent, by their biochemical reactions (Popoff and LeMinor, 1992; Popoff *et al.*, 1992).

The O antigen is part of the lipopolysaccharide component of the cell wall that also contains lipid A and a core portion. The O antigen, or O-specific side chain consists of repetitive oligosaccharide units of which the type, order and repetition of sugar moieties differ between serovars. These differences and those in the flagella antigens are used to type *Salmonella* into serovars. At least 67 different O antigens are currently known and are identified by the Arabic numerals 1 to 67. Some of these occur singly (e.g. 11), while others occur in combination (e.g. 1, 4, 5, 12; and 6, 7).

The H antigens are heat-labile and form an integral part of the flagella in those serovars which possess them. The antigens are designated by a combination of letters of the alphabet and numerals (e.g. a to z, z1 to z32 and 1 to 7). Two antigenic forms (also referred to as "phases") of the flagella may occur in culture. A culture may therefore contain cells in which the flagella are all in the same phase, or cells which possess flagella of both phases. Most of the serovars contain flagella of two phases, but in some (e.g. *S.* Dublin) the flagella occur in only one phase.

In order to establish the complete antigenic composition of any *Salmonella* serovar, antigens of both flagellar phases must be known, as well as the O antigens (Le Minor, 1984). This technique is perform by testing suspensions of the bacteria against antisera produced in rabbits against individual O and H antigens by means of a series of slide agglutination tests (Edwing, 1986). When a bacterial culture is mixed with a specific antiserum directed against bacterial surface components, the cells are bound together through antigen-antibody bonds to form aggregates (agglutination). This is usually visible to the naked eye as clumps in the suspension. By mixing specific antisera with a *Salmonella* culture, the O- and H antigens are determined. On the basis of the observed agglutination pattern, the serotype is determined using the Kauffmann-White Scheme. In this scheme the antigenic formula has three parts, as described above:



### *1.1.4.4. Phage typing*

Bacteriophage typing schemes for a number of important *Salmonella* serovars have been developed and are used internationally for epidemiological studies. The available information on the distribution of *Salmonella* serovar and phage types along the food chain varies greatly between countries. Phage typing of *S.* Enteritidis and *S.* Typhimurium is predominantly carried out according to the Colindale scheme.

In accordance with the standardized protocol, eighteen hour cultures on Blood agar plates were inoculated into 3 mL of a phage broth (double concentration nutrient broth with 0.85 % NaCl). After a 1.5 h incubation with vigorous shaking, the broth was poured onto phage agar plates. After the removal of excess broth from the plates, 10 typing phages were spotted per plate using a micropipette. Dried plates were incubated overnight at 37º C, and the phage lysis pattern of each culture was compared with a published pattern list (Kim *et al.*, 2008).

### *1.1.4.5. Molecular techniques*

Traditional methods for *Salmonella* isolation, confirmation and serotyping are too laborious and time consuming (Van de Giessen, 1996). In recent years, several advances in diagnostic technology have been developed and mean an important advance in infectious disease studies. These molecular techniques are based on the Polymerase Chain Reaction and are characterized by being simple, rapid and discriminative (Fernandez-Cuenca, 2004).

The Polymerase Chain Reaction (PCR) name derives from one of its key components, a DNA polymerase used to amplify a piece of DNA by *in vitro* enzymatic replication. As PCR progresses, the DNA generated is used as a template for replication. This sets in motion a chain reaction in which the DNA template is exponentially amplified (Feder *et al.*, 2001). With PCR it is possible to amplify a single piece of DNA across several orders of magnitude, generating millions or more copies of

the DNA piece. PCR can be extensively modified to perform a wide array of genetic manipulations.

### 1.1.5. Immunology against *Salmonella*

The immune response against *Salmonella* depends on the host and serotype involved. Cellular immune response is considered more important for the development of a protective immune response to *S.* Enteritidis than antibodies (Desmidt *et al.*, 1998).

On the basis of experimental studies related with poultry immunology, it is thought that infections at an early age include a greater risk of evolving into a carrier state, with birds becoming life-long infected. Berndt *et al.* (2004) reported that *Salmonella* bacteria that reach the intestinal tract can cross the intestinal epithelium after attachment to the mucosa. From there, they reach the lamina propria, where they replicate or proceed to deeper tissues, presumably carried within non-activated macrophages. After reaching the blood stream, they infect organs, liver and spleen. Van Immerseel *et al.* (2002) detected the bacteria 3 hours post infection in the caecal lumen and 9 h post-infection in the caecal lamina propria. Moreover, colonization of liver and spleen started more than 1 day post-inoculation. Their results suggested that initially an antigen non-specific inflammatory response mediates the clearance of bacteria in the lamina propria of neonatal chicks, thereby reducing entry of bacteria into the blood stream. Macrophages and granulocytes are the primary effector cells in this process. Attraction and activation of T-cells will further reduce bacterial invasion. Moreover, T-cells will contribute to an antigen-specific B-cell response. B-cells are already organized in follicular aggregates on day 3 post-challenge. A peak by intestinal IgA against *S.* Enteritidis paralleled the reduction of faecal excretion, indicating that the IgA response might play a role in the clearance of the bacteria from the gut (Desmindt *et al.*, 1998). Van Immerseel *et al.* (2002) also observed that structural maturation of the mucosa-associated lymphoid tissues is antigen-driven, since B-cells organize in a follicular pattern.

### 1.1.6. Antimicrobial resistance

Since the beginning of the 1990s, strains of *Salmonella* which are resistant to a range of antimicrobials, including first-choice agents for the treatment of humans, have emerged and are threatening to become a serious public health problem. This resistance results from the use of antimicrobials both in humans and animal husbandry. Multi-drug resistance to "critically important antimicrobials" is compounding the problems (WHO, 2005).

The antimicrobials most widely regarded as optimal for the treatment of salmonellosis in adults is the group of fluoroquinolones. They are relatively inexpensive, well tolerated, have good oral absorption and are more rapidly and reliably effective than earlier drugs. Third-generation cephalosporins are widely used in children with serious infections, as quinolones are not generally recommended for this age group. The earlier drugs chloramphenicol, ampicillin, amoxycillin and trimethoprim-sulphamethoxazole are occasionally used as alternatives (WHO, 2005).

Enter-net provided data on antimicrobial resistance for *Salmonella* isolates from human salmonellosis in 2006 (EFSA, 2008). *S.* Enteritidis resistance to nalidixic acid increased from 13.4 % in 2005 to 14.8 % in 2006, in sulphonamides from 6.4 % in 2005 to 8.0 % in 2006, and from 5.1 % in ampicillin in 2005 to 8.1 % in 2006, whereas resistance to ciprofloxacin remained generally at a low level (0.6 %). For *Salmonella* Typhimurium, the highest levels of resistance were observed for sulphonamide, tetracycline, ampicillin and streptomycin (59.7 %, 56.3 %, 55.6 % and 51.9 %, respectively). Compared to 2005 Enter-net data, these represent significant increases in resistance to sulphonamides and streptomycin.

When fluoroquinolones were first licensed for human therapy, no immediate rise in *Salmonella* resistance was observed. In contrast, when fluoroquinolones were subsequently licensed for use in food animals, the rates of fluoroquinolone-resistant *Salmonella* in animals and food, and then subsequently in human infections, rapidly increased in several countries (WHO, 2005).

EFSA (2008) reported the occurrence of antimicrobial resistance in *S.* Typhimurium and *S.* Enteritidis from animals. In broilers, the highest proportions of resistant isolates were reported for *S.* Typhimurium. The highest levels of antimicrobial resistance for *S.* Enteritidis were reported for nalidixic acid (overall average 27.5 %) which may reflect widespread use of quinolones in poultry production. For *S.* Typhimurium, the highest levels of resistance were reported for streptomycin (overall average 26.4 %) and tetracycline (overall average 27.3 %). In broiler meat, the highest proportions of resistant isolates were observed for nalidixic acid, streptomycin and tetracycline. Resistance to nalidixic acid was remarkably high and may indicate widespread use of quinolones for poultry (EFSA, 2008).

The emergence of multidrug-resistant strains of *Salmonella* with resistance to fluoroquinolones and third-generation cephalosporins is a serious development, which results in severe limitation of the possibilities for effective treatment of human infections (WHO, 2005).

## 1.2. *Salmonella* epidemiology in humans

### 1.2.1. Human clinical aspects

*Salmonella* is one of the major bacterial causes of gastroenteritis worldwide (Barrow *et al.*, 2003). The organism may also be transmitted through direct contact with infected animals or faecal-contaminated environments and humans. The most common presentation of non-typhoidal *Salmonella* infection is acute gastro-enteritis. Onset of intestinal salmonellosis usually occurs between a few hours and three to four days following ingestion of the infectious agent (Van de Giessen, 1996). Human salmonellosis is usually characterized by acute fever, abdominal pain, nausea, and sometimes vomiting (WHO, 2005). Symptoms are often mild and most infections are self-limiting, lasting a few days (Scherer *et al.*, 2008). However, in some patients, the infection may be more serious and the associated dehydration can be life threatening. In these cases, as well as when *Salmonella* causes bloodstream infection, effective

antimicrobials are essential for treatment. Salmonellosis has also been associated with long-term and sometimes chronic effects e.g. reactive arthritis (EFSA, 2009).

### 1.2.2. Salmonellosis in humans

There are numerous food-borne sources of *Salmonella,* including a wide range of domestic and wild animals and a variety of foodstuffs covering both food of animal and plant origin. *Salmonella* infection occurs when organisms are introduced in food preparation areas and allowed to multiply on food, e.g. due to inadequate storage temperatures, or because of inadequate cooking or cross contamination of ready-to-eat food (EFSA, 2009).

Millions of human salmonellosis cases are reported worldwide every year and the disease results in thousands of deaths (WHO, 2005). *Salmonella* causes an estimated 1.4 million illnesses each year in the USA (Mead *et al.*, 1999). In 2007, a total of 155,540 confirmed cases of human salmonellosis were reported by the European Surveillance System from 30 countries (27 EU Member States and three non-Member States, Figure 1). Salmonellosis notification rates in humans have decreased since 2005: from 173,879 (38.2 / 100,000) confirmed cases in 2005 to 164,011 (35.8 / 100,000) in 2006 and to 151,995 (31.1 / 100,000) in 2007. This represents a 7.3 % decrease from 2006. In Spain, confirmed cases of human salmonellosis have decreased since 2003: from 8,558 cases in 2003 to 7,109, 6,048, 5,117 and 3,658 in 2004, 2005, 2006 and 2007, respectively. The latest data published in Spain were from January to April 2008, when 800 cases of salmonellosis were declared (BES, 2008).


**Figure 1. Salmonellosis notification rates in humans in the EU, 2007 (per population of 100,000; EFSA, 2009).**

The age distribution of *Salmonella* cases in 2007 closely parallels that seen in 2006. Out of 151,995 reported confirmed cases, age data were available for 86.0 % of cases. The highest notification rate was for 0 to 4 years old (125.4 /100,000) which is almost three times higher than that of the next highest notification rate age group (5 to 14 years old) and almost six to nine times higher than for those aged 15 and over (Figure 2).

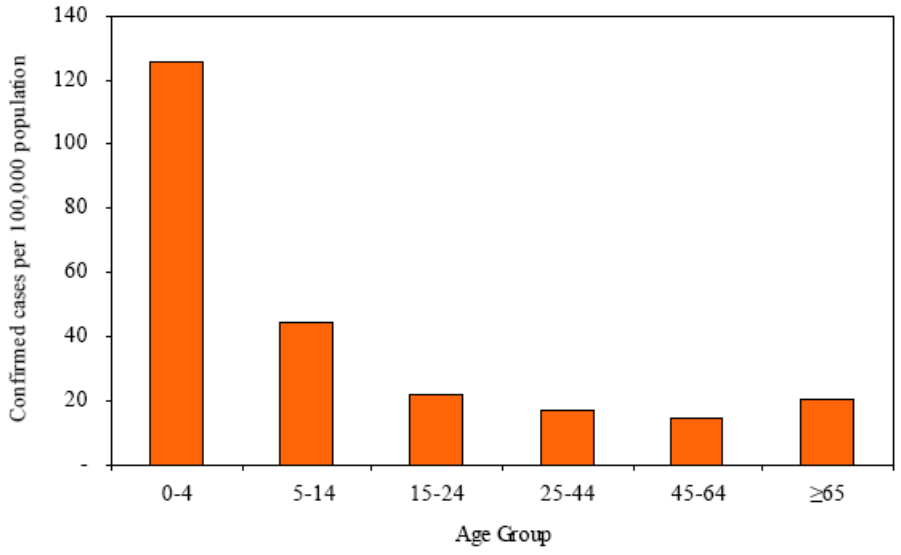
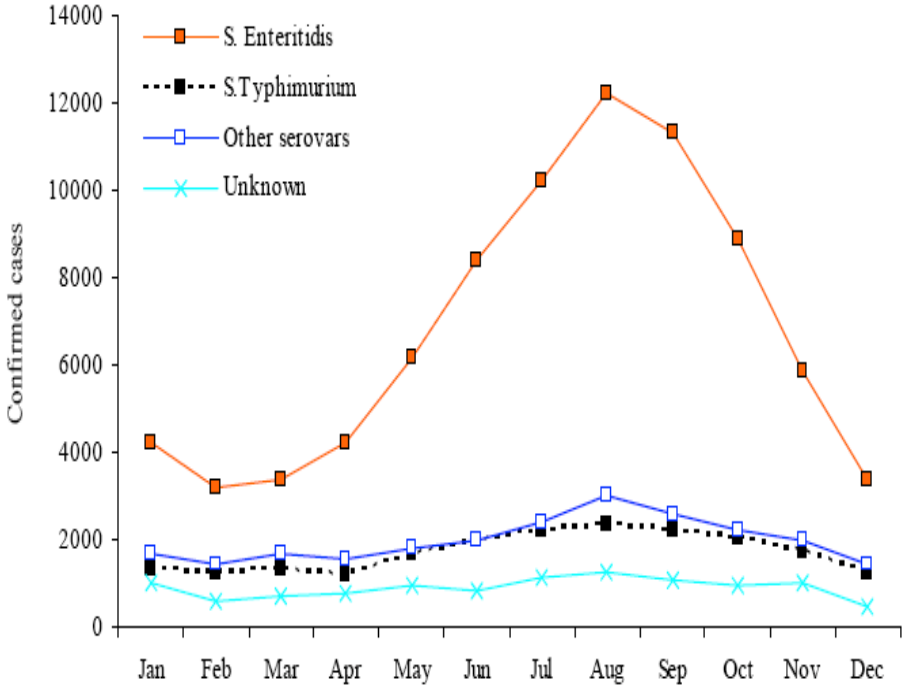



**Figure 2. Incidence of reported confirmed cases of human salmonellosis in reporting Member States and relative frequency of age group (date 2007; The EFSA Journal, 2009).**

A peak in the number of reported cases occurs in summer and autumn, with a rapid decline in the winter months (Figure 3). This pattern supports the influences of temperature and behaviour (i.e. food consumption habits such as barbequing) on *Salmonella* notification rates. This seasonal variability has been observed in earlier reports, yet when further analysing specific serovar case counts per month, *S*. Enteritidis demonstrates a much more prominent summer / autumn peak than other serovars.




Source: Belgium, Cyprus, the Czech Republic, Denmark, Estonia, Finland, France, Germany, Hungary, Ireland, Italy, Luxembourg, Latvia, Malta, the Netherlands, Portugal, Slovakia, Slovenia, Spain, Sweden and the United Kingdom (N=137,584).

**Figure 3. Number of reported confirmed salmonellosis cases in humans by month and serovar (date 2007; The EFSA Journal, 2009).**

As in previous years, the two most common *Salmonella* serovars involved in human outbreaks were *S*. Enteritidis and *S*. Typhimurium, representing 81.0 % of all known types (7.2 % were unknown), compared to 86.0 % in 2006 (EFSA, 2009). The top ten serovars were the same as for 2006, with the remaining same eight serovars, each representing one percent or less of the known top serovars, as in the previous year (Table 1). In Spain, *S*. Enteritidis and *S*. Typhimurium were the most prevalent serovars involved in human outbreaks (41.0 % and 19.0 %).

**Table 1. Distribution of confirmed salmonellosis cases in humans by serovars (10 most common serovars, date 2006-2007).**

| 2007 | | | 2006 | | |
|---|---|---|---|---|---|
| **Serotype** | **n** | **%** | **Serotype** | **n** | **%** |
| Enteritidis | 81,472 | 64.5 | Enteritidis | 90,362 | 71.0 |
| Typhimurium | 20,781 | 16.5 | Typhimurium | 18,685 | 14.7 |
| Infantis | 1,310 | 1.0 | Infantis | 1,246 | 1.0 |
| Virchow | 1,068 | 0.8 | Virchow | 1,056 | 0.8 |
| Newport | 733 | 0.6 | Newport | 730 | 0.6 |
| Stanley | 589 | 0.5 | Hadar | 713 | 0.6 |
| Hadar | 479 | 0.4 | Stanley | 522 | 0.4 |
| Derby | 469 | 0.4 | Derby | 477 | 0.4 |
| Kentucky | 431 | 0.3 | Agona | 367 | 0.3 |
| Agona | 387 | 0.3 | Kentucky | 357 | 0.3 |
| Other | 18,562 | 14.7 | Other | 12,790 | 10.0 |
| **Total** | **126,281** | | **Total** | **127,305** | |
| Unknown | 9,814 | | Unknown | 17,359 | |

Source: The EFSA Journal, 2009.

The most frequently reported phage type of *S.* Enteritidis in 2007 was PT4, which was also the most frequent in 2006 (EFSA, 2009). The top six most common phage types remained the same between 2006 and 2007, though PT8 surpassed PT1 in 2007, and two new additions, PT12 and PT1b, were added to the top ten list of *S.* Enteritidis phage types. PT 193 was, in 2007, the most common phage type of *S.* Typhimurium, followed by DT104. Six of the top ten *S.* Typhimurium phage types in 2007 were the same as in 2006. The reporting of phage types for these two serotypes increased substantially compared to 2006. However, 22.0 % of the *S.* Enteritidis and 43.0 % of *S.* Typhimurium phage types were still reported as unknown (EFSA, 2009).

## 1.3. *Salmonella* epidemiology in broiler production

### 1.3.1. Avian salmonellosis

Poultry and many other animals are often unapparent carriers, latently infected, or, less frequently, clinically ill. Poultry are commonly infected with a wide variety of *Salmonella* serovars. Infection is mostly confined to the gastrointestinal tract, and the birds often excrete *Salmonella* in their faeces and form a large reservoir and source of contamination for other animals and the environment (Poppe, 1999). In the past decade,

one *Salmonella* serotype, *Salmonella* Enteritidis, has emerged as a major serotype causing human salmonellosis (Cogan and Humphrey, 2003). Until the early 1970s, *S.* Gallinarum and *S.* Pullorum were common in poultry flocks. These serovars cause invasive disease such as typhoid and pullorum in hens and fowl, respectively, but only rarely cause illness in humans (Bullis, 1977). Culling of seropositive hens, followed by vaccination, resulted in the virtual eradication of these diseases by the mid-1970s (Baumler *et al.*, 2000). It has been postulated that the eradication of these bacteria left a niche that was filled by the antigenically similar *S.* Enteritidis. Up until this time, *S.* Enteritidis had been found occasionally in poultry and eggs but had caused only a small number of cases in humans. *Salmonella* Enteritidis may have found its way into poultry from the rodent population associated with hen houses (Cogan and Humphrey, 2003).

The fact that *S.* Enteritidis is able to infect poultry without causing observable disease, particularly in laying hens, probably assisted its spread (Cooper *et al.*, 1989), as this made detection more difficult. As *S.* Enteritidis infections of chickens increased, so did those in humans. During the early 1980s, a rapid increase in the number of *S.* Enteritidis infections in Europe and North America was observed, with PT4 being the most commonly isolated type. It has been postulated that this may have been because of infected layer breeder hens from Europe (Ward *et al.*, 2002) or to the declining number of hens with immunity to *S.* Pullorum, which would also protect against *S.* Enteritidis infection (Baumler *et al.*, 2000). It has also been suggested that the rapid spread of *S.* Enteritidis throughout Europe and the United States could indicate the emergence and expansion of a new more virulent, strain of the bacterium because of the recent acquisition of the ability to enter and persist in poultry (Rabsch *et al.*, 2001). In Australia, *S.* Enteritidis is present at levels comparable with those prepandemic in Europe. It is probable that this strain is a different clone to that now prevalent in Europe (Cox, 1995), supporting the theory that the pandemic was caused by the expansion of a new strain of the bacterium. It is more likely that all of these factors, in combination, played a role in the development of the international epidemic (Cogan and Humphrey, 2003).

### 1.3.2. *Salmonella* prevalence in broiler production

In 2007, the European Food Safety Authorities reported the results of the European Union-wide baseline survey. This study was carried out in 2005 and 2006 to determine the *Salmonella* prevalence in commercial flocks with at least 5,000 birds. The prevalence of the flocks was assessed by collecting faeces samples from the litter with five pairs of sock swabs within 3 weeks before leaving for slaughter (EC, 2005). First, the floor area of the houses was divided into five equal sectors and one pair of sock swabs was used in each sector for sampling. Samples were taken by walking over the chosen sector and each pair of sock swabs with faecal material fixed was analysed as an individual sample. This sampling procedure will theoretically provide 95.0 % confidence of detection of 1.0 % within flock prevalence, assuming the test is 100.0 % sensitive (EC, 2005). A total of 6,325 holdings corresponding to 7,440 flocks with validated results were included in the survey analyses.

The Community-observed prevalence of *Salmonella*-positive flocks was 23.7 %. This means that in the European Union one in four broiler flocks raised over the one year period of the baseline survey was *Salmonella*-positive. The *Salmonella* prevalence varied widely amongst the Member States, from 0.0 % to 68.2 %. A total of 11.0 % of the broiler flocks was estimated to be positive for *Salmonella* Enteritidis and/or *Salmonella* Typhimurium, the two most common serovars found in *Salmonella* infection cases in humans. The Member State-specific observed flock prevalence of *S.* Enteritidis and/or *S.* Typhimurium also varied greatly, from 0.0 % to 39.3 %. The five most frequently isolated *Salmonella* serovars from broiler flocks in the European Union were, respectively, in decreasing order, *S.* Enteritidis, *S.* Infantis, S. Mbandaka, *S.* Typhimurium and *S.* Hadar. All these serovars, with the exception of *S.* Mbandaka, are frequent causes of *Salmonella* infections in humans within the European Union. *S.* Enteritidis was the most common serovar and was detected in 37.0 % of the *Salmonella* positive flocks. *S.* Infantis also accounted for an important proportion of positive flocks (20.0 %). The serovar distribution varied amongst the Member States, many of them having a specific distribution pattern of their own (Figure 4, EFSA 2007).

In Spain, prevalence of *Salmonella*-positive flocks was 41.2 %. A total of 28.2 % of the broiler flocks was estimated to be positive for *Salmonella* Enteritidis and/or *Salmonella* Typhimurium. Nevertheless, *S.* Enteritidis and *S.* Typhimurium were isolated from 29.5 % and 0.3 % of the flocks, respectively. The most frequently isolated *Salmonella* serovars from broiler flocks in the Spain were, in decreasing order, *S.* Enteritidis, *S.* Hadar and *S.* Ohio (EFSA, 2008). The Regional Ministry of Environment and Rural and Marine Affairs of the Valencia Region (2009) reported a prevalence of 9.9 % *Salmonella*-positive broiler flocks in 2008. Nevertheless, the most prevalent serotypes isolated were not yet reported.


**Figure 4. Distribution pattern of most frequently *Salmonella* serovars isolated (the percentage of the *Salmonella* positive units) in the European Union broiler flocks during 2005-2006 (The EFSA Journal, 2007).**

### 1.3.3. *Salmonella* prevalence in broiler meat

*Salmonella* prevalence in broiler meat and products in 2007 was reported by the European Food Safety Authority in 2009. In 2007, 21 Member States and one non-Member State reported research covering approximately 585,000 units of broiler meat

and products. The type of products sampled varied and the analyses were either performed on single samples or on a batch of broiler meats.

Most of the countries providing data on *Salmonella* in fresh broiler meat in 2007 reported positive samples. The bacteria were detected in all Member States except in Finland. Spain, Greece and Hungary recorded the highest levels of contamination. At slaughterhouse, the reported proportion of positive samples varied from 1.0 % to 43.5 %, and at processing *Salmonella* was detected in 0.0 % to 55.6 % of the samples. At retail level, the range was from 2.3 % to 11.6 %. Denmark, Finland, Ireland, Sweden and Norway have had programmes for the control of *Salmonella* in live broilers for a numbers of years, and have reported very low levels in broiler meat from several years. Also, Sweden in 2007 had no cases of *Salmonella* in tested samples (EFSA, 2009). Eleven Member States reported specific data on *Salmonella* serovar distribution in broiler meat. Overall, *S.* Kentucky was the most frequent serovar reported from broiler meat in 2007 (EFSA, 2009). As in previous years, *S.* Enteritidis, *S.* Infantis, *S.* Typhimurium and *S.* Paratyphi B var. Java were among the most common serovars (Table 2).

**Table 2. Distribution of *Salmonella* serovars in broiler meat in the EU (date 2007, The EFSA Journal, 2009).**

| Countries | No. of isolates serotyped | % positive | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | S. Kentucky | S. Enteritidis | S. Paratyphi B var. Java | S. Infantis | S. Typhimurium | S. Hadar | S. Virchow | S. Agona | S. Ohio | S. Indiana | Other serovars, non-typeable, and unspecified |
| **Total no. of isolates** | **1,494** | **262** | **247** | **153** | **105** | **107** | **70** | **69** | **49** | **29** | **27** | **376** |
| Austria | 96 | 1.0 | 35.4 | - | 21.9 | 1.0 | 3.1 | - | - | - | 4.2 | 33.3 |
| Czech Republic | 53 | 3.8 | 34.0 | - | - | 3.8 | 1.9 | - | 9.4 | 15.1 | 3.8 | 28.3 |
| Germany | 266 | - | 26.3 | 25.2 | 8.6 | 7.9 | 2.3 | 1.5 | - | 7.1 | 5.3 | 15.8 |
| Ireland | 332 | 77.7 | 4.2 | - | 0.9 | 0.6 | - | 0.6 | 10.3 | - | 0.3 | 5.1 |
| Italy | 201 | - | 10.0 | - | 1.5 | 9.5 | 14.9 | - | - | - | - | 64.2 |
| Latvia | 21 | - | 95.2 | - | - | - | - | - | - | - | - | 4.8 |
| Luxembourg | 21 | - | 19.0 | 14.3 | 4.8 | 33.3 | 4.8 | - | - | - | - | 23.8 |
| Netherlands | 134 | - | 3.0 | 61.9 | 9.7 | 1.5 | - | 4.5 | 0.7 | 1.5 | 4.5 | 12.7 |
| Poland | 283 | - | 13.8 | - | 13.8 | 18.0 | 5.3 | 8.5 | 2.5 | - | - | 38.2 |
| Romania | 75 | - | 21.3 | - | 2.7 | - | 18.7 | 44.0 | - | - | - | 13.3 |
| Slovakia | 13 | 7.7 | 61.5 | - | - | 15.4 | - | - | 15.4 | - | - | - |
| **Proportion of serotyped isolates** | | **17.5** | **16.5** | **10.2** | **7.0** | **7.2** | **4.7** | **4.6** | **3.3** | **1.9** | **1.8** | **25.2** |

Note: Data are only presented for sample size ≥10. The serovar distribution (% isolates) was based on the number of reported serotyped isolates, including non-typeable and unspecified isolates. Ranking was based on the sum of all reported serovars. Some countries may not have a strict separation of serotypes achieved from meat and farm level

Domestic processing of broiler meat is an important concern for human outbreaks. In Spain, 100.0 % of *Salmonella* outbreaks declared in 2007 were domestic (EFSA, 2009). Broiler meat is typically consumed in a well cooked form; properly prepared broiler meats do not pose a health risk for consumers. *Salmonella* infection risk arises from undercooking of the broiler meat or from cross-contamination from raw broiler meat to other dishes during preparation in the kitchen. All the above considerations suggest that good kitchen hygiene and thorough cooking of broiler meat will prevent or reduce the risk of *Salmonella* outbreaks.

### 1.3.4. Main sources of *Salmonella* contamination in the poultry sector

Many epidemiological studies have demonstrated the wide variety of routes by which *Salmonella* can be disseminated within integrated poultry companies (Rose *et al.*, 1999:2000; Heyndrickx *et al.*, 2002; Davies and Breslin, 2003; Namata *et al.*, 2008). Prevention of *Salmonella* contamination in poultry products requires detailed knowledge of the most important sources associated with its presence in the production system (Slader *et al.*, 2002).

Vertical transmission of *S.* Enteritidis and *S.* Typhimurium from the parent flock to day-old chicks leaving the hatchery has often been reported and implemented as a main controlling factor in many eradication programmes (Bisgaard, 1992; Limawongpranee *et al.*, 1999). *S.* Enteritidis is related to the extraordinary biology of the infection in the avian host. It has now been clearly established that these strains can cause lifelong colonization of the peri-reproductive tissues of the hens from which the eggs can be colonized before the shell is formed (EFSA, 2007).

Horizontal transmission in hatcheries and at farm level during the rearing period is of greater importance in broiler production. In addition, it leads to the isolation of a greater variety of *Salmonella* serovars (Bailey *et al.*, 2001:2002). The horizontal infection of poultry could be via digestive or respiratory tract. When the bacteria are transmitted orally, the digestive tract may reduce their number because of gastric secretions, flora, defensins or mucus, until the caeca colonization (Figure 5). Once the

bacteria reach the caeca, they may attach to the epithelia and multiply to high numbers in a relatively short period of time (Cox *et al.*, 1996). In this situation, the birds will be excreting large numbers of *Salmonella*e in their caecal droppings, a situation which will result in the contamination of other birds and the house environment (Cox *et al.*, 1996). Moreover, the bacteria could infect the chicken via the respiratory tract, reach the lungs and penetrate the mucosa until the internal organs without defensive barriers (Figure 5, Cox *et al.*, 1996).

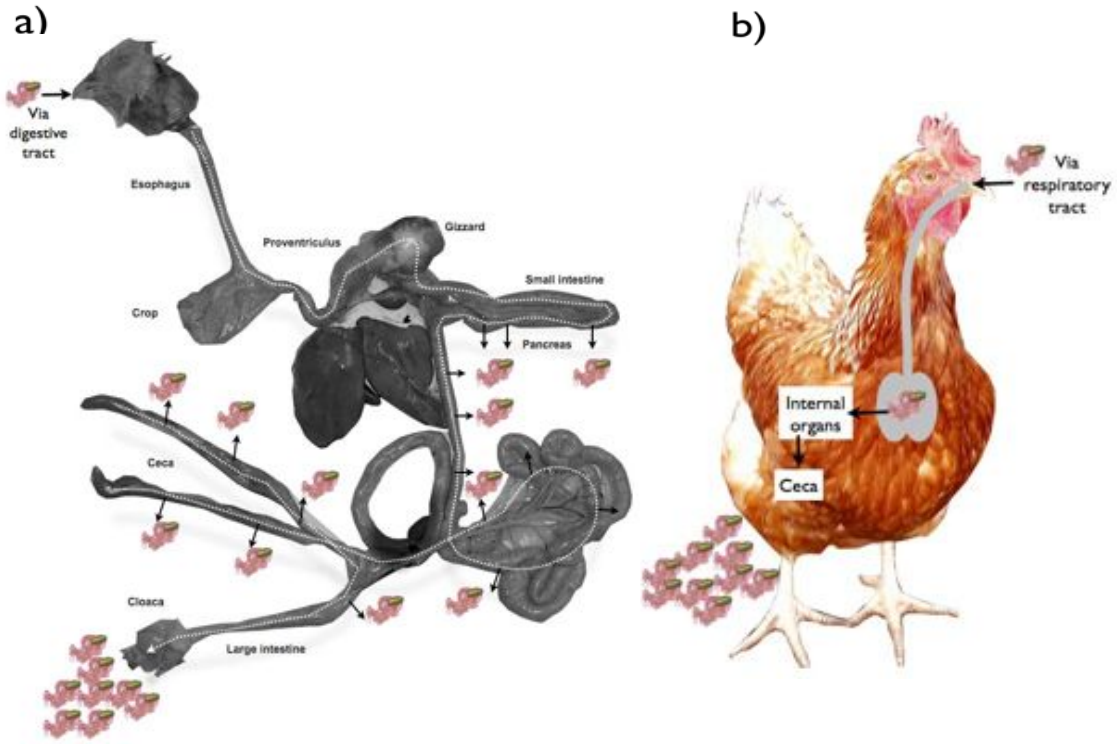



**Figure 5. Poultry horizontal infection with *Salmonella.* a) Via digestive. b) Via respiratory tract.**

Several authors have studied and identified the main risk factors for horizontal transmission, and include inadequate management factors as inaccurate house cleaning and disinfection between consecutive flocks, contamination of feed and water, *Salmonella* status of day old chicks, bedding status and inaccurate disinfection of farming clothes and boots (Rose *et al.*, 1999; Heyndricks et al 2002; Rojas *et al.*, 2002; Davies and Breslin, 2003; Doyle and Erickson, 2006). Other risk factors are: the size of the farm, flock density, rearing of flocks in wet seasons or the presence of carriers on the farms, such as rodents, litter-beetles, wild birds and flies (Chriél *et al.*, 1999; Davies

*et al.*, 2001; Davies and Breslin 2003; Carrique-Mas *et al.,* 2008). Contamination of *Salmonella* negative flocks during transport to the slaughterhouse through contaminated slaughter-trucks has been also described by several authors (Ramesh *et al.*, 2002; McCrea *et al.*, 2006).

Historically, in most broiler organizations, when cleaning and disinfection was carried out ineffectively, carry-over of infection was the predominant risk factor in broiler production (Baggesen *et al.*, 1992). Davies and Wray, (1996) reported that persistence of *Salmonella* environmental contamination after disinfection occurred for at least 1 year in an empty poultry building. Rose *et al.* (2000, France) in broiler houses and Davies and Breslin (2003, United Kingdom) in laying houses also observed inaccurate cleaning and disinfection procedures applied in broiler houses against *Salmonella*. Every year, many resources are spent on cleaning and disinfection of animal houses, both as a general means to minimize infection pressure and to target specific organisms (Gradel *et al.*, 2004). Disinfection as a routine part of the management procedures has probably increased with the intensification of animal husbandry. Moreover, more focus has been placed on this topic with the implementation of programmes against specific microorganisms (Gradel *et al.*, 2004).

Feed was often considered as a key route for introduction of new *Salmonella* infections into poultry flocks (Davies *et al.*, 1997; Heyndrickx *et al.*, 2002). Several reports reveal that pelleted poultry feed subjected to a heat treatment (60-80 ºC) reduces the *Salmonella* contamination rate of the feed (Himathongkham *et al.*, 1996). Nevertheless, when feed remain in close contact with house environment, it could be an important source of *Salmonella* spreading. Heyndrickx *et al.*, (2002) reported a significant relation between the flock status and the high contamination level of the feed from feeders within the chicken houses. Moreover, feed contamination could be a result of a contaminated house environment and/or poultry and rodents shedding *Salmonella*.

Water is also an important vehicle of enteric pathogens such as *Campylobacter* species or *E. Coli* (LeJeune *et al.*, 2001; Kemp *et al.*, 2005). Lahellec *et al.* (1986) suggested contaminated water as an important source of *Salmonella* contamination. Different authors described the importance in bacteria spread of water pipes (Davies and

Wray, 1996) and water drinkers (Lahellec *et al.*, 1986). However, different authors reported that water infection source is not common in poultry production, although it has been reported (Kinde *et al.*, 1996; Heyndrickx *et al.*, 2002).

Day-old chicks infected with *Salmonella* were reported as a major *Salmonella* contamination risk factor by several authors (Christensen *et al.*, 1997; Hoover *et al.*, 1997; Rose *et al.*, 1999; Cox *et al.* 2000). *Salmonella* can be transmitted vertically from infected breeders to the day-old chicks (Chriél *et al.*, 1999) or transmitted horizontally during hatching, loading and transport to the farm (Cox *et al.*, 1996; Cason *et al.*, 1994). The fact that a *Salmonella* control of day-old chicks was not requested by the farmer was associated with the risk to a flock of becoming contaminated, favouring *Salmonella* recontamination of broiler houses (Rose *et al.* 1999). Thus, the production companies have to deliver day-old chicks systematically *Salmonella* free to their integrated farms (Rose *et al.*, 1999).

Farmer management is usually attributed as a factor in *Salmonella* persistence and recontamination of the houses (Rose *et al.*, 1999). Wet or dirty environment, or the bedding quality and dirty plumage may facilitate the growth of *Salmonella* (Chriél *et al.*, 1999). Contaminated boots and clothing used within houses are potential entering sources of *Salmonella* and are allowed to recontaminate the broiler houses by the farmers or outside visitors (Davies *et al.*, 1997; Rose *et al.*, 1999; Gradel *et al.*, 2002). Farmers who have a general sense of order and system are more successful in eliminating an incoming *Salmonella* infection than the less-diligent farmers (Gradel *et al.*, 2003). Bedding also has to be correctly managed, because straw piled up outside the farm could be contaminated by wild animals. Rojas *et al.* (2002) reported that broilers often ingest litter during rearing, constituting an important source of chicken infection.

Another important reason for the persistence of *Salmonella* in poultry production is the presence of farm pests, particularly in commercial laying farms, where rodents have been shown to present a major risk in relation to the carry-over of *Salmonella* between flocks (Davies and Wray, 1995; Davies *et al.*, 2001; Carrique-Mas *et al.*,

2008). Otherwise, in broiler production it is difficult for mice to consistently occupy the house with nowhere for them to avoid pecking from the chickens (Gradel *et al.*, 2003). Free-living mice tend to be territorial, so do not disseminate any infection widely (Pocock *et al.*, 2001). However, they can become infected with as few as 15 cells and may therefore be responsible for amplifying a low level of environmental contamination which might otherwise have posed a low risk of direct infection of poultry (Davies and Breslin, 2003). Mice can also acquire the infection from inaccessible parts of the house and then deposit contaminated droppings directly into feeding systems and onto egg belts (Davies and Breslin, 2003). Rose *et al.* (2000) reported that the risk for *Salmonella* persistence in broiler units was two times higher when rodents were observed by the farmers. Nevertheless, other pests such as flies are common and can multiply in houses throughout the year; they can travel up to 32 km and so could transmit *Salmonella* widely (Graczyck *et al.*, 2001). Beetles, especially carnivorous species such as *Alphitobius* and *Carcinops*, are important predators of fly eggs and larvae and can also carry *Salmonella* for short periods, but probably constitute a smaller risk than flies (Gray *et al.*, 1999). Otherwise, Jones *et al.* (1991) suggested that litter beetles may be capable of carrying *Salmonella* and perpetuating the infection in poultry houses. Wild birds, especially where there are large breeding populations with access to animal faecal waste, may be reservoirs and vectors of *Salmonella* (Literak *et al.*, 1996), but like mice they are more likely to be found on farms where livestock are already infected (Craven *et al.*, 2000).

Chriél *et al.* (1999) reported that the flock size, number of houses located on the farm and the annual number of productions within the house had no significant association with the risk of *Salmonella* contamination. Angen *et al.* (1996) evaluated chicken density, flock size and size of the house, and found no association with the *Salmonella* status of the flocks. Similar non-significant findings have been described by Waldroup *et al.* (1992), who analysed the effect of animal density on the infection pressure in broilers after rearing them under different densities. Seasonality had been reported as an important risk factor for *Salmonella* contamination (Chriél *et al.*, 1999). Broiler flocks that hatched in summer period showed a lower risk of being infected with *Salmonella*, compared to chickens hatched in the early winter period (Chriél *et al.*, 1999). This variation corresponds with earlier described seasonal patterns for

*Salmonella* contamination in broilers, indicating a higher risk during wet and cold period (Davies and Wray, 1996).

Contamination of *Salmonella* poultry flocks during transport to the slaughterhouse and processing at the slaughter plant have been observed as apparent sources of *Salmonella* contamination (Doyle and Erickson, 2006). The importance of transport in broiler flock contamination is described below.

### 1.3.5. Importance of the *Salmonella* status of the flock

*Salmonella* species are common contaminants in poultry. Typically, these pathogens are carried in the animal intestinal tract asymptomatically; however, they can be shed in faeces in large populations (Poppe, 1999). Chickens are often unapparent carriers, which has contributed to hindering the diagnosis of *Salmonella* at farm level (Doyle and Erickson, 2006). Nevertheless, *Salmonella* infection can be directly diagnosed at farm level or at the abattoir by isolating *Salmonella* with various established bacteriological methods or by serodiagnosis using ELISA based on lipopolysaccharide antigens (Christensen *et al.*, 2002). Culture methods are laborious, time-consuming, and costly. Serological techniques have proven to be practical and cost-effective methods and therefore more suitable for routine diagnosis (Mousing *et al.*, 1997). However, serological screening methods indicate exposure to *Salmonella* but cannot differentiate between acute or subclinical infection (Lo Fo Wong *et al.*, 2003). On the other hand, several studies reported that litter sampling utilizing several pairs of overshoes provided the highest sensitivity for determining the *Salmonella* status of the broilers during rearing (Skov *et al.*, 1999; Buhr *et al.*, 2007). So, important aspects in *Salmonella* surveillance and monitoring programmes are the type of sample and the time of sampling to determine the flock status with the highest sensitivity (Heyndrickx *et al.*, 2002).

From a consumer's viewpoint, continuing efforts are needed to reduce the incidence of *Salmonella* in poultry production. In this regard, information on the infection dynamics of *Salmonella* during the rearing and faecal shedding rates of

*Salmonella* can be a useful tool (Scherer *et al.*, 2008). Vaccination and hygienic measures have considerably reduced the vertical transmission of the infection from the parent flocks (Van Immerseel *et al.*, 2004). Nevertheless, on commercial broiler farms, two of the major problems are contaminated houses and infected day-old-chicks (Rose *et al.*, 2000; Davies and Breslin, 2003). Young chickens (less than 2 weeks) are extremely susceptible to infection by *Salmonella* spp. (Bailey *et al.*, 2001). When day-old-chick flocks are contaminated with the bacteria, there is a rapid spread of *Salmonella* throughout the house, as the rest of the birds ingest the bacteria, being infected in few days (Heyndrickx *et al.*, 2002). These results may be explained because the enteric immune system does not fully mature until some weeks after hatching (Beal *et al.*, 2004). Van Immerseel *et al.* (2004) reported that young chicks infected with *Salmonella* resulted in persistent excretion for at least 18 weeks of rearing. In this study, faecal shedding of infected chickens, determined with cloacal swabs, was higher in the first weeks of rearing and then became negative a few weeks post infection. Most birds sampled showed intermittent shedding during the study period. Similar results were obtained by Beal *et al.*, (2004), who reported that irrespective of age at exposure, *Salmonella* infection of young birds persists until between 10 and 12 weeks, well beyond the slaughter age for broiler chickens. It is unclear why the infection resulted in a persistent excretion of *Salmonella*, but this may be related to differences in development of immunity after infection (Van Immerseel *et al.*, 2004). These conclusions have implications for the broiler sector and indicate the need to remain *Salmonella* free throughout the rearing period (Beal *et al.*, 2004).

*Salmonella* shedding is not delimited to the hatchery and rearing period (McCrea *et al.*, 2006). Feed withdrawal, holding and transportation from farm to slaughterhouse are known to be stressful for poultry (Slader *et al.*, 2002). Stress causes a disturbance of intestinal functions and may lower the resistance of the live animal and increase spreading of intestinal bacteria (Scherer *et al.*, 2008). Stern *et al.* (1995) reported that transport to the processing plant increased the prevalence of positive birds due to faecal contamination of skin and feathers by neighbouring infected birds during shipping. Related with this hypothesis, Rigby and Pettit (1980) reported that prolonged crating of the animals was a contributor to the contamination of processed broiled carcasses. Moreover, the use of contaminated trucks during transport is a great concern as

*Salmonella* could infect free flocks (Slader *et al.*, 2002; Heyndickx *et al.*, 2002). The number of contaminated crates at the farms and the results of washing experiments demonstrated that cleaning and disinfection procedures at slaughterhouses had little effect against *Salmonella*. One reason is the remaining organic matter detected regularly on truck crates after washing (Slader *et al.*, 2002). On the other hand, Corry *et al.* (2002) suggested that there was limited evidence for infection or contamination of birds from dirty crates. They reported that this may happen because it was not possible to examine the crates immediately before the birds were loaded. Nevertheless, serovars isolated before cleaning were those present in the flock at the farm.

At the processing plant, several studies suggested that processing procedures increase contamination by *Salmonella* (Corry *et al.*, 2002; Slader *et al.*, 2002; McCrea *et al.*, 2006). Along the production line, as processing continues the product comes into contact with surfaces that may have built up residual tissue debris containing *Salmonella*s that grow and contaminate other carcasses. Carramiñana *et al.* (1997) observed that some contributory factors may have arisen from heavily contaminated water used for scalding. They also reported that *Salmonella* serotypes isolated from faeces were later detected in matched carcasses and livers indicating a cross-contamination of carcasses by endogenous microflora in bird faeces. Due to the previous considerations, faecal shedding throughout transport provided cross-contamination between carcasses and equipment during processing, increasing the contamination status of the final food products (McCrea *et al.*, 2006).

In conclusion, several authors reported that lowering the farm prevalence of the bacteria that are potential food pathogens and reducing stress during loading practices and transport has been suggested as an important strategy for lowering the risk of contaminated meat products entering the food chain (McCrea *et al.*, 2006; Belles, 2007).

### 1.3.6. *Salmonella* persistence in broiler production

Several studies were carried out related to factors that can influence *Salmonella* persistence, as reported above. However, inaccurate cleaning and disinfection and extermination of rodents were two of the most important factors related with current *Salmonella* persistence in poultry production (Rose *et al.*, 2000; Davies *et al.*, 2001; Davies and Breslin, 2003; Carrique-Mas *et al.*, 2008). Several hypotheses explain why cleaning and disinfection is insufficient to remove *Salmonella* from hatcheries, rearing houses, slaughter truck crates and processing lines, such as resistance to the disinfectant (Gradel *et al.*, 2005), inaccurate concentration of disinfectant (Ramesh et al, 2002), inadequate temperature (Gradel *et al.*, 2004), water hardness (Davison *et al.*, 1996), biofilm development (Ramesh *et al.*, 2002) and presence of remains of organic matter (feed, fats, egg yolk, Gradel *et al.*, 2004).

Repeated use of the same types of antibiotics is known to favour the development of antibiotic resistance, but less is known about the use of disinfectants (Gradel *et al.*, 2004). A few disinfectant types are commonly used in the poultry sector (such as glutaraldehyde, formaldehyde and peroxygen, Gradel *et al.*, 2004). Theoretically, this could induce resistance to the disinfectant used, which could explain the persistence of *Salmonella* (Gradel *et al.*, 2005). However, there is a lack of studies published on the role of the mechanisms that explain the resistance to disinfectants commonly used in the agricultural sector (Gradel *et al.*, 2005).

It has also been demonstrated that cellulose production and biofilm formation may be important for the survival of *Salmonella* on surface environments; in fact, cellulose-deficient *Salmonella* strains did not develop biofilm (Latasa *et al.*, 2005). Biofilms are defined as a large number of bacteria surrounded by an exopolysaccharide matrix. In recent years, biofilm development mechanisms have been studied by several authors (Bonafonte *et al.*, 2000; Cucarella *et al.*, 2001; Solano *et al.*, 2002; Garcia *et al.*, 2004; Latasa *et al.*, 2006). Lasa (2007) reported that life in a biofilm state protects the bacteria against environmental insults like chemical sanitizers, which are generally unable to eliminate most biofilm-associated bacteria. These authors also reported that

microorganisms that live in a biofilm state are 1,000 times more resistant than microorganisms in suspension. Holah *et al.* (1990) reported that the most effective disinfectants against bacterial cells in suspension may not be as effective when treating bacterial cells embedded in a Biofilm. Ramesh *et al.* (2002) tested 13 commercial disinfectants against *Salmonella* biofilms on metallic surfaces such as transport containers. This study suggested that use of hypochlorite (0.05 %) and alkaline peroxide compound (1.0 %) applied under the prescribed regimen could result in effective elimination of *Salmonella* in biofilm state. Nevertheless, this study was done *in vitro* conditions and was not applied under field conditions.

Another hypothesis for the survival of the bacteria on surface environments is that poultry houses have inaccessible equipment and considerable amounts of organic matter and high contents of protective compounds (fats, carbohydrates and proteins) from which *Salmonella* are difficult to remove (Gradel *et al.*, 2004). On one hand, different studies have shown that formaldehyde and glutaraldehyde are effective disinfectants to use in presence of organic matter, unlike other disinfectants (Davies and Wray, 1995; Gradel *et al.*, 2004). On the other hand, disinfectants such as peroxygen had been suggested to have low efficacy against bacteria because of their susceptibility to organic matter (Russell and Chopra, 1996; Amass *et al.*, 2001). There are also considerable differences in the effectiveness of different approved products within the same chemical group because of variations in the individual ingredients and formulations. Flock owners, particularly large integrated companies and their veterinary advisers, should therefore ensure that sufficient in-house trials are carried out to establish that the concentration and application rate of the disinfectants used are sufficient to eliminate *Salmonella* from contaminated environment within the houses (Davies and Breslin, 2003).

The efficacy of a proper disinfection procedure regarding *Salmonella* in poultry houses is often ruined by the presence of *Salmonella*-infected mice remaining or returning to the house after cleaning and disinfection (Davies and Wray, 1995:1996). The role of rodents has been reported in several studies (Kinde *et al.*, 1996; Rose *et al.*, 2000; Davies *et al.*, 2001; Gradel *et al.*, 2003; Davies and Breslin, 2003; Carrique-Mas *et al.*, 2008). Rodents can acquire the infection from inaccessible parts of the house and

then deposit contaminated droppings directly into feeding systems and onto egg belts (Davies and Breslin, 2003). Moreover, mice produce contaminated droppings for 2 months after infection and the droppings are actively sought out by birds when mixed in their food or bedding (Davies and Wray, 1995). Droppings can contain up to $2.3x10^5$ cfu *Salmonella* per dropping, more than sufficient to infect newly hatched chicks (Henzler and Opitz, 1992). Dead mice can also be found in poultry houses which have been cleaned and disinfected, and the *Salmonella* in these carcasses may be a hazard for the new flock. Carcasses contain higher levels of bacteria than droppings and may be pecked by chickens. In this way, chickens become infected by the bacteria (Davies and Wray, 1995).

## 1.4. Study cornerstone

*Salmonella* has long been recognized as an important zoonotic pathogen of economic significance in animals and humans. There are numerous sources of human salmonellosis, although eggs and poultry meat are considered the most common source of human infection (EFSA, 2009). According to Jimenez and Martin (2004), 75.0 % of the human salmonellosis outbreaks in Spain are related with eggs and poultry meat consumption. In this sense, legislators are working to minimize *Salmonella* prevalence in poultry sectors with the introduction of a National Control Programme to reduce the incidence of the bacteria in poultry flocks. The programme for broiler flocks sets out measures to reduce the prevalence of *S*. Enteritidis and *S*. Typhimurium, the strains which pose the highest human health risk, to 1.0 % or less by 31 December 2011 (EC, 2007). However, greater efforts in poultry production are needed, because the official survey on the prevalence of *Salmonella* in broiler flocks reported that in Spain the currently prevalence is around 41.2 % (EFSA, 2007).

Poultry is an important production sector in Spain and *Salmonella* control programmes could entail important economic losses in 2011. Productivity in the Spanish poultry sector has been enhanced over recent decades (MARM, 2008). Nowadays, broiler rearing is one of the most important livestock activities in Spain. Fresh poultry meat is the most frequently consumed fresh meat and comes second after

pork meat in total meat consumed in Spain (MARM, 2008). The Region of Valencia is the third broiler producer in Spain after Catalonia and Andalusia, with 15.8 % of broiler meat production (Martinez *et al.*, 2008). The Region has a total of 493 broiler farms throughout the territory, with 13,900,079 fattening places (Martinez *et al.*, 2009).

Before *Salmonella* prevalence in broiler flocks (EFSA, 2007) and the Community objectives for *Salmonella* control on poultry farms were established (EC, 2007), no official data were available regarding the *Salmonella* situation in broiler production in Spain. In this context, in 2005 the Regional Ministry of Environment and Rural and Marine Affairs of the Valencia Region invested public funding in a project involving the study of the main sources of *Salmonella* contamination in broiler flocks reared in the Valencia Region and the study of *Salmonella* epidemiology in field conditions. These studies were intended to anticipate the National Control Programme for *Salmonella* in broiler production, with the aim of applying suitable measures in line with the future community objectives.

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# CHAPTER 2. OBJECTIVES

This study started in October 2005 and finished in October 2007. Over two years, 65 flocks from different farms were intensively sampled during the rearing period. Broiler farms were distributed throughout the Region of Valencia.

The main objectives of this study were:

(i) To determine the main sources for *Salmonella* contamination in broiler flocks and determine the main serotypes involved in broiler production in the Region of Valencia.

(ii) Assess the main risk factors for *Salmonella* contamination of the flock at the end of the rearing period.

(iii) Select the best moment of sampling to assess the *Salmonella* status of the broiler flocks.

(iv) Assess the influence of live transport to the slaughterhouse on *Salmonella* detection.

(v) Evaluate biofilm development capacity of strains isolated in poultry samples.

(vi) Evaluate the most common disinfectants used in poultry against *Salmonella* biofilm and non-biofilm strains in field conditions.

# CHAPTER 3. EXPERIMENTAL DESIGN

# 3.1. Main sources of *Salmonella* contamination in broiler production

## 3.1.1. Abstract

Prevention of *Salmonella* contamination of poultry products requires detailed knowledge of the main sources associated with its presence in the production system. The aims of this study were to determine the main sources of *Salmonella* contamination in broiler production, assess the risk factors for *Salmonella* contamination of broiler flocks at the end of the rearing period, and determine the serotypes involved in broiler production system of the Valencia Region. A total of 65 different broiler houses from different farms were sampled in the Valencia Region between October 2005 and October 2007. Each house was visited 11 times during the rearing period. First, when the previous flock was taken to the slaughterhouse, samples of dust, surfaces and faeces were collected; after cleaning and disinfection, samples of dust and surfaces were also taken. On the first day of rearing, samples of water, bedding, farming boots, meconiums, delivery-box liners and feed were collected. During rearing, 6 visits took place and feed samples were taken. On slaughter day, samples of dust, surfaces, water, feed and faeces were also collected. Finally, two days after slaughter, carriers (rodents, flies and beetles) were trapped. All samples were analysed according to ISO 6579:2002 (Annex D) and positive samples were serotyped in accordance with Kauffman-White-Le-Minor technique. Our results showed that all different types of samples collected were contaminated with *Salmonella* (ranged between 1.5 % and 38.6 %), except for some samples of feed collected from the truck. The most contaminated samples related with poultry production were: delivery-box liners, faeces samples, dust samples, farming boots and feed from feeders. However, the most important risk factors for *Salmonella* contamination of the flocks at the end of the rearing period were contaminated feed from feeders, *Salmonella* status of the house after cleaning and disinfection and *Salmonella* status of day-old chick flocks. Twenty-one different serotypes were isolated from the samples analysed. The most prevalent were in decreasing order: *S.* Enteritidis (52.9 %), *S.* Hadar (17.8 %), *S.* Virchow (8.9 %) and *S.* Ohio (5.4 %). The study suggested that there are many sources for *Salmonella*

contamination and persistence in broiler production. So, whole production chain needs to be controlled to eradicate the bacteria from the primary production.

### 3.1.2. Introduction

Salmonellosis is an important public health concern associated with food consumption of animal origin (EFSA, 2009). According to Jiménez and Martín (2004), eggs and poultry meat are involved in 75.0 % of human salmonellosis outbreaks in Spain, constituting an important threat to Public Health. In recent years, Food Safety has become an important concern for European society and governments, so that many stricter and stronger regulations were imposed throughout the production chain with the aim of guaranteeing and increasing consumer confidence in food from animal sources (EC 2003:2007).

Many epidemiological studies have reported the wide variety of routes by which *Salmonella* can be disseminated within integrated poultry companies in Europe (Rose *et al.*, 1999:2000; Hendrickx *et al.*, 2002; Davies and Breslin, 2003; Namata et al, 2008). However, to our best knowledge, in Spain there are no studies related with risk factors for *Salmonella* contamination in broiler flocks. Historically, an inaccurate cleaning and disinfection has been reported as an important risk for *Salmonella* persistence in poultry houses (Davies and Breslin, 2003; Rose *et al.*, 2003; Gradel *et al.*, 2005). Moreover, removing rodents and insects during production break is also an important factor in *Salmonella* control (Davies and Breslin, 2003). These authors reported that the presence of contaminated carriers, especially rodents, is involved in recontamination of houses after cleaning and disinfection. Rose *et al.* (2000) suggested that two of the most important risk factors are the *Salmonella* status of the previous flock and day-old chick flocks. Infection in day-old flocks could be vertical from infected breeder flocks or horizontally transmitted during hatching, loading and transport to the farm (Cox *et al.*, 1990; Cason *et al.*, 1994; Chriél *et al.*, 1999) and, at farm level, from the house environment (Rojas *et al.*, 2002; Davies and Wray, 1996).

In addition, Heyndrickx *et al.* (2002) reported that feed and water in broiler houses are risk factors significantly related to the status of the flock. The role of feed and feed ingredients in the spread of *Salmonella* through the poultry industry has received a great deal of attention (Bailey *et al.*, 2001). Less than one *Salmonella* per gram of feed has been shown to establish colonization in 1 to 7 day-old chicks (Waldroup *et al.*, 1992). Livestock drinking water has also been considered as a major source of exposure of food-borne pathogens (LeJeune *et al.*, 2001).

Other factors, such as the flock size, number of houses located on the farm and the annual number of productions within the house had been reported as posing no significant risk of *Salmonella* contamination (Chriél *et al.*, 1999). Moreover, Waldroup *et al.* (1992) reported that the effect of animal density on the infection pressure is not significant for *Salmonella* contamination of the flock.

The objectives of this study were (i) to determine the main sources for *Salmonella* contamination in broiler production, (ii) assess the main risk factors for *Salmonella* contamination of broiler flocks at the end of the rearing period, and (iii) determine the main serotypes involved in poultry production systems of the Valencia Region.

### 3.1.3. Material and methods

#### Study sample

Over two years (October 2005 and October 2007), 65 commercial broiler farms from the Valencia Region were sampled. Only one flock was studied on each farm. These farms belong to 5 companies, which have the majority of the poultry slaughtered in the Valencia Region. To participate in the study, farms had to be commercial broiler farms with chickens reared on the floor. The location of the farms and the day of placing the chicks were provided by the companies. All the farm owners were willing to cooperate during the lifespan of the flock.

**Moment of sampling for *Salmonella* in broiler flocks**

Each farm was visited eleven times during the flock lifespan (Figure 6). The first visit occurred when the previous flock left for the slaughterhouse (before cleaning and disinfection, C&D). The next visit was after C&D. Then, the farm was visited again just before placing day-old chicks (Day 1). During the rearing period, each farm was visited six times. Finally, last visits were on slaughter day, between day 42 and day 49 of rearing (common fattening period) and two days after slaughter.

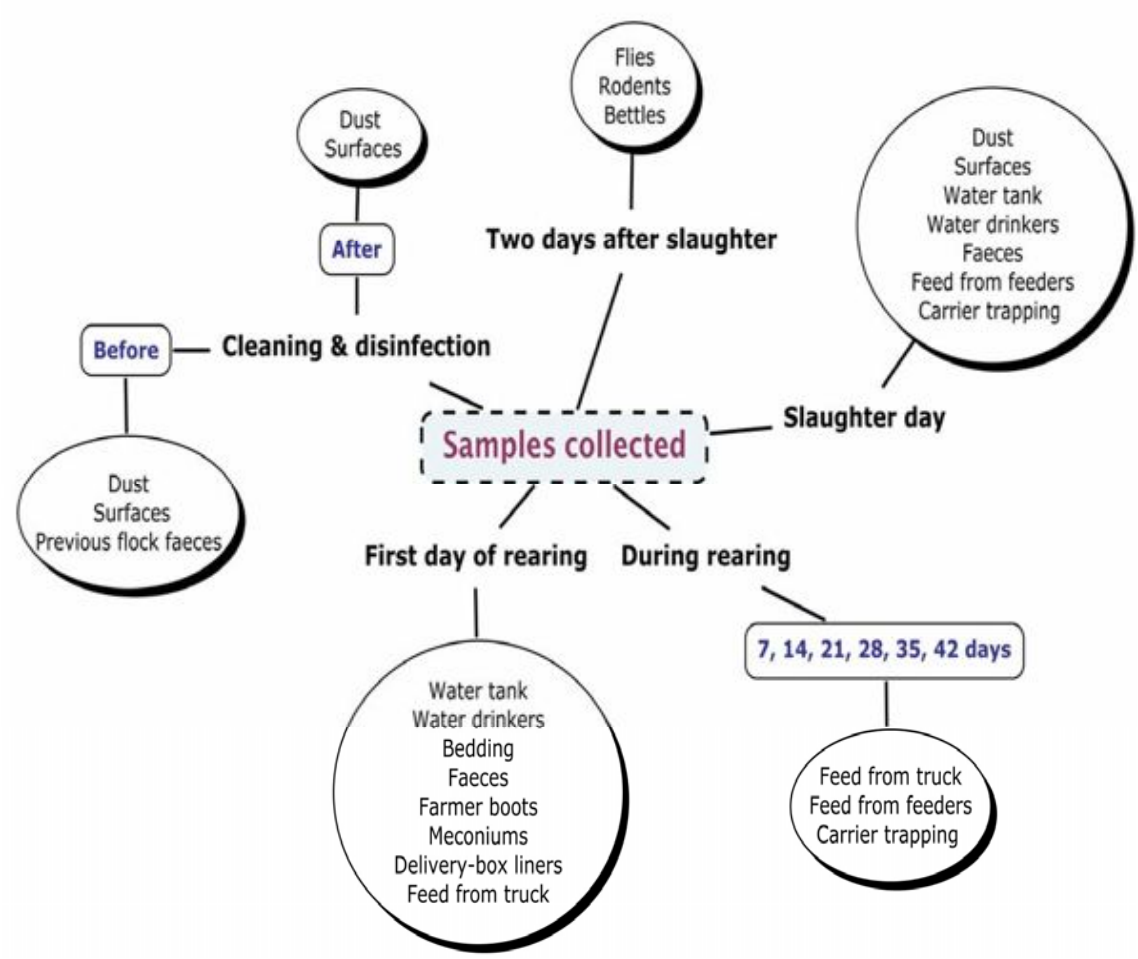


**Figure 6. Sampling visits and samples collected during the lifespan of each broiler flock.**

***Before cleaning and disinfection:*** To assess the *Salmonella* status of the previous flock, faeces were taken from the litter with five pairs of sock swabs as recommended by the European Food Safety Authority (EC, 2005). First, the floor area of the houses was divided into five equal sectors and one pair of swab-socks was used

in each sector for sampling. Faeces were taken by walking over the chosen sector and each pair of swab-socks with faecal material fixed was analysed as an individual sample (Figure 7).


**Figure 7. Sampling method in accordance with the EC (2005). a) Faeces were taken by walking over the chosen sector. b) Each pair of swab-socks with faecal material fixed was removed with sterile gloves. c) Each pair of socks was analysed as an individual sample.**

Then, 100 g of dust (250 mL) was collected from different points of the house, the sample was homogenized at the laboratory and 25 g were analysed (Figure 8). Finally, a surface sample was taken from wall crevices and floor joints with sterile wet gauze pads (AES laboratories®, Bruz Cedex, France). Broiler houses were declared contaminated if at least one of the samples taken tested positive for *Salmonella*.

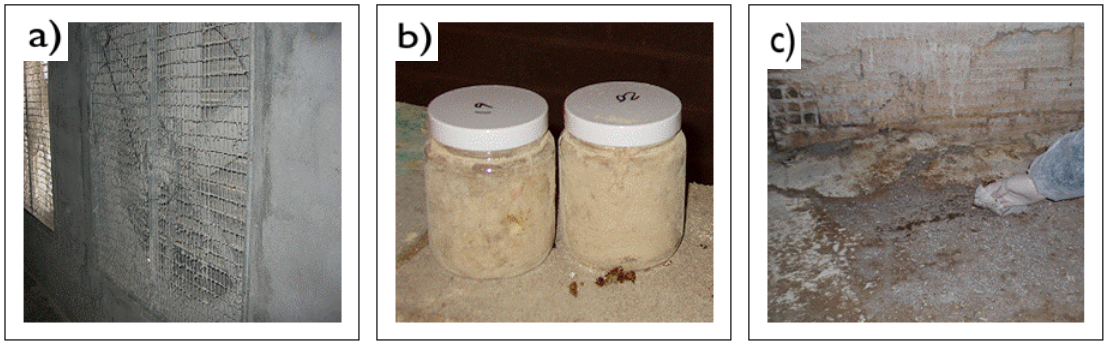


**Figure 8. Environmental sampling. a) Fan which is a suitable point to take dust samples. b) Dust samples taken at the farm. c) Surface samples collected with a sterile wet gauze pad.**

***After cleaning and disinfection:*** *Salmonella* status of the house was assessed taking samples of dust as described above. However, surface samples were taken with sterile wet gauze pads with disinfectant neutralizer (AES laboratories®, Bruz Cedex,

France). Data concerning the C&D procedures applied in each broiler house were collected. Broiler houses were declared contaminated if at least one of the samples taken tested positive for *Salmonella*.

***First day of rearing:*** Two water samples were taken (500 mL): one from the tank and another from final dispenser lines (Figure 9). Water samples were homogenized at the laboratory and 25 mL was analysed from each source.

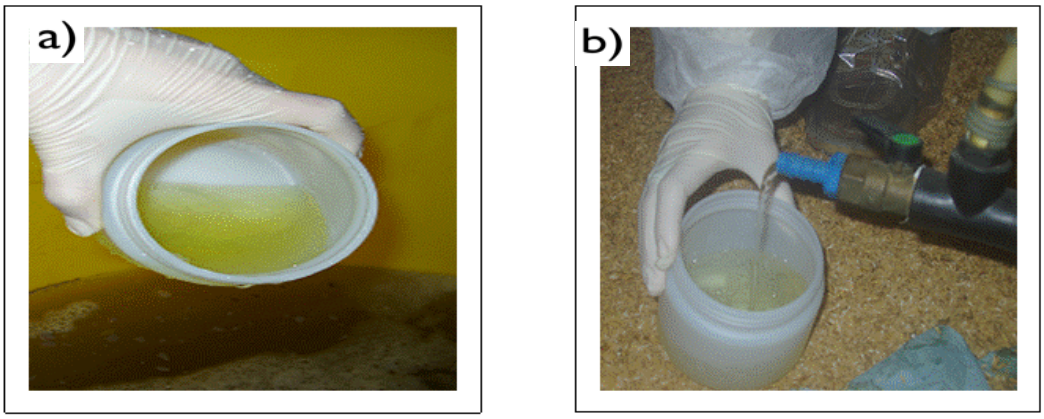


**Figure 9. Water sampling. a) Water sample collected from a water tank. b) Water sample collected from final dispenser lines.**

When the feed-truck arrived at the farm, one sample was collected directly from the truck (500 g, Figure 10). Then, the feed sample was homogenized at the laboratory and 25 g was analysed. Moreover, one sterile jar of bedding was filled from 6 different points of the house (500 g). Finally, farming boots were swabbed with sterile wet gauze pads with disinfectant neutralizer (AES laboratories®, Bruz Cedex, France).

**Figure 10. Feed sample taken directly in a sterile pot from the truck.**

In order to determine the *Salmonella* status of day-old chick flocks, in accordance with the Commission Regulation (EC, 2003), meconiums were obtained from 250-300 chicks (Figure 11). At same time, when present, 10 chick delivery-box liners were collected by placing the whole consignment into sterile bags. Day-old chick flocks were declared contaminated if at least one of the samples taken tested positive for *Salmonella*.

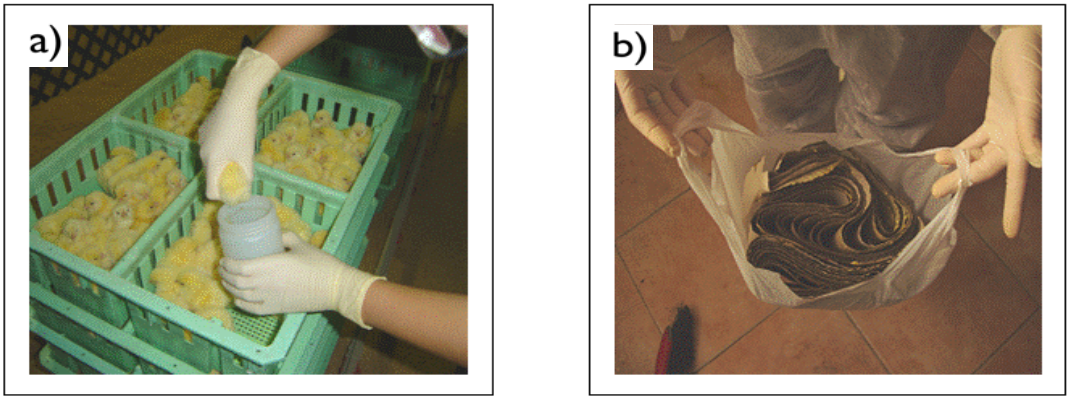


**Figure 11. Testing *Salmonella* status of day-old chick flocks. a) Meconium sampling. b) Delivery-box liners sample collected at farm level.**

***During the rearing period:*** Feed samples where taken every time that the composition changed and a new truck arrived at the farm: age 1 (delivered first 14 days of rearing), age 2 (delivered from day 14 to day 21), age 3 (delivered from day 21 to day

35) and age 4 (delivered from day 35 until slaughter). When the feed-truck arrived at the farm, one sample was collected directly from the truck (500 g). Then, after one week in close contact with house environment and chickens, one feed sample was also collected from the end of feed liners (feeders, 500 g, Figure 12). Then, the samples were homogenized at the laboratory and 25 g was analysed. Moreover, in the last week of rearing, fly feeder traps (Econex®, Castellon, Spain) with insecticide (Agita® 1GB of Novartis, Barcelona, Spain and Quick Bayt® of Bayer, Barcelona, Spain) were installed inside the house for 7 and 10 days to trap flies (Figure 13).

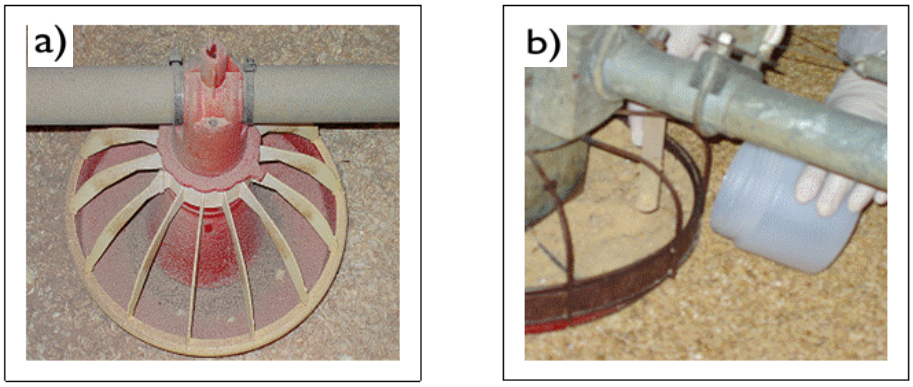


**Figure 12. Feed sampling after one week in close contact with the house environment. a) Remainder feed in broiler feeder. b) Feed sampling from feeders.**

***At the end of rearing period:*** samples of dust, surfaces, water (from the tank and from final dispenser lines) and feed from feeders were taken as reported above. Also, faeces from the litter were taken in accordance with the European Food Safety Authority guidelines (EC, 2005, Figure 7). When the flock left for the slaughterhouse, rodent traps (Cage All®, Tom cat® and T-Rex® form Bell, USA) and beetle traps (25 cm PVC tubes with corrugated cardboard inside) were installed on the litter for two days (Figure 13).

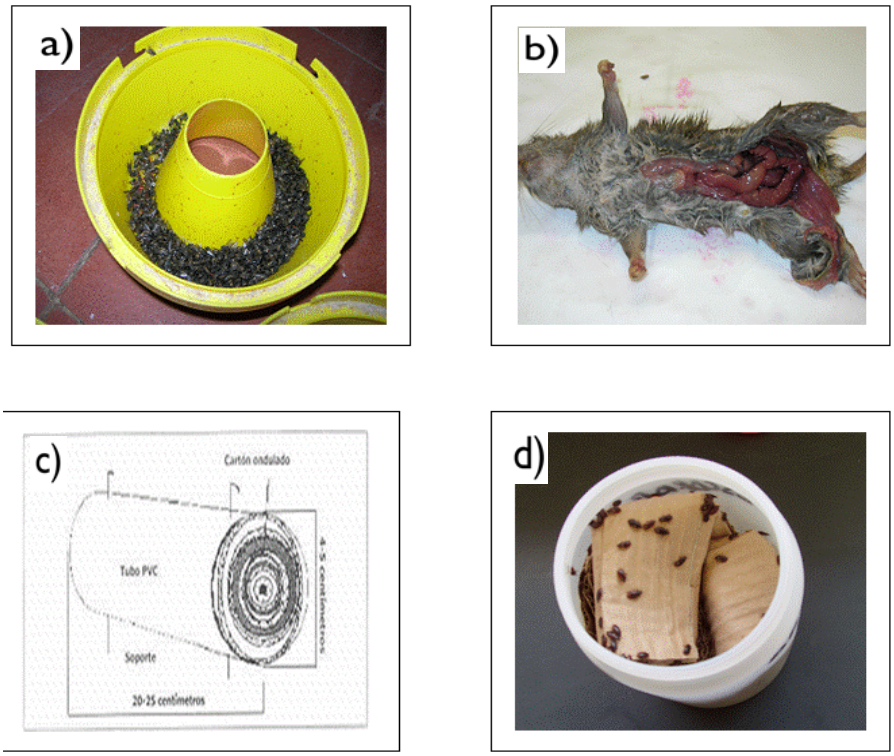


**Figure 13. Carrier sampling in broiler houses a) Fly feeder traps with flies. b) Intestines of rodent carcasses removed aseptically for culture. c) Beetle trap scheme (REA, 2003). d) Beetle sample collected from the bedding with beetle traps.**

***Two days after slaughter:*** Flies, rodents and beetles traps were collected. Flies and beetles captured were analysed as a pool. Liver, spleen and intestines of rodent carcasses were removed aseptically for culture (Figure 13).

### *Salmonella* isolation

The samples were collected directly into 500 mL sterile sample jars and analysed according to ISO 6579:2002 (Annex D). First, the samples were pre-enriched in 1:10 vol/vol Buffered Peptone Water (BPW, Scharlau®, Barcelona, Spain) and then incubated at 37±1 ºC for 18±2 h. The pre-enriched samples were transferred onto Semi-Solid Modification Rappaport Vassiliadis (MSRV, Difco®, Valencia, Spain) agar plate (0.1 mL) and incubated at 41.5±1 ºC for 24-48 h. The culture obtained in MSRV was inoculated onto Xylose-Lysine-Desoxycholate (XLD, Liofilchem®, Valencia, Spain) and Xylose-Lysine-Tergitol-4 (XLT4, Biokar Diagnostics®, Pantin Cedex, France) and incubated at 37±1 ºC for 24-48 h. After incubation, 5 colonies of *Salmonella* were

streaked onto the surface of pre-dried nutrient agar plates (Scharlab®, Barcelona, Spain) 37±1 ºC for 24±3 h. Then, a biochemical test API (API-20®, bioMerieux, Madrid, Spain) was done to confirm *Salmonella* spp (Figure 14). Moreover, *Salmonella* strains isolated were serotyped by the Ministry of Environment and Rural and Marine Affairs Reference Laboratory (Algete, Madrid, Spain) in accordance with Kauffman-White-Le-Minor technique. According to this technique, each strain has to be mixed with polyvalent and monovalent antisera until the antigenic formula is determined. One drop of antisera has to be mixed with the strain in circular movements. If an agglutination reaction was observed, the reaction was considered positive. If agglutination was not observed, the reaction was considered negative.

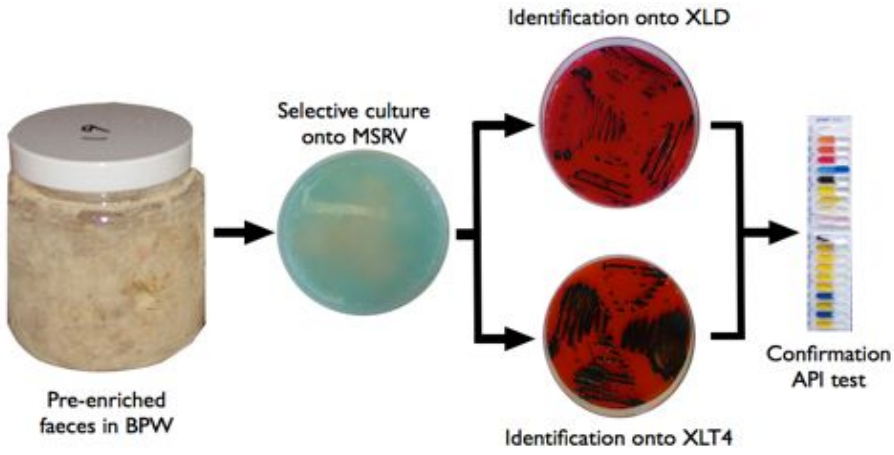


**Figure 14. ISO 6579:2002 (Annex D) scheme. BPW: Buffered Peptone Water. MSRV: Modified Semisolid Rappaport-Vassiliadis. XLD: Xylose-Lysine-Desoxicolate. XLT4: Xylose-Lysine-T-4.**

## Statistical analysis

The prevalence of *Salmonella* contamination according to the type of sample collected and the moment of sampling (previous flock leaving, first day of rearing, during rearing and at the end of rearing) were compared by a Chi-square Test. On the other hand, a two-stage procedure was used to assess the relationship between samples collected and *Salmonella* status of the flock at the end of rearing period. The unit of observation was the flock. A flock was declared contaminated by *Salmonella* if one or more samples taken from the house at the end of rearing period tested positive. The outcome variable was thus dichotomous (contaminated flock *vs.* non-contaminated flock). Logistic regression analysis was used according to the method described by

Rose *et al.* (1999). In the first stage, a univariable analysis was performed to relate *Salmonella* contamination of the flock to each sample. Only factors associated with *Salmonella* contamination of the flock were considered for the next analysis (Chi-square Test, $P \leq 0.25$). The second stage involved a logistic multiple-regression model which included all factors that passed the first screening test. The contribution of each factor to the model was tested using a Chi-squared Test. The variable with the highest *P* was removed and the logistic regression was rerun. This process was continued until a model was obtained with all factors significant at $P \leq 0.05$. Statistical analyses were performed using a commercially available statistics package (Statgraphics Plus, Version 5.1, STSC Inc., Rockville, MD, USA).

### 3.1.4. Results

During this study, 65 broiler houses from different farms were sampled to determine *Salmonella*. A total of 2,036 samples were taken at different times of the rearing period and the total prevalence of positive samples was 13.6 %.

**Environmental *Salmonella* contamination of broiler houses during the rearing period**

When the previous flock left for the slaughterhouse, *Salmonella* prevalence of the samples according to the moment of sampling (before and after C&D) were statistically different (P=0.009). The results showed that 41.3 % and 20.0 % of the broiler houses were positive for *Salmonella* before and after C&D, respectively. Before C&D, houses were contaminated (from highest to lowest) with faeces from the previous flock (28.6 %), dust (24.6 %) and surfaces (15.2 %, Table 3). No significant differences were found between *Salmonella* contamination and the type of sample collected before C&D (P=0.202). After C&D, 20.0 % of the houses remained positive for *Salmonella* in dust and surfaces (12.3 % and 10.8 %, respectively. Table 3). No traces of faeces from the previous flock were observed.

**Table 3. Percentage of *Salmonella*-positive houses by samples collected the day of previous flock leaving for the slaughterhouse.**

| | Before C&D | | | After C&D | | |
|---|---|---|---|---|---|---|
| **Samples** | **n** | ***Salmonella* (%)** | **S.E** | **n** | ***Salmonella* (%)** | **S.E** |
| **Dust** | 57 | 24.6 | 5.7 | 65 | 12.3 | 4.1 |
| **Surfaces** | 59 | 15.2 | 4.7 | 65 | 10.8 | 3.9 |
| **P.F.F** | 63 | 28.6 | 5.7 | - | - | - |

n: Number of houses sampled. P.F.F: Previous flock faeces. S.E: Standard error. *Salmonella* (%): Percentage of *Salmonella* positive houses.

C&D procedures were managed by the farmer in all cases. Prior to disinfection, houses were washed using a pressure washer and allowed to dry. The disinfectant active ingredients used were: glutaraldehyde, formaldehyde and a formaldehyde-glutaraldehyde compound. No statistical differences were found in the efficacy of each treatment on the levels of contamination after C&D (P=0.990).

On the first day of rearing, houses were contaminated as described above in dust and surfaces at different rates. Moreover, houses were contaminated (from highest to lowest) in farming boots (19.7 %), bedding (7.7 %), water from drinkers (3.2 %) and water from the tank (1.5 %). Later, when chicks arrived from the hatchery, 31.2 % of the flocks were determined positive in meconiums (14.1 %) and/or delivery-box liners (32.0 %, only 40 flocks presented delivery liners). Therefore, after day-old chicks' arrival, 43.0 % of houses were again contaminated with the bacteria. Significant differences were found between *Salmonella* contamination and the type of sample collected on the first day of rearing (P=0.010, Table 4).

**Table 4. Percentage of *Salmonella*-positive houses by samples collected on first day of rearing.**

| Samples | n | *Salmonella* (%) | S.E. |
|---|---|---|---|
| **W. tank** | 65 | 1.5[a] | 1.5 |
| **W. drinkers** | 63 | 3.2[a] | 2.2 |
| **Boots** | 61 | 19.7[b] | 5.1 |
| **Bedding** | 65 | 7.7[a] | 3.3 |
| **Day-old chick** | 64 | 31.2[b] | 5.8 |

n: Number of houses sampled. W.: Water. S.E: Standard error. *Salmonella* (%): Percentage of *Salmonella* positive houses.

Significant differences were also found between *Salmonella* contamination of feed samples collected from the truck and those collected from feeders (P=0.000). The majority of feed samples collected from the truck were negative. Feed for age 1 and 4 arrived at the farm *Salmonella*-free and feed for age 2 and 3 were contaminated with the bacteria in 3.7 % and 4.8 % of cases, respectively. However, after one week of feed arrival at the farm, *Salmonella* prevalence in samples collected from feeders was 22.4 %, 15.5 %, 18.7 % and 7.0 % for ages 1, 2, 3 and 4, respectively (Figure 15).

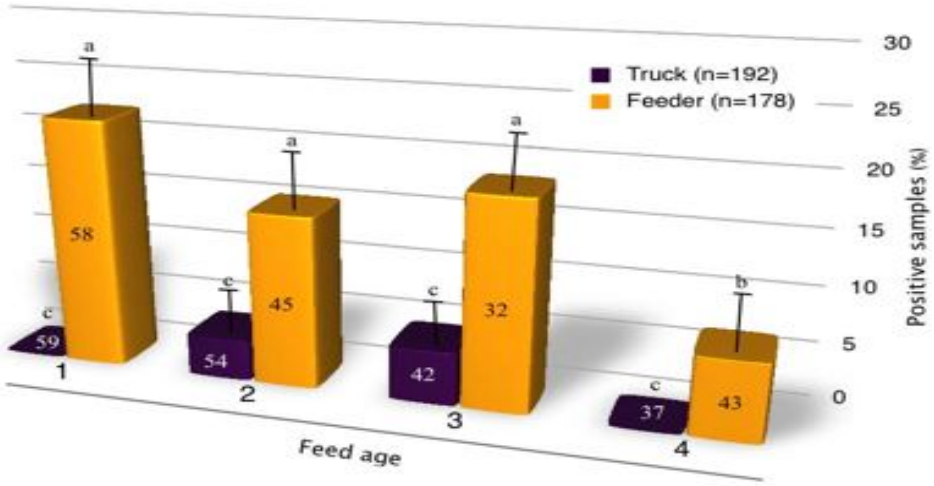


**Figure 15. Percentage of *Salmonella*-positive feed samples collected from the truck and from feeders throughout the rearing period. [a, b] Different superscripts within columns of feed from feeders indicate a significant difference (*P≤0.05*). [c] Superscripts within columns of feed from truck indicate that there is no significant difference (*P>0.05*). Data inside the bars are the number of feed samples collected.**

At the end of rearing period, 49.2 % of broiler houses assessed were environmental contaminated with *Salmonella*. Significant differences were found

between *Salmonella* contamination and the type of sample collected ($P$=0.000, Table 5). Houses were contaminated (from highest to lowest) in faeces (33.9 %), dust (25.4 %), surfaces (11.1 %), water from drinkers (3.2 %) and water from the tank (1.5 %).

**Table 5. Percentage of *Salmonella*-positive houses by samples collected at the end of the rearing period.**

| Samples | n | *Salmonella* (%) | S.E |
|---|---|---|---|
| **Dust** | 65 | 25.4[b] | 5.5 |
| **Surfaces** | 65 | 11.1[c] | 4.0 |
| **W. tank** | 65 | 1.5[a] | 6.0 |
| **W. drinkers** | 63 | 3.2[ac] | 1.5 |
| **Faeces** | 62 | 33.9[b] | 2.2 |

n: Number of houses sampled. W: Water. S.E: Standard error. *Salmonella* (%): Percentage of *Salmonella* positive houses. [a-c] Different superscripts within columns indicate a significant difference ($P \leq 0.05$).

The total prevalence of positive carriers trapped was 14.3 %. No significant differences were found between *Salmonella* contamination and the carrier trapped ($P \leq 0.225$). The prevalence was (from highest to lowest): litter beetles (17.4 %), flies (13.6 %) and rodents (5.4 %).

## Main risk factors for *Salmonella* contamination in broiler flocks at the end of the rearing period

The results of this study suggested that contaminated feed collected from feeders, *Salmonella* status of the house after C&D and *Salmonella* contamination of day-old chick flocks were the main risk factors related to *Salmonella* contamination of the flock at the end of the rearing period ($P$=0.000, Table 6).

**Table 6. Relationship between the house status after cleaning and disinfection and the samples collected on first day of rearing in *Salmonella* status of the flock at the end of rearing period.**

| Samples | First day of rearing | | |
|---|---|---|---|
| | n | P-value | 95 % C.I |
| **House after C&D** | 65 | 0.001 | 0.000-0.484 |
| **Tank water** | 65 | 0.999 | |
| **Drinker water** | 63 | 0.099 | |
| **Bedding** | 65 | 0.999 | |
| **Farming boots** | 61 | 0.729 | |
| **Day-old chicks** | 64 | 0.028 | 0.004-1.020 |
| **Feed from truck** | 65 | 0.417 | |
| **Feed from feeders** | 65 | 0.000 | 0.000-1.000 |

Logistic-regression model: Model P-value=0.000; Model deviance=28.3 %. Percentage of deviance explained by the model=60.6 %.

In contrast, factors such as water, feed collected from the truck, bedding and farming boots do not seem to be related to *Salmonella* contamination of the flock at the end of the rearing period (Table 6).

## Serotypes isolated in broiler production related samples

In this study, a total of 259 *Salmonella* strains were isolated and 21 different serotypes were determined. The most prevalent serovar isolated was *S.* Enteritidis (52.9 %), followed by *S.* Hadar (17.8 %), *S.* Virchow (8.9 %) and *S.* Ohio (5.4 %, Figure 16). The rest of the serovars isolated (15.1 % of the total) were in decreasing order: *S.* Mbandaka (3.1 %), *S.* Senftenberg (2.7 %), *S.* Infantis (1.2 %), *S.* Typhimurium (1.2 %), *S.* Altona (1.2 %), *S.* Bonn (0.8 %), *S.* Brikama (0.8 %), *S.* Muenchen (0.8 %), *S.* Diarizonae (0.4 %), *S.* Ordonez (0.4 %), *S.* London (0.4 %), *S.* Mikawasima (0.4 %), *S.* Enterica (0.4 %), *S.* Goldcoast (0.4 %), *S.* Havana (0.4 %), *S.* Menden (0.4 %), and *S.* Muenster (0.4 %).

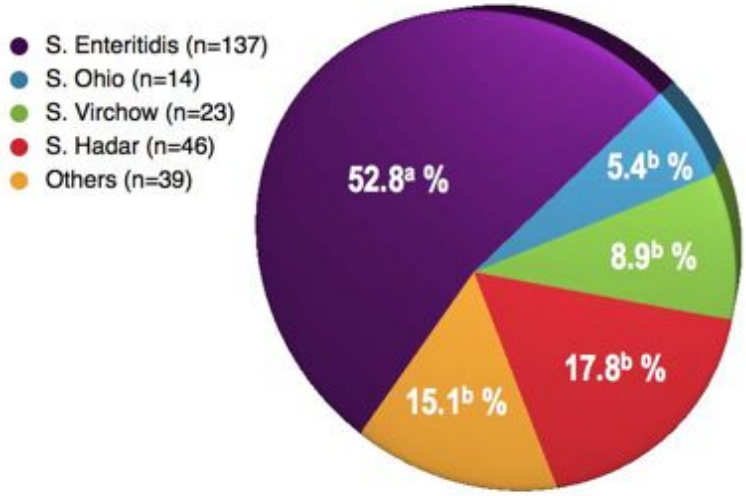


**Figure 16. Most prevalent serotypes isolated from poultry samples. [a, b] Different superscripts indicate significant differences ($P \leq 0.05$). Numbers within graphic showed the isolation percentage of each serotype.**

### 3.1.5. Discussion

Prevention of *Salmonella* contamination in poultry products requires detailed knowledge of the most important risk factors associated with its presence in the production system. This study suggested that delivery-box liners, faeces, dust, farming boots and feed from feeders are in decreasing order the most contaminated samples related with poultry production during the rearing period (Wray and Davies, 1994; Davies and Wray, 1996; Rose *et al.*, 1999; Davies *et al.*, 2001; Heyndrickx *et al.*, 2002). Nevertheless, feed from feeders, *Salmonella* status of the house after C&D, and *Salmonella* status of day-old chicks are the main risk factors related to the *Salmonella* status of the flock at the end of the rearing period (Rose *et al.*, 1999:2000; Heyndrick *et al.*, 2002). There is a significant relation between the flock status and the high contamination level of *Salmonella* collected from feeders (Heyndrickx *et al.*, 2002). Several hypotheses could explain feed contamination during rearing. Results showed that dust was highly contaminated after C&D, and so may settle in open feeders and contaminate *Salmonella*-free feed. Moreover, rodents that could acquire the infection from inaccessible parts of the house may deposit contaminated droppings directly into feeding systems (Davies and Breslin, 2003). Another hypothesis is when day-old chick flocks arrived at the farm highly contaminated with the bacteria. Broiler chicks gain

access to feeders and tainted feed in early weeks, coinciding with the highest excretion of *Salmonella* in faeces (Van Immerseel *et al.*, 2004). Then, the rest of the birds ingest the bacteria and the whole flock may be infected in a few days (Heyndrickx *et al.,* 2002).

On the other hand, the status of the house before placing the chicks is an important risk factor for *Salmonella* contamination of broiler flocks, being an important source of new flock infection (Rose *et al.*, 1999). The importance of dust as a *Salmonella* reservoir between flocks has been reported by several authors (Rose *et al.*, 2000; Davies and Breslin, 2003; Mueller-Doblies, 2009). Thus, when growing started, contaminated dust could infect feeders, litter, carriers, ventilation systems and finally the growing flock (Rose *et al.*, 1999:2000; Davies and Breslin, 2003). Several hypotheses are related with the high persistence of *Salmonella* after C&D (breeder houses, hatcheries, broiler houses…), such as the lack of scientific literature on disinfection in the agricultural sector (Ramesh *et al.*, 2002). Davies *et al.* (2001) reported that occasional inconsistencies in application of regimes which are effective against the bacteria led to the persistence of *Salmonella* infection. Consequently, it is important for C&D to be supervised adequately to ensure that procedural errors do not take place.

Several authors determined *Salmonella* status of day-old chicks as an important risk factor involved in *Salmonella* contamination of the flock (Christensen *et al.*, 1997; Rose *et al.*, 1999; Cardinale *et al.*, 2004). Although broiler houses are properly disinfected, there is an important risk of environmental contamination when chicks are delivered to the farm shedding the bacteria in faeces (Van Immerseel *et al.*, 2004). On the other hand, samples protected from chickens and environmental crossed contamination (water tank, final water lines, bedding and feed from trucks) showed lower prevalence and do not seem to be related with *Salmonella* contamination of the flock at the end of the rearing period.

Farming management has been reported as an important factor for *Salmonella* contamination. Rose *et al.* (1999) reported that farmers are able spread the bacteria with

their boots and clothes between consecutive flocks. Our results suggested that despite the high rates of contamination from farming boots, boots are not related with the contamination of the flock at the end of rearing. The role of pests in *Salmonella* persistence in poultry houses has been reported in several studies (Davies and Wray, 1995; Kinde *et al.*, 1996; Rose *et al.*, 2000; Davies *et al.*, 2001; Davies and Breslin, 2003; Gradel and Rattenborg, 2003). According to these authors, our results suggested that carriers from broiler houses are frequently contaminated with the bacteria. So, pest control management has to be implemented effectively to minimize the chance of flock infection (Davies and Breslin, 2003).

The main important serotype isolated from broiler production in this study was *S.* Enteritidis, in agreement with European reports (EFSA 2007). Moreover, contamination with *S.* Enteritidis is an important threat to food safety, especially regarding egg production and broiler meat in Spain (Jiménez y Martín, 2004). *S.* Hadar and *S.* Virchow also showed high prevalence, in line with European reports (EFSA 2007). While the Community reduction target will most likely be set for a transitional period only for *S.* Enteritidis and *S.* Typhimurium, it is recommended that Member States address in their national *Salmonella* control programmes also other serovars when these serovars are of Public Health importance in their country (EFSA, 2007). If not, prevalent serovars, such as *S.* Hadar and *S.* Virchow in Spain, could fill the niche left by *S.* Enteritidis and *S.* Typhimurium, as reported before for other serotypes involved in poultry production (Cogan and Humphrey, 2003).

In conclusion, the most contaminated samples related with poultry production throughout the rearing period are delivery-box liners, faeces, dust, farming boots and feed from feeders. Moreover, the main risk factors for *Salmonella* contamination of broiler flocks at the end of the rearing period are feed from feeders, *Salmonella* status of the house after C&D, and *Salmonella* status of day-old chicks. Nevertheless, results suggested that there are many sources for *Salmonella* persistence in poultry houses and the whole production chain has to be controlled to eradicate the bacteria from the primary production. The most prevalent serovar isolated from broiler production in the Valencia Region is *S.* Enteritidis, followed by *S.* Hadar and *S.* Virchow.

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## 3.2. *Salmonella* detection in faeces during broiler rearing and after live transport to the slaughterhouse

### 3.2.1. Abstract

Eggs and poultry meat are a common source of human salmonellosis. Contamination of poultry or poultry meat may occur throughout the production chain. Nevertheless, in Spain non contaminated broiler meat may be sold for human consumption as of 2011. The aim of this study was to assess *Salmonella* detection from faeces samples during the rearing, and assess the influence of live transport to the slaughterhouse on *Salmonella* detection. During this study, 65 flocks were sampled at weekly intervals from first day of rearing until slaughter. Samples of faeces were taken from the litter using five pairs of cellulose sock swabs attached to boots and applied over the length of the house (EC, 2005). To assess *Salmonella* detection rates before and after live transport to the slaughterhouse, faeces samples were collected. Before loading, faeces samples were taken as described above with five pairs of cellulose sock swabs (EC, 2005). After transport, two pooled faeces samples were taken directly from the truck (200-300 g each; EC, 2005). All samples were analysed in accordance with ISO 6579:2002 (Annex D). Results showed that regardless of whether broiler flocks arrived at the farm already shedding the bacteria in faeces, or were infected during rearing, both groups described the same detection pattern, with the highest detection in faeces at 14$^{th}$ day of rearing (50.5 % and 34.5 %, respectively). Moreover, *S*. Enteritidis was the most prevalent serotype isolated during rearing (66.7 %), followed by *S.* Virchow (13.7 %), *S.* Hadar (9.4 %) and *S.* Ohio (2.8 %). On the other hand, before loading and after transport to the slaughterhouse 15.4 % and 41.2 % of faeces samples collected were determined positive, respectively. In addition, a change in the serotype pattern was also observed. *S.* Enteritidis remains the most prevalent serotype isolated (54.5 %). *S.* Hadar doubled the excretion rates (39.3 %), and *S.* Virchow and *S*. Ohio were not isolated after transport.

### 3.2.2. Introduction

Consumption of poultry products contaminated with *S. enterica* is one of the most common sources of human gastroenteritis, especially in eggs and poultry meat (EFSA, 2009). Contamination of poultry or poultry meat may occur throughout the production chain and continuous efforts are therefore needed to reduce the incidence of *Salmonella* in broiler production (Heyndrickx *et al.*, 2002).

Vaccination and hygienic measures have considerably reduced the vertical transmission of the infection from the parent flocks (Van Immerseel *et al.*, 2004). Nevertheless, on commercial broiler farms, two of the major problems are contaminated houses and infected day-old chicks (Rose *et al.*, 2000; Davies and Breslin, 2003). When day-old chick flocks are contaminated with the bacteria, a rapid spread of *Salmonella* throughout the house and feeders is observed, for example in early weeks, when broiler chicks gain access to feeders and taint the feed, coinciding with the highest excretion of *Salmonella* in faeces (Van Immerseel *et al.*, 2004). Then, the rest of the birds ingest the bacteria and the whole flock may be infected in few days (Heyndrickx *et al.,* 2002). Van Immerseel *et al.* (2004) reported that young chicks infected with *Salmonella* resulted in persistent excretion for at least 18 weeks of rearing. Similar results were obtained by Beal *et al.* (2004), who reported that irrespective of age at exposure, *Salmonella* infection of young birds persists until between 10 and 12 weeks, well beyond the slaughter age for broilers.

Important aspects in *Salmonella* surveillance and monitoring programmes are the type of sample and the time of sampling to determine the flock status with the highest sensitivity (Heyndrickx *et al.*, 2002; Carrique-Mas and Davies, 2008). Both aspects have to be considered in control programmes based on faecal samples analysis (Heyndrickx *et al.*, 2002; Buhr *et al.*, 2007). Usually, cloacal swabs are used to monitor the *Salmonella* status of the chickens (Van Immerseel *et al.*, 2004). However, *Salmonella* shedding is intermittent and special care must be taken, because this technique is not reliable to detect carrier birds (Van Immerseel *et al.*, 2004). Scherer *et al.* (2008) used serological examination techniques in pigs, which are more suitable for

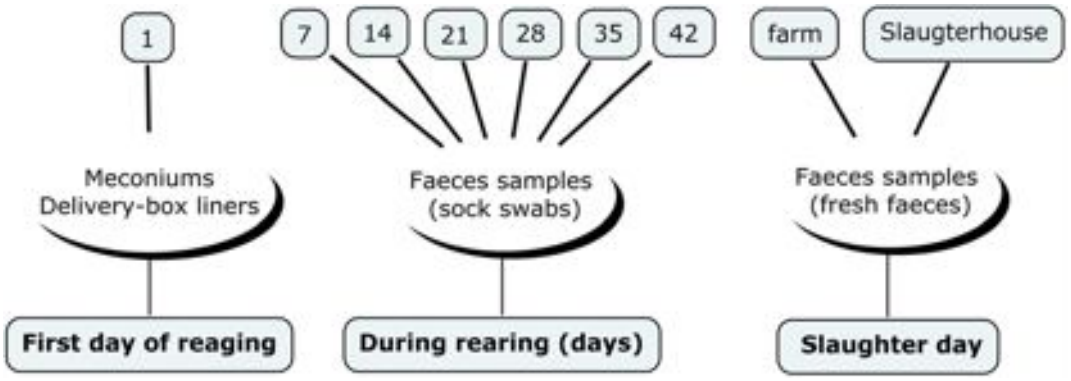
routine diagnosis. However, these methods indicate exposure to *Salmonella* and cannot differentiate between shedding or exposed flocks (Lo Fo Wong *et al.*, 2003). Previous studies reported that litter sampling utilizing several pairs of overshoes provided the highest sensitivity for determining the *Salmonella* status of the broilers during rearing (Buhr *et al.*, 2007).

It is well known that *Salmonella* contaminated faeces are an important source of environmental contamination and chicken infection (Rose *et al.*, 1999). However, this problem does not seem to be restricted to the hatchery and rearing period (McCrea *et al.*, 2006). Feed withdrawal, loading and transportation from farm to slaughterhouse are known to be stressful for animals (Slader *et al.*, 2002; Burkholder *et al.*, 2008). Several authors reported that stress causes a disturbance of intestinal functions and may lower the resistance of the live animal and increase the spread of intestinal bacteria (Mulder, 1995; Burkholder *et al.*, 2008; Scherer *et al.*, 2008). Slader *et al.* (2002) reported that transport to the slaughterhouse increased the prevalence of positive birds due to faecal contamination of skin and feathers by neighbouring infected birds during shipping. On the other hand, transport to the slaughterhouse in contaminated trucks is a great concern as *Salmonella* may be introduced into a *Salmonella* free flock (Slader *et al.,* 2002; Heyndickx *et al.*, 2002). Due to these considerations, faecal shedding throughout transport facilitates cross-contamination between carcasses and equipment during processing, increasing the contamination status of the final food products (McCrea *et al.*, 2006). These authors suggested that lowering the farm prevalence of the bacteria and stress during transport are important strategies to lower the risk of contaminated meat products entering the food chain.

The objectives of this study were: (i) to assess *Salmonella* detection rates from faeces samples a long the rearing period, and (ii) assess the influence of live transport to the slaughterhouse on *Salmonella* detection.

### 3.2.3. Material and methods

***Study sample*** Over two years (October 2005 and October 2007), 65 commercial broiler flocks from the Valencia Region were sampled. Each flock belonged to one farm. Farms were affiliated with five production companies which handle the majority of the poultry slaughtered in the Valencia Region.




Numbers within table: days of rearing. Farm: Samples collected at farm level. Slaughterhouse: Samples collected after transport to the slaughterhouse.

**Figure 17. Sampling visits and samples collected during the lifespan of each broiler flock.**

#### Experiment 1

***Data collection*** Each farm was visited at weekly intervals until slaughter (Figure 17). The first visit was on day-old chick delivery day. When chickens arrived, 10 delivery-box liners were pooled in two batches. At the same time, meconiums were obtained by pressing chicks' abdomens (250-300 chicks per batch). Day-old chick flocks were declared infected if at least one of the three samples tested positive for *Salmonella*. For 6 weeks (common fattening period), faeces samples were taken from the litter at weekly intervals. Faeces samples from each flock were collected with five pairs of sock swabs in compliance with European Food Safety Authority guidelines (EC, 2005). First, the floor area of the houses was divided into five equal sectors and one pair of sock swabs was used in each sector for sampling (Figure 18). Samples were taken by walking over the chosen sector and each pair of sock swabs with faecal material fixed was analysed as an individual sample. This sampling procedure will

theoretically provide 95.0 % confidence of detection of 1.0 % within flock prevalence, assuming the test is 100.0 % sensitive (EC, 2005).

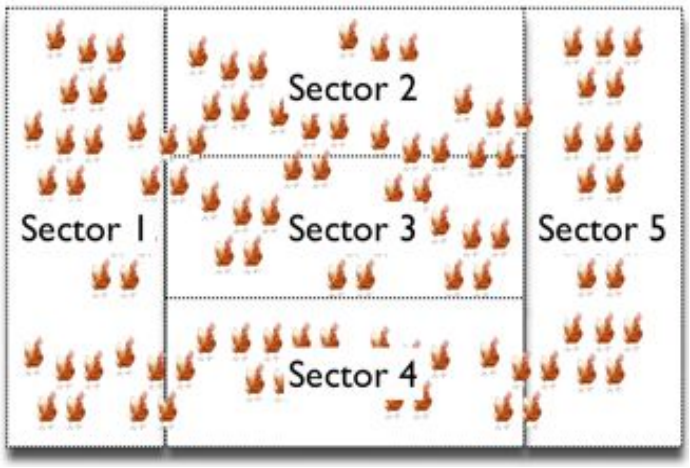



**Figure 18. Floor area of the houses divided into five equal sectors for faeces sampling with sock swabs (EC, 2005).**

## Experiment 2

***Data collection*** The transport period influence on *Salmonella* faecal detection was studied taking faeces samples from 59 broiler flocks before and after transport to the slaughterhouse. Before loading, five cellulose sock swabs were taken from each flock as described above (EC, 2005). One flock was declared *Salmonella* positive if at least one of the samples tested positive for the bacteria. When the slaughter truck arrived at the farm, containers and platform were sampled with sterile wet gauze pads with disinfectant neutralizer (AES laboratories®, Bruz Cedex, France, Figure 19).

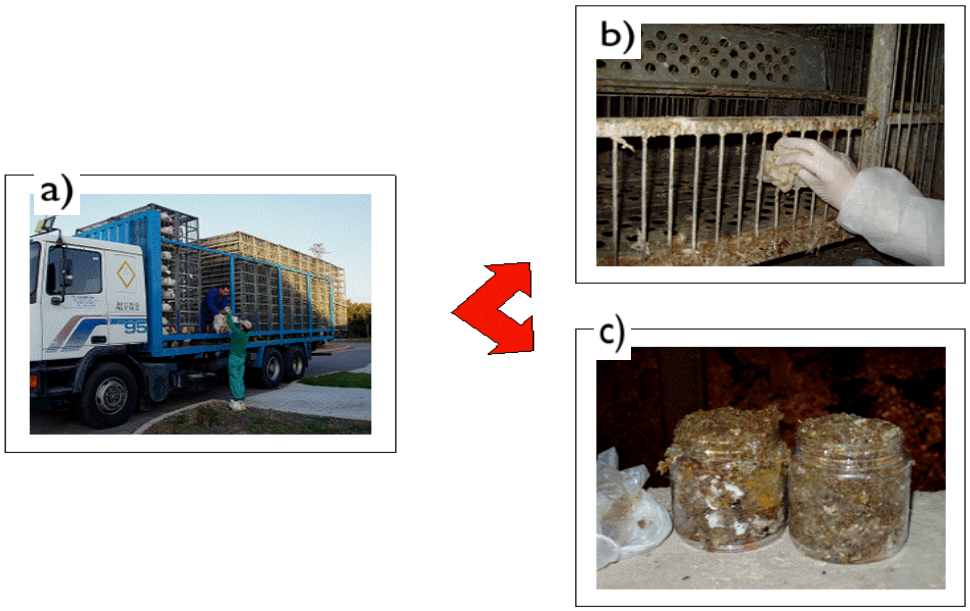



**Figure 19. Slaughter truck sampling. a) Containers and platform sampling with sterile wet gauze pads with disinfectant neutralizer. b) Pooled faeces samples collected directly from the truck after transport to the slaughterhouse.**

On reaching the abattoir, two pooled faeces samples were taken directly from the truck (200-300 g each; EC, 2005, Figure 19). A flock was declared infected if at least one of the samples tested positive for *Salmonella*. Loading and transportation period of the flocks studied ranged between 4 and 7 hours.

## Bacteriological analysis

Samples from experiments 1 and 2 were collected directly into sterile sample pots and analysed according to ISO 6579:2002 (Annex D). First, the samples were pre-enriched for 18 h in BPW 2.5 % (1:10 vol/vol, Scharlau®, Barcelona, Spain) at 37 ºC. Afterwards, selective culture was carried out by transferring 0.1 mL of incubated broth to a MSRV (Difco®, Valencia, Spain) agar plates and incubated at 42 ºC for 24-48 h. The culture obtained in MSRV was inoculated onto XLD (Liofilchem®, Valencia, Spain) and XLT4 (Biokar Diagnostics®, Pantin Cedex, France) and incubated at 37 ºC for 24-48 h. After incubation, 5 typical colonies of *Salmonella* were streaked onto the surface of pre-dried nutrient agar plates (Scharlab®, Barcelona, Spain) and incubated at 37 ºC for 24 h. Then, an API biochemical test (API-20®, bioMerieux, Madrid, Spain)

was done to confirm *Salmonella spp*. Moreover, *Salmonella* strains isolated were serotyped by the Ministry of Environment and Rural and Marine Affairs Reference Laboratory (Algete, Madrid, Spain) in accordance with Kauffman-White-Le-Minor technique.

### Statistical Analysis

On the first day of rearing, two levels were tested to evaluate the *Salmonella* detection from faeces according to the status of day-old chicks (free or infected flocks). *Salmonella* detection according to the status of day-old-chick flocks and the moment of sampling (days 1, 7, 14, 21, 28, 35 and 42) were compared by a Chi-square Test. The *Salmonella* detection patterns in both groups of day-old chick flocks throughout the rearing period were compared using a cross tabulation Chi-square Test. On the other hand, the relationship between *Salmonella* detection rates before and after transport to the slaughterhouse was analysed by Chi-square Test. Flocks transported with trucks that arrived contaminated from the slaughterhouse were discarded from the statistical analysis. Also, serotypes present in broiler production during the rearing period and after transport to the slaughterhouse were analysed using a Chi-square Test. Statistical analyses were performed using a commercially available statistics package (Statgraphics Plus, Version 5.1, STSC Inc., Rockville, MD, USA). All tests were carried out using a significance level of $P \leq 0.05$.

## 3.2.4. Results

### Experiment 1

On the first day of rearing, 20 flocks were positive and 45 were negative. Day-old chick flocks were positive in meconiums and paper liners in 14.0 % and 32.0 % (only 40 flocks presented paper liners first day of rearing), respectively. A total of 1,977 faeces samples (pairs of sock swabs) were taken during this experiment in both groups (day-old chick flocks free and infected). *Salmonella* detection according to the

status of day-old chick flocks (free or infected) and the moment of sampling (days 1, 7, 14, 21, 28, 35 and 42) were statistically significant ($P \leq 0.05$, Figure 20). Regardless of whether the flocks arrived infected from the hatchery or became infected at the farm, no differences between both *Salmonella* detection patterns were observed ($P=0.7955$). As shown in Figure 20, in both groups the bacteria detection in faeces increased during the first 3 weeks of rearing, with a maximum on day 14 and a decrease on day 28. For *Salmonella*-positive day-old flocks, the detection rate at day 28 and 42 were the same. However, there was a significant increment on day 35 ($P \leq 0.05$, Figure 20). For the *Salmonella*-negative day-old flocks, the detection rate in faeces on days 28 and 35 were the same. Then, there was a slight decrease at the end of rearing. Regardless of the moment of flock contamination (at the hatchery or during rearing), by the end of rearing the rate of *Salmonella* detection in faeces was similar (around 17.4 %).

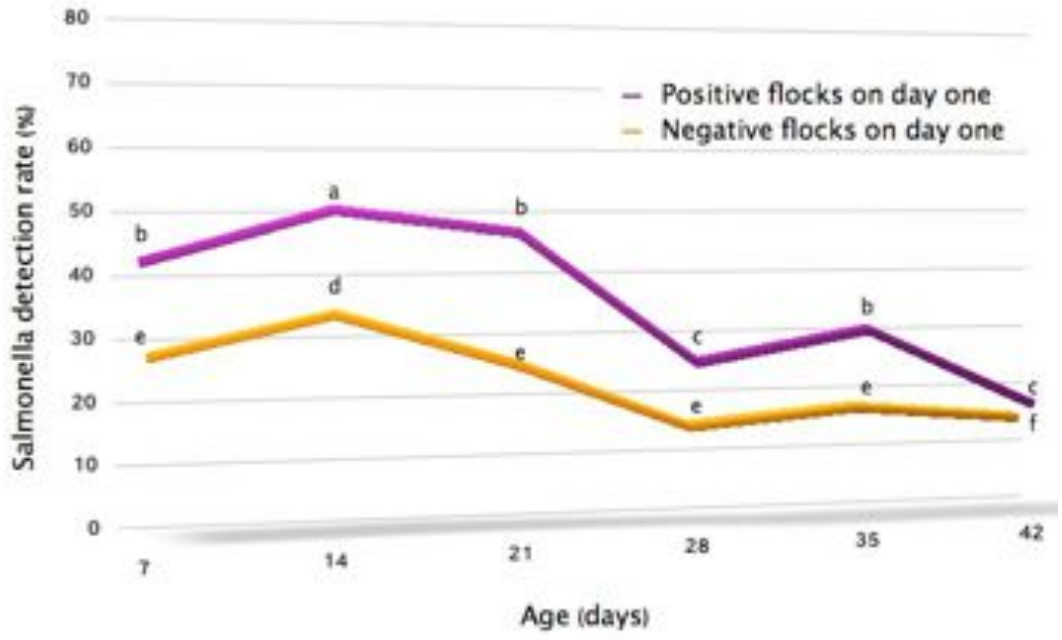



**Figure 20. Percentage of positive faeces samples in each positive flock during the rearing period in days. [a-c] Different superscripts within line of day-old infected chicks indicate a significant difference ($P \leq 0.05$). [d-f] Different superscripts within line of day-old free chicks indicate a significant difference ($P \leq 0.05$).**

During the rearing period, 531 *Salmonella* strains from 15 different serotypes were isolated. The most prevalent serotypes (92.6 % of positive samples) were in decreasing order: *S.* Enteritidis (66.7 %), *S.* Virchow (13.7 %), *S.* Hadar (9.4 %) and *S.* Ohio (2.8 %). The remaining serotypes isolated (7.4 % of positive samples) were: *S.*

Mbandaka (1.3 %), *S.* Typhimurium (0.9 %), *S.* Brikama (0.9 %), *S.* Senftenberg (0.9 %), *S.* Goldcoast (0.7 %), *S.* Altona (0.5 %), *S.* Havana (0.5 %), *S.* Agona (0.5 %), *S.* Heidelberg (0.5 %), *S.* Infantis (0.5 %) and *S.* Brandenburg (0.2 %). The pattern of the most prevalent serotypes was significantly different during the rearing period ($P \leq 0.05$). As shown in Figure 21, *S.* Enteritidis was isolated among 64.8 % and 75.9 % of samples analysed. Chickens maintained *S.* Enteritidis serotype in faeces constant without fluctuation throughout the rearing period. As in *S.* Enteritidis, *S.* Hadar with rates between 5.0 % and 12.8 % was constantly present in samples collected during the rearing. On the other hand, *S.* Virchow, with rates between 0.0 % and 23.9 %, was not isolated in day-old chicks. Nevertheless, this serotype increased at weekly intervals until 35 days of life when the detection rate was highest (23.9 %). *S.* Ohio pattern was opposite to *S.* Virchow. The first day of chicken rearing, *S.* Ohio rate was the second highest behind *S.* Enteritidis (20.0 % and 65.0 %, respectively). However, *S.* Ohio rates decreased at weekly intervals until 28 days of rearing when the excretion of this serotype stopped completely (Figure 21).

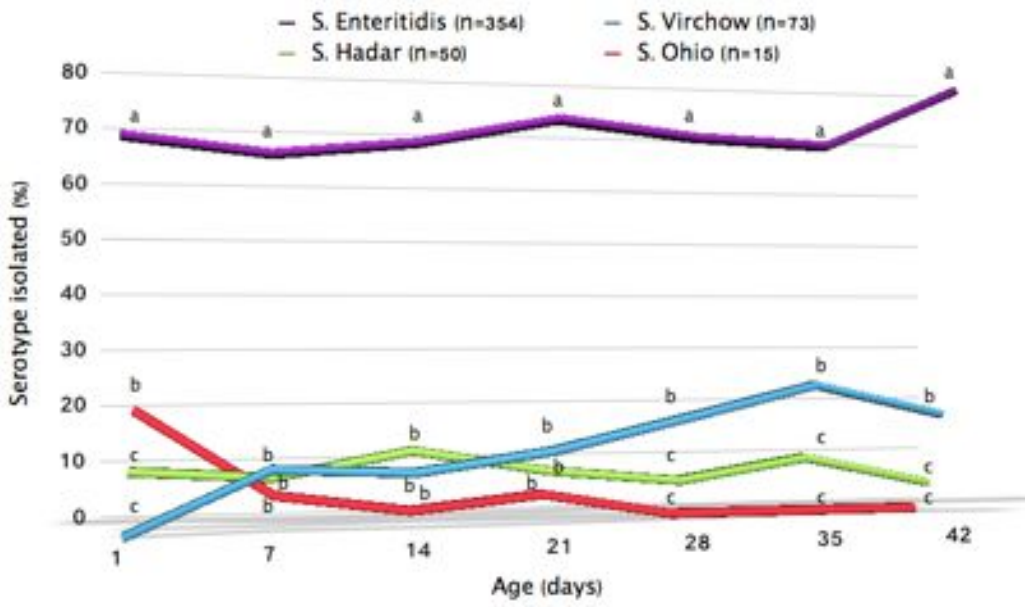



**Figure 21. Percentage of *Salmonella* serotypes isolated in broiler faeces samples in each positive flock during the rearing period in days. [a-c] Different superscripts within lines according with different days of age indicate a significant difference ($P \leq 0.05$).**

## Experiment 2

On slaughter day, 18 transport trucks sampled were contaminated with the bacteria (30.5 %). *Salmonella* detection rates in faeces on last day of rearing (with sock swabs) and *Salmonella* detection at the slaughterhouse (from fresh faeces) showed statistical differences ($P \leq 0.05$). According to the samples collected, before loading and after transport to the slaughterhouse, samples were contaminated at 15.4 % and 41.2 %, respectively. Moreover, 50.0 % of the flocks determined negative at farm level had positive faeces in crate upon arrival at the slaughterhouse plant.

The analysis of different serotypes before loading showed the presence of three serotypes: *S.* Enteritidis, *S.* Hadar and *S.* Virchow (Figure 22). After transport to the slaughterhouse, *S.* Enteritidis was the most prevalent serotype isolated, but decreased significantly (from 64.7 % to 54.5 %). *S.* Hadar doubled the presence in faeces (from 17.6 % to 39.3 %) and *S.* Virchow was not isolated after transport. Finally, two serotypes, *S.* Typhimurium (3.0 %) and *S.* Mbandaka (3.0 %) were isolated after live transport; however these serotypes were not isolated before loading.

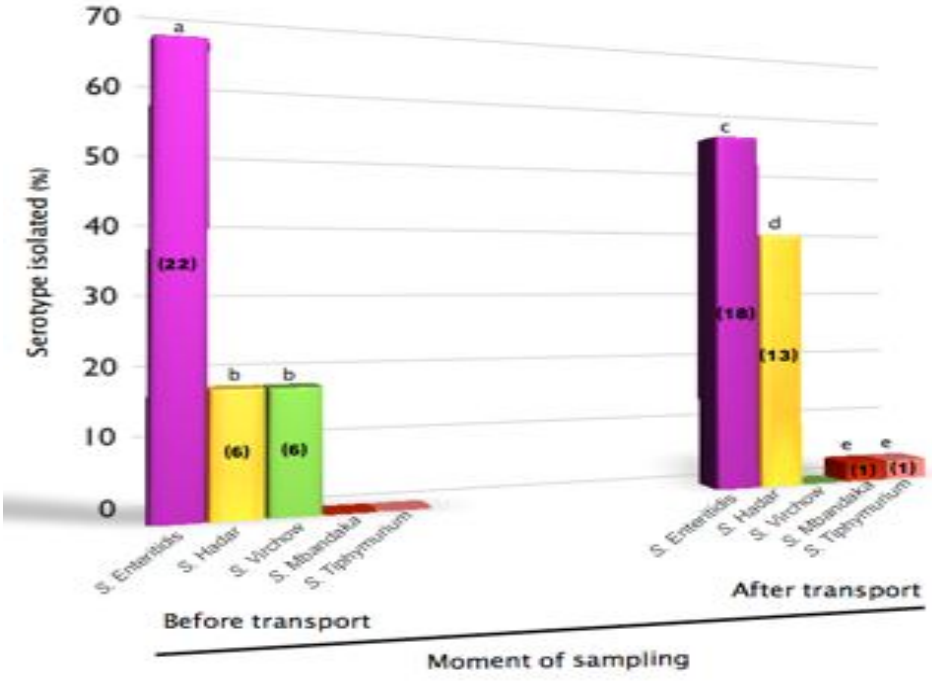



**Figure 22. Percentage of *Salmonella* serotypes isolated in broiler faeces samples in each positive flock before and after transport to the slaughterhouse. [a-c] Numbers within columns with different superscripts differ (*P≤0.05*). Data inside the bars are the number of serotypes isolated.**

### 3.2.5. Discussion

Previous results showed that infected flocks that shed *Salmonella* in faeces on the first days of rearing kept shedding the bacteria until slaughter, and so were a potential route of processing contamination (Bailey *et al.*, 2001: 2002; Van Immerseel *et al.*, 2004). This event has to be taken in account, because to eradicate *Salmonella* from poultry products, the whole production system must be *Salmonella* free (Rose *et al.*, 2000; Davies and Breslin, 2003). An important aspect in *Salmonella* surveillance and monitoring programmes by the government and/or poultry companies are the type of sample and the time of sampling to determine the *Salmonella* status of the flock with the highest sensitivity (Heyndrickx *et al.*, 2002; Carrique-Mas and Davies, 2008). The European Commission (EC, 2005) reported faeces sampling from the litter, utilizing several pairs of overshoes, within 3 weeks before slaughter, as the official sampling method in the baseline survey on the prevalence of *Salmonella* in broiler flocks of *Gallus gallus*. This study suggested that *Salmonella* variations in faeces detection rates collected with sock swabs throughout the rearing period occurred independently of the fact that day-old flocks arrived infected from the hatchery or became infected at the farm. Moreover, our results revealed that the highest detection trend from faeces occurred on 14$^{th}$ day of rearing. Later, *Salmonella* detection decreased and became intermittent until day of slaughter. This fact could be explained because the highest *Salmonella* excretion occurs around 2 weeks of rearing, coinciding with an immature immune system (Berndt *et al.*, 2004; Van Immerseel *et al.*, 2004). However, an increase in *Salmonella* detection was observed at 35 days of rearing in day-old infected flocks. This increase seems to be related with the removal of antibiotic growth promoters from diets that induce a disturbance of the bacterial flora in chickens gut (Francesch, 2007). Another hypothesis could be as a result of the stress induced during thinning. This practice induced carrier flocks to shed the bacteria at higher rates and increased *Salmonella* detection (Mulder, 1995; Corry *et al.*, 2002). For all these reasons, evaluating the farm status within 3 weeks before slaughter-age could underestimate the prevalence of the flock. Probably, around 2 weeks of rearing should be considered the best moment to determine the *Salmonella* status, because of the higher detection in samples. Moreover, the sooner the flock can be detected positive for

the bacteria, the fewer expenses will be involved in carrying out control and eradication measures such as flock slaughter.

However, *Salmonella* surveillance and control programmes do not stop at farm level. Control should be considered until the end of the processing plant and markets. The importance of transport in *Salmonella* spread is frequently ruled out (Belles, 2007). Broiler transport to the processing plant was shown to increase *Salmonella* prevalence in faeces, favouring the risk of final food product contamination (Slader et al, 2002; Heyndrickx *et al.*, 2007). Several reports showed that stressful situation such as transport to the slaughterhouse can induce the carrier flocks to shed *Salmonella* at higher rates because of a disturbance in intestinal functions that may increase the spread of intestinal bacteria (Mulder, 1995; Corry *et al.*, 2002). Moreover, as in this study, several authors reported that transport trucks were found to be contaminated at high frequency with the bacteria, being an important source of *Salmonella* contamination of broiler flocks (Slader *et al.*, 2002; Heyndickx *et al.*, 2002; Marin *et al.*, 2009). Results showed that after transport substantial number of negative flocks had *Salmonella* positive faeces, even though trucks were properly disinfected. Consequently, poultry determined negative at farm level could enter the processing plant carrying the bacteria, internally or externally. However, the main problem of slaughterhouse analyses is the time necessary to determine a positive sample according to the official ISO method 6579:2002 (Annex D). It is well known that 24 hours after slaughter, carcasses are in markets ready for human consumption. Consequently, more modern, practical, cost-effective and suitable techniques for routine diagnosis should be developed to determine the status of the flocks in a short period of time and with the highest sensitivity.

The main serotype involved in human *Salmonella* outbreaks is *S.* Enteritidis (EFSA, 2009). *S.* Enteritidis was the most prevalent serotype isolated in this study during rearing and after transport to the slaughterhouse according to previous reports (Carramiñana *et al.*, 1997; EFSA, 2007). Carramiñana *et al.* (1997) suggested that recent acquisition of virulence factors, in combination with existing mechanisms of invasiveness and pathogenicity, may contribute to the development of systemic infection with *S.* Enteritidis and its consequent prevalence in poultry in the

slaughterhouse. Moreover, our data suggest that only four serotypes from the fifteen isolated during rearing were isolated in faeces at the slaughterhouse (*S.* Enteritidis, S. Hadar, *S.* Typhimurium and *S.* Mbandaka). Carramiñana *et al.* (1997) reported that serotypes isolated from faeces were later detected in carcasses and livers, indicating a contamination by endogenous microflora in bird faeces. To our best knowledge, there is a lack of scientific literature related with the identity and movement of specific serotypes of *Salmonella* throughout the rearing period (Bailey *et al.*, 2002). Nevertheless, rapid spread of a limited number of successful *Salmonella* serotypes in the primary sector, such as *S.* Enteritidis, has been suggested as the most important cause of salmonellosis worldwide (Wegener *et al.*, 2003).

In conclusion, regardless of whether broiler flocks reached the farm already shedding *Salmonella* in faeces or were infected during rearing, the maximum rate of *Salmonella* detection is around 14$^{th}$ day of rearing. A significant increase in *Salmonella* detection rates is observed after transport to the slaughterhouse. The patterns of most prevalent serotypes vary throughout the rearing period and change after transport to the slaughterhouse, although *S.* Enteritidis is the most prevalent serotype isolated during rearing and after transport.

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# 3.3. Biofilm development capacity of *Salmonella* strains isolated in broiler risk factors and their resistance to disinfectants

## 3.3.1. Abstract

Prevention of *Salmonella* contamination of poultry products requires detailed knowledge of the most important risk factors associated with its presence in the production system. An inaccurate cleaning and disinfection between flocks has been described as an important risk factor for *Salmonella* persistence in poultry production (Rose *et al.*, 1999; Rose *et al.*, 2000). The aim of this study was to determine the biofilm development capacity of the strains isolated from poultry samples on 44 broiler farms. So, glutaraldehyde (50.0 % vol/vol), formaldehyde (37.0 % vol/vol) and hydroxide peroxide (35.0 % vol/vol) were applied to evaluate their capacity to remove *Salmonella*, biofilm and non-biofilm strains, isolated from each risk factor in an artificial contamination test in field conditions. Samples of faeces, dust, surfaces, meconiums, delivery-box liners, water tank, water dispensers, litter, vectors (rodents, flies and beetles) and surfaces of the slaughter trucks were taken throughout the rearing period. All samples were analysed in accordance with ISO 6579:2002 (Annex D). To evaluate biofilm development, a screening method based on the fluorescence of *Salmonella* colonies on calcofluor agar plates was used. In the artificial contamination test, the chemical solutions were prepared at a concentration of 1.0 % and applied at exact times (1, 15 or 60 min). Our results showed that irrespective of the origin of different *Salmonella* strains, around 50.0 % of the different serotypes were able to produce biofilm. Finally, the use of glutaraldehyde, formaldehyde and peroxygen at a concentration of 1.0 % in field conditions are inadequate for *Salmonella* elimination irrespective of the serotype, biofilm development capacity and disinfectant contact time.

### 3.3.2. Introduction

It is well known that *Salmonella* elimination from poultry houses is a hard task (Davies and Breslin, 2003; Gradel *et al.*, 2005). However, why can *Salmonella* not be removed from the sheds? What are the sources of these bacteria? The main risk for *Salmonella* contamination of poultry flocks is the *Salmonella* status of the previous flock (Rose *et al.*, 2000), an inefficient cleansing and disinfection during the period between flocks (Rose *et al.*, 2003; Davies and Breslin, 2003; Gradel *et al.*, 2005), *Salmonella* status of day-old chicks (Cardinale *et al.*, 2004), contaminated litter (Rojas *et al.*, 2002), feed and water (Heyndrickx *et al.*, 2002), presence of contaminated carriers (Davies and Breslin, 2003; Carrique-Mas *et al.*, 2008), rodents, flies and beetles, and inadequate abattoir truck disinfection (Ramesh *et al.*, 2002). It has also been demonstrated that cellulose production and biofilm formation may be important for the survival of *Salmonella* on surface environments; in fact, cellulose-deficient *Salmonella* strains did not develop biofilm (Latasa *et al.*, 2005). Lasa (2007) reported that life in a biofilm state protects the bacteria against environmental insults like chemical sanitizers, which are generally unable to eliminate most biofilm-associated bacteria. In recent years, biofilm development mechanisms have been studied (Bonafonte *et al.*, 2000; Cucarella *et al.*, 2001; Solano *et al.*, 2002; Garcia *et al.*, 2004; Latasa *et al.*, 2006). Calcofluor white has been widely used to identify mutants defective in the production of exopolysaccharides in different bacteria species (Leigh *et al.*, 1985; Ramaswamy *et al.*, 1997; Thomashow *et al.*, 1987). Solano *et al.* (2002) showed that all biofilm developer *Salmonella* strains fluoresced strongly under long-wave UV light on calcofluor agar plates and none of the non-biofilm developer strains were able to fluoresce under similar conditions.

The poultry industry is intensive and consistently applies an all-in all-out system with the aim of minimizing infection pressure and targeting specific organisms like *Salmonella*. Disinfecting during production break is therefore a routine part of the management of poultry houses. Several chemical agents are commercially available for *Salmonella* elimination. However, different studies revealed that cleansing and disinfection methods were ineffective against *Salmonella* in a field situation (Ramesh *et al.*, 2002). Rose *et al.* (2000, France) in broiler houses and Davies and Breslin (2003,

United Kingdom) in laying hen houses, showed high prevalence of *Salmonella* in environment samples after cleansing and disinfection, proving that disinfection was ineffective against the bacteria in a field situation. Most common disinfectant active ingredients used in poultry houses in eastern Spain were glutaraldehyde, formaldehyde and peroxygen (Martinez *et al.*, 2008). Several related studies with these disinfectants have shown the effectiveness of formaldehyde and glutaraldehyde against *Salmonella* (*in vitro*, Ramesh *et al.*, 2002; Gradel *et al.*, 2003:2004). Nevertheless, poultry houses have inaccessible equipment and considerable amounts of organic matter and high contents of protective compounds (fats, carbohydrates and proteins) from which *Salmonella* are difficult to remove (Gradel *et al.*, 2004). On the other hand, water hardness, low temperatures and biofilm development also decrease the efficacy of disinfectants (Taylor *et al.*, 1996; Gradel *et al.*, 2004; Lapidot, 2006).

The main aims of this study were (i) to evaluate biofilm development capacity of strains isolated in broiler production related samples and (iii) evaluate disinfectants used in poultry against *Salmonella* in field conditions.

### 3.3.3. Material and methods

All chemicals, unless otherwise stated, were reagent grade and purchased from Sigma-Aldrich Química S.A. (Alcobendas, Madrid, Spain). Over two years (October 2005 and October 2007), 44 commercial broiler farms from the Valencia Region were sampled. Farms were affiliated with five production companies which handle the majority of poultry slaughtered in the Region.

#### Experiment 1

***Risk factor sampling in broiler flocks.*** Samples of faeces, dust, surfaces, meconiums, delivery-box liners, water tank, water dispensers, litter and vectors (rodents, flies and beetles) were taken in all the flocks throughout the rearing period

(Figure 23). Before transportation to the slaughter plant, the abattoir trucks, containers and platforms were sampled.

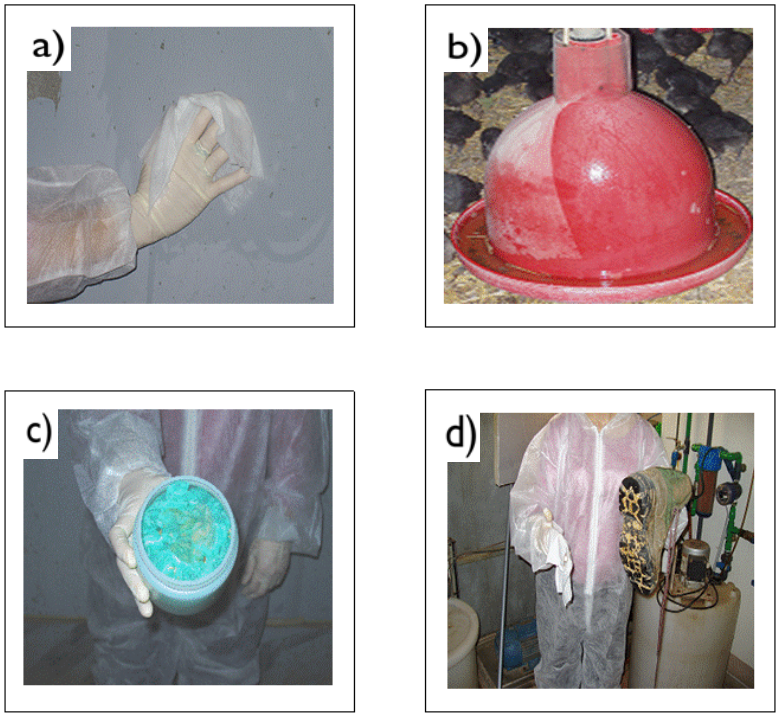


**Figure 23. Environmental sampling. a) Surfaces sampling with sterile wet gauze pads. b) Water samples collected from dispensers. c) Faeces sampling. d) Farming boot sampling.**

Samples of previous flock faeces were taken using five pairs of cellulose swab-socks attached to boots and applied over the length of the house (EC, 2005). First, the floor area of the houses was divided into five equal sectors and one pair of sock swabs was used in each sector for sampling. The faeces were taken by walking over the chosen sector and each pair of swab-socks with faecal material fixed was analysed as an individual sample. Before and after cleansing and disinfection, samples of surfaces (wall, floor, feeders and dispensers) were collected with sterile wet gauze pads with disinfectant neutralizer (AES laboratories®, Bruz Cedex, France). Dust samples were also collected in different places on the farm (25-30 g). Before chicks were placed, 10 chick delivery-box liners were pooled in two batches. At same time, meconiums were obtained from 300 chicks. Then, feed samples were collected from the truck and feeders (about 25-30 g). Water was sampled from the tank and final dispenser lines (20-30 mL). Bedding samples were also taken from different points of the house (25-30 g) and farming boots were sampled with sterile wet gauze pads with disinfectant neutralizer. During rearing period, chicken faeces were collected every week using five pairs of cellulose sock swabs (as reported above). At the end of the rearing period,

abattoir transport containers and truck platform surface samples were obtained with gauze pads wet with disinfectant neutralizer. In addition, after the chickens left, water samples from tanks and dispensers, dust and surfaces were taken again. Samples from carriers (flies, rodents and beetles) were also collected. Fly feeder traps (Econex®, Castellón, Spain) with insecticide (Agita® 1GB of Novartis and Quick Bayt® of Bayer, Barcelona, Spain) were installed inside the shed over a week long period to cage flies. Rodent traps (Cage All®, Tom cat® and T-Rex® form Bell, Pensacola, USA) and beetle traps (25 cm PVC tubes with corrugated cardboard inside) were installed on the litter for two days at the end of the rearing period to cage rodents and beetles. Liver, spleen and intestines of rodent carcasses were removed aseptically for culture and beetles captured were analysed as a pool.

***Salmonella isolation.*** Samples were collected directly into sterile sample pots and analysed according to ISO 6579:2002 (Annex D). First, the samples were pre-enriched in 1:10 vol/vol BPW (Scharlau®, Barcelona, Spain) and then incubated at 37±1 ºC for 18±2 h. The pre-enriched samples were transferred onto MSRV (Difco®, Valencia, Spain) agar plate (0.1 mL) and incubated at 41.5±1 ºC for 24-48 h. The culture obtained in MSRV was inoculated onto XLD (Liofilchem®, Valencia, Spain) and XLT4 (Biokar Diagnostics®, Pantin Cedex, France) and incubated at 37±1 ºC for 24-48 h. After incubation, 5 colonies of *Salmonella* were streaked onto the surface of pre-dried nutrient agar plates (Scharlab®, Barcelona, Spain) 37±1 ºC for 24±3 h. Then, a biochemical test API (API-20®, bioMerieux, Madrid, Spain) was done to confirm *Salmonella* spp. Moreover, *Salmonella* strains isolated were serotyped by the Ministry of Environment and Rural and Marine Affairs Reference Laboratory (Algete, Madrid, Spain) following the Kauffman-White-Le-Minor technique.

***Biofilm development screening.*** In order to study the frequency of biofilm formation capacity, we analysed the ability of *Salmonella* isolates of different origins from poultry risk factors. To evaluate biofilm development, a screening method based on the fluorescence of *Salmonella* colonies on calcofluor agar plates was used (Solano *et al.*, 2002). The calcofluor agar plate components were Lauria-Bertoni (LB, Sigma Aldrich, Alcobendas, Madrid, Spain) agar with $MgCl_2$ (0.1 M, Merck®, Madrid, Spain) $Cl_2Ca$ (0.5 M, VWR®, Barcelona, Spain), NaOH (1 M, Merck®), Hepes (1 M) and

Fluorescent brightener (1.0 %). Calcofluor solution and calcofluor plates should be protected from the light and kept in darkness at 4 ºC. Every *Salmonella* strain isolated from poultry was inoculated onto calcofluor agar plates in duplicate, including positive control (SE 3934 AbapA :: km) and negative control (SE 3934 Acsg D) for biofilm development in every plate. Cells were grown on LB calcofluor agar plates at room temperature for 48 h in darkness. Fluorescence was observed under a 366 nm UV light source (Figure 24).

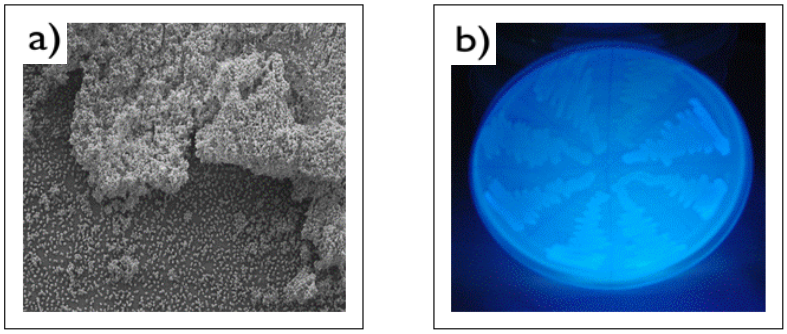


**Figure 24. *Salmonella* biofilm developer strains. a) Scanning electron microscopy photography of *Salmonella* Enteritidis biofilm (Lasa, 2007). b) Fluorescence of *Salmonella* colonies on calcofluor agar plates.**

## Experiment 2

***Disinfection Procedures.*** The disinfectants selected in this experiment were: glutaraldehyde (50.0 % vol/vol, Scharlau®), formaldehyde (37.0 % vol/vol, Scharlau®) and hydrogen peroxide (35.0 % vol/vol, Scharlau®). Milli-Q sterile water was used for controls and to dilute disinfectant solutions. The chemical solutions were prepared at a concentration of 1.0 % (vol/vol) according to Gradel *et al.* (2004) and then were evaluated through an artificial contamination test in field conditions. All disinfectant solutions were made on the day of use. The five most important *Salmonella* serovars for Public Health isolated from poultry houses in experiment 1 (*S.* Enteritidis, *S.* Typhimurium, *S.* Hadar, *S.* Virchow and *S.* Infantis) were used in this experiment. When possible, two pools of ten strains of each *Salmonella* serotype were made. For each serotype, the first pool consisted of *Salmonella* strains able to develop biofilm and the second pool was of non-biofilm strains. The biofilm development capacity was assessed on calcofluor agar plate in experiment 1. Every

*Salmonella* pool was grown separately in 25 mL of LB broth for 18 to 24 h at 37 ºC, until stationary phase (Gradel *et al.*, 2004). The concentration of each broth culture was measured using a Thoma-Zeiss counting cell chamber (Marienfield, Germany) and adjusted to $10^9$ bacteria/mL.

The infection procedure was performed inside an experimental poultry house in the Centro de Tecnología Animal (CITA, IVIA, Segorbe, Spain, Figure 25), to mimic the real conditions of poultry production. The experimental house was tested for *Salmonella* before the experiment in accordance with ISO 6579:2002 (Annex D). The material of the disinfection test was the cement floor of the house, because house floors have shown a high tendency to resist *Salmonella* disinfection (Davies and Wray, 1995; Davies and Breslin, 2001). Each disinfectant was tested on ten surfaces (5 serotype × biofilm formation capacity). The surfaces were marked on the cement floor (20 cm diameter circle drawn in the centre) and each circle was divided into 4 parts (A, B, C, D). Each area was swabbed to determine if there was *Salmonella* contamination before *Salmonella* inoculation. Then, one millilitre of *Salmonella* culture was seeded onto the surfaces and spread over the 10 cm diameter circle with a sterile towel. All test surfaces were allowed to dry under ambient conditions for 3 days.

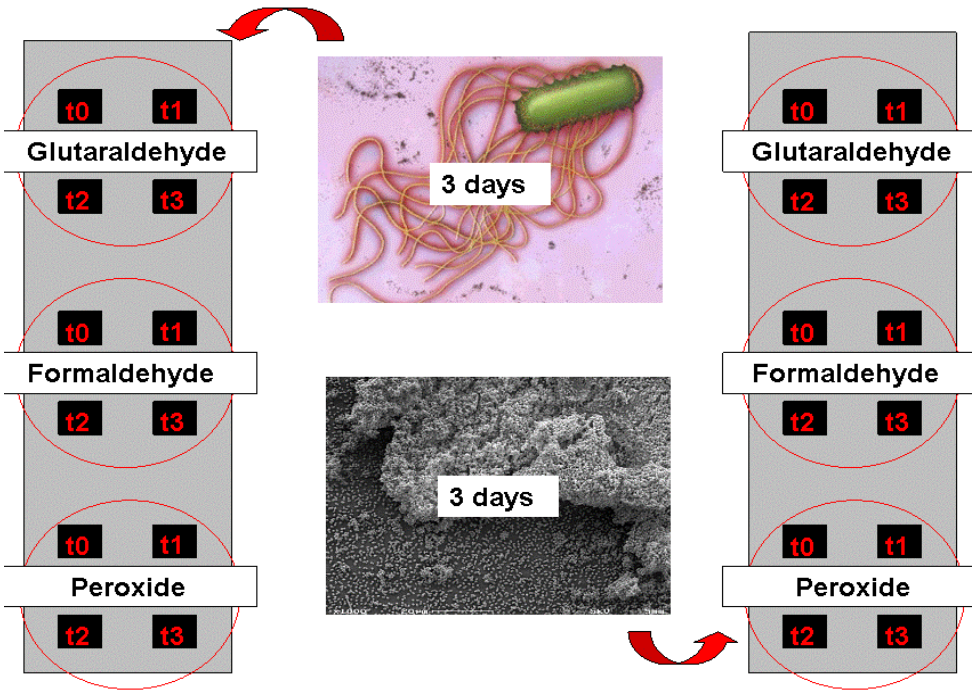


**Figure 25. Experimental design of artificial contamination and disinfection test in field conditions.**

Before disinfectants were applied, each area A was swabbed to establish initial *Salmonella* growth. Finally, disinfection treatments were applied with atomizer onto the dried surfaces and then each area (B, C and D) was swabbed with neutralizer at exact times (B=1, C=15 or D=60 min) to determine if *Salmonella* was removed or not after disinfection. Every sample was analysed in accordance with ISO 6579:2002 (Annex D). Each treatment (biofilm formation capacity × disinfectant × serotype × time) was evaluated three times.

**Statistical Analysis**

Two statistical procedures were used to assess the relationship between *Salmonella* strains with biofilm development capacity isolated in poultry and their resistance against disinfectants used in field conditions. *Salmonella* presence in broiler and laying hen production and the effect of isolated serotypes in biofilm development were analysed by Chi-square Test. Then, logistic regression analyses were used to investigate relationships between biofilm (development and non-development strains), serotypes (*S.* Enteritidis, *S.* Typhimurium, *S.* Hadar, *S.* Virchow and *S.* Infantis), disinfectants (glutaraldehyde, formaldehyde and hydrogen peroxide) and contact time (1, 15 or 60 min) and their interactions. The interactions were not significant and so were excluded from the analysis. Statistical analyses were performed using a commercially available statistics package (Statgraphics Plus, Version 5.1, STSC Inc., Rockville, MD, USA). All tests were carried out using a significance level of $P \leq 0.05$.

### 3.3.4. Results

**Experiment 1**

Among 44 broiler flocks sampled, 27.2 % of samples tested positive (2,678). *Salmonella* presence according to risk factors was statistically significant ($P \leq 0.05$, Table 7). Samples from day old chicks and chicken faeces showed high *Salmonella* prevalence (34.2 and 35.1 %, respectively, Table 7), whereas source feed, water tank

and water showed lower percentages of *Salmonella* (1.5, 2.3 and 4.6 %, respectively, Table 7).

**Table 7. *Salmonella* presence in broiler houses samples obtained from different risk factors.**

| Samples | *Salmonella* presence (%) | |
|---|---|---|
| | n | Broilers |
| Water tank | 86 | $2.3^{a}$ |
| Water drinker | 86 | $4.6^{ab}$ |
| Origin feed | 135 | $1.5^{a}$ |
| Feeder feed | 122 | $20.5^{de}$ |
| Dust | 125 | $28.8^{ef}$ |
| Surfaces | 125 | $15.2^{cde}$ |
| Faeces | 1522 | $35.1^{f}$ |
| Vectors | 35 | $11.4^{bcd}$ |
| P.F.F | 206 | $23.8^{def}$ |
| Day old Chick | 76 | $34.2^{f}$ |
| Farming boots | 40 | $15.0^{cde}$ |
| Trucks | 76 | $25.0^{def}$ |
| Litter | 44 | $7.0^{abc}$ |

n: Numbers of samples collected in broiler houses. P.F.F: Previous flock faeces. $^{a\text{-}f}$ Numbers within columns with different superscripts are statistically different ($P \leq 0.05$).

Strains isolated from risk factors analysed were not statistically significant to biofilm formation capacity (Table 8). Around 50.0 % of strains isolated showed the biofilm production ability, with the exception of feeders where *Salmonella* biofilm-developer strains increased up to 84.2 %.

**Table 8. Biofilm development capacity of *Salmonella* strains isolated in broiler houses samples obtained from different risk factors.**

| Samples | Biofilm development (%) | |
|---|---|---|
| | n | Broilers |
| Water drinker | - | - |
| Feeder feed | 19 | 84.2 |
| Dust | 28 | 46.4 |
| Surfaces | 13 | 54.0 |
| Faeces | 451 | 57.6 |
| Vectors | - | - |
| P.F.F | 37 | 43.0 |
| Day old Chick | 16 | 43.8 |
| Trucks | 14 | 57.1 |

n: *Salmonella* positive samples collected in broiler houses. P.F.F: Previous flock faeces.

In our study, 12 different serotypes were isolated in broiler flocks. The most prevalent serovars isolated were *S.* Enteritidis (66.0 %), *S.* Virchow (13.0 %), *S.* Hadar (12.0 %) and *S.* Ohio (2.6 %). The study of serotypes which had the highest capacity of biofilm development showed statistical differences ($P \leq 0.05$, Figure 26). *S.* Enteritidis and *S.* Ohio were stronger biofilm producer strains than *S.* Hadar (Figure 26).

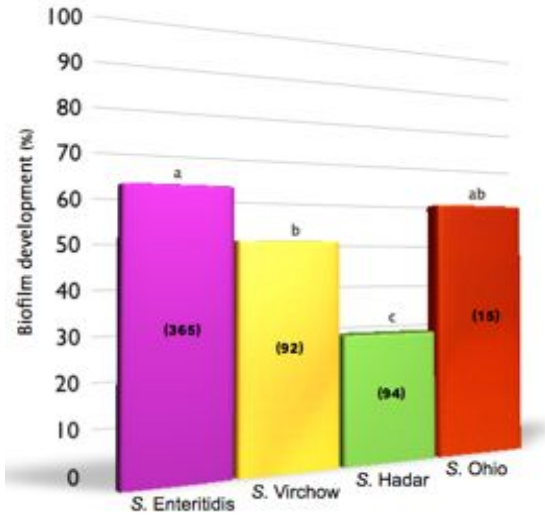


**Figure 26. Percentage of biofilm development capacity of *Salmonella* serotypes isolated in broiler houses samples obtained from different risk factors. [a-d] Numbers within columns with different superscripts are statistically different (*P≤0.05*).**

## Experiment 2

The study of biofilm protection against disinfectants showed no significant differences between biofilm and non-biofilm formation capacity of the strains (88.1 *vs.* 87.0 %, respectively). *S.* Enteritidis and *S.* Virchow were more susceptible to disinfectants than *S.* Typhimurium, *S.* Hadar and *S.* Infantis (Figure 27).

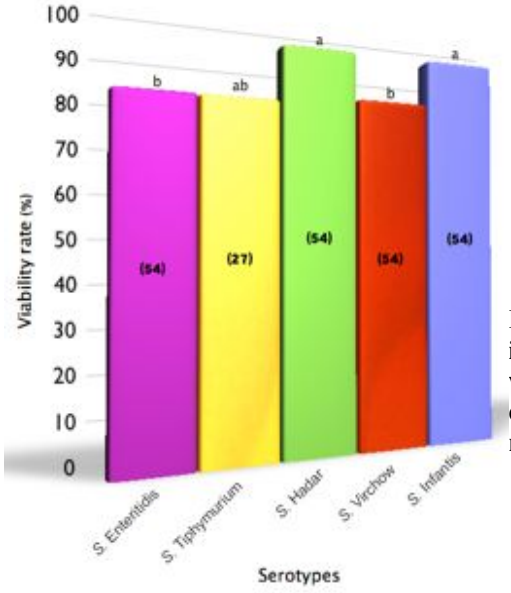


**Figure 27. Viability rate of *Salmonella* serotypes isolated in poultry after disinfection. [a,b] Columns within bars with different superscripts are statistically different (*P≤0.05*). Data inside the bars are the number of samples analysed.**

The most effective disinfectant against *Salmonella* in field conditions was glutaraldehyde ($P \leq 0.05$, Figure 28) which removed nearly 30.0 % of *Salmonella* strains. Formaldehyde and hydrogen peroxide had less capacity to eliminate the bacteria, 6.2 % and 1.2 %, respectively (Figure 28).

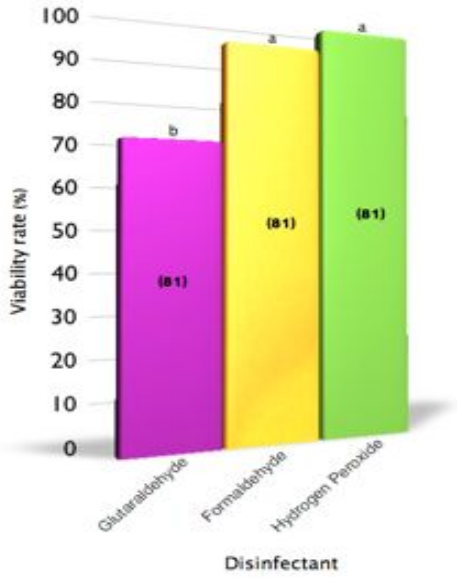


**Figure 28. Viability rate of *Salmonella* serotypes isolated in poultry according with different disinfectants applied. [a,b] Numbers within columns with different superscripts are statistically different ($P \leq 0.05$). Data inside the bars are the number of samples analysed.**

Each disinfectant was more effective in eliminating *Salmonella* in the first minute of contact time ($P \leq 0.05$, Figure 29).

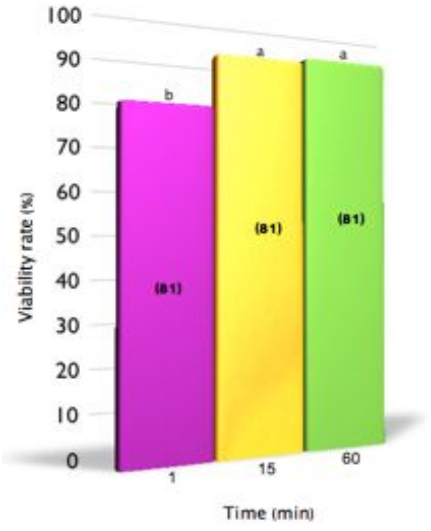


**Figure 29. Viability rate of *Salmonella* serotypes isolated in poultry according with contact time in minutes. [a,b] Numbers within columns with different superscripts differ ($P \leq 0.05$). Data inside the bars are the number of samples analysed.**

### 3.3.5. Discussion

Prevention of *Salmonella* contamination in poultry products requires detailed knowledge of the most important risk factors associated with its presence in the production system. Our results showed that dust, surfaces and faeces are the poultry samples most *Salmonella* contaminated, in agreement other authors (Irwin *et al.*, 1994; Wray and Davies, 1994; Davies and Wray, 1996). These data suggest that an inadequate cleaning and disinfection procedure could infect the following flock (Rose *et al.*, 2000). When the new flock is contaminated, there is a rapid spread of *Salmonella* throughout the house environment. Then, the rest of the birds may be infected in few days (Heyndrickx *et al.*, 2002). So, the presence of several contaminated feeders samples observed in this study was probably a consequence of high levels of *Salmonella* in environmental and carrier samples (Davies and Breslin, 2003). On the other hand, samples protected from environmental crossed contamination (water tank, final water lines and feed origin) were less contaminated with the bacteria than those in direct contact with the environment. Day-old chick flocks samples were highly contaminated, in agreement with several authors (Christensen *et al.*, 1997; Rose *et al.*, 1999; Cardinale *et al.*, 2004). If the cleaning and disinfection process was effective and removed *Salmonella* from the poultry houses, the presence of several infected chicks could increase this risk of contamination when chicks arrived at the farm (Van Immerseel *et al.*, 2004). Also, an incorrect management was identified as a risk factor, data in accordance with those previously reported (Davies *et al.*, 1997; Rose *et al.*, 1999). Farmers are able to spread the bacterium with their boots and clothes between consecutive flocks. As Davies and Breslin (2003) indicated, the pest control management has to be implemented effectively to minimize the chance of flock infection, because carriers are able to taint the feed and house surfaces. According with Ramensh *et al.* (2002) slaughter-trucks arrived at the farms with high levels of contamination, favouring *Salmonella* contamination of the houses and chickens during transport to the abattoir.

The main important serotype isolated from broiler production in this study was *S.* Enteritidis, in agreement with the European report on *Salmonella* prevalence in Spain (EFSA 2007). Moreover, contamination with *S.* Enteritidis is an important threat to

food safety, especially regarding egg production and meat from poultry (EFSA, 2009). Also, *S.* Virchow and *S.* Hadar in broilers, in line with European reports (EFSA 2007).

Several hypotheses are related with this high persistence of *Salmonella* in poultry houses, such as absence of standardized cleaning and disinfection guidelines (Gradel *et al.*, 2004), lack of scientific literature related with disinfection in the agricultural sector (Ramesh *et al.*, 2002), absence of official methods for testing disinfectants (Lasa, 2007), inaccurate use of disinfectants (Davies and Breslin, 2003), incorrect hardness and temperature of cleaning water (Leriche *et al.*, 1995; Taylor *et al.*, 1996), high contents of protective compounds in poultry houses (fats, carbohydrates and proteins, Gradel *et al.*, 2004) and biofilm development (Alvarez *et al.*, 1997). Our results showed that irrespective of their origin, around 50.0 % of the *Salmonella* strains isolated from each risk factor were able to produce biofilm. To date, to our best knowledge, there are not many studies describing biofilm development capacity in *Salmonella* strains isolated from poultry. Costerton *et al.* (1978) appreciated that, in most natural environments, growth as a biofilm is the prevailing microbial lifestyle. Moreover, biofilm may confer mechanical, chemical or biological protection within the natural habitat (Solano *et al.*, 2002). The importance of biofilms in poultry has only been reported as an important risk factor for *Salmonella* in abattoir trucks (Ramesh *et al.*, 2002) and water systems (Gradel *et al.*, 2004). However, our results revealed that the disinfectants used in infected poultry houses are more important than *Salmonella* biofilm development capacity. The application of glutaraldehyde, formaldehyde and hydrogen peroxide (1.0 % vol/vol) was insufficient to remove *Salmonella* from the poultry houses in field conditions, irrespective of the strain capacity of biofilm development, *Salmonella* serotype and the disinfectant contact time. Glutaraldehyde, formaldehyde and hydrogen peroxide are most common disinfectants active ingredients used in cleaning and disinfection procedures in poultry farms (Gradel *et al.*, 2004, Martinez *et al.*, 2008). Previous reports showed the efficacy of these disinfectants at a concentration of 1.0 % was (best first): formaldehyde> glutaraldehyde> hydrogen peroxide (Gradel *et al.*, 2004). The low efficacy of peroxygen was probably related with its susceptibility to organic matter (Russell and Chopra, 1996; Gradel *et al.*, 2004). Formaldehyde was reported to be more effective than glutaraldehyde in field conditions (Davies and Wray, 1995; Davies *et al.*, 1998; Davies and Breslin, 2003; Gradel *et al.*,

2004) but did not guarantee total *Salmonella* elimination. Studies mimicking poultry house disinfection with other conditions are encouraged. Moreover, field condition tests are difficult to standardize and this may affect the reproducibility (Rebrouck, 1999).

In conclusion, nearly 50.0 % of the strains isolated from poultry risk factors are able to develop biofilm. The use of glutaraldehyde, formaldehyde and peroxygen at a concentration of 1.0 % in field conditions is insufficient to eradicate *Salmonella*. However, more studies are necessary to find the correct concentrations and application of disinfectants in field conditions and compare this effect with biofilm or non-biofilm strains.

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# CHAPTER 4. GENERAL DISCUSSION

*Salmonella* has long been recognized as an important zoonotic pathogen of economic significance in animals and humans. Although there are numerous sources of human salmonellosis, eggs and poultry meat are considered the most common source of human infection (EFSA, 2009). According to Jimenez and Martin (2004), 75.0 % of the human salmonellosis outbreaks in Spain are related with eggs and poultry meat consumption. Legislators are therefore working to minimize *Salmonella* prevalence in poultry sectors with the introduction of a National Control Programme to reduce the incidence of the bacteria in broiler flocks. The programme sets out measures to reduce the prevalence of *S*. Enteritidis and *S*. Typhimurium, the strains which pose the highest human health risk, to 1.0 % or less by 31 December 2011 (EC, 2007). However, the official survey on the prevalence of *Salmonella* in broiler flocks reported that in Spain the current prevalence is around 41.2 % (EFSA, 2007).

It is well known that *Salmonella* control is complicated because there are numerous potential sources of contamination in an integrated poultry operation. For example, breeder flocks, hatcheries, chicks, feed, rodents, wild birds, insects, transportation, farm environment and processing plant environment (Bailey *et al.*, 2002). All sources of *Salmonella* are potentially important, but it is necessary to characterize the relative importance of the different sources under specific management and environmental conditions. Neighbouring countries have studied the main risk factors for *Salmonella* contamination in poultry flocks for several years (Rose *et al.*, 1999:2000; Corry *et al.*, 2002; Heyndrickx *et al.*, 2002; Namata *et al.*, 2008). However, to the best of our knowledge, in Spain there have been no studies related with risk factors for *Salmonella* contamination in broiler flocks. While in neighbouring countries vertical transmission from the parent flock to day-old chicks, hatcheries, cleaning and disinfection during period between flocks, feed, water, dust, faeces, bedding, farming clothes and boots, and rodents have been reported as the major risk factors, this study suggested that, under our production conditions, feed from feeders, the *Salmonella* status of the house after C&D and *Salmonella* status of day-old chick flocks are the most important risk factors related with *Salmonella* status of the flock at the end of the rearing period. During rearing, *Salmonella*-free feed may be contaminated by several sources in the house such as house environment, chicken faeces and rodents' droppings (Heyndricks *et al.*, 2002; Davies and Breslin, 2003; Carrique-Mas *et al.*, 2008). The

importance of inaccurate C&D procedures in *Salmonella* source persistence between flocks has been reported by several authors (Rose *et al.*, 2000; Davies and Breslin, 2003). When growing period starts, contaminated dust could infect litter, carriers, ventilation and finally the growing flock (Rose *et al.*, 1999:2000; Davies and Breslin, 2003). The main source of day-old flock contamination seems to be at the hatcheries for two reasons: the newly hatched chicks are more susceptible to colonization and hatcheries often serve as reservoirs for the bacteria (Bailey, 2002). Consequently, it is necessary to assess the main sources of *Salmonella* contamination at the hatcheries and then, implement suitable measures of control and eradication at the beginning of the production system. The main problem of bacterial infection is that irrespective of age at exposure (at the hatchery or at farm level) their shedding in faeces may persists between 10 and 12 weeks, well beyond the slaughter age for broiler chickens (Beal *et al.*, 2004). Consequently, birds will arrive infected with the bacteria at the processing plant, favouring cross-contamination of the carcasses (Ramesh *et al.*, 2002).

FAO-OMS (2001) demonstrated that lowering the on-farm prevalence of the bacteria is an important strategy for reducing the bird contamination entering the processing plant and lowering the risk of contaminated meat products entering the food chain. This hypothesis is especially significant for broiler production. For this reason, control of the main risk factors should be a useful tool for lowering the on-farm prevalence. However, important aspects in *Salmonella* surveillance and monitoring programmes by the government and/or poultry companies are the type of sample and the time of sampling to determine the flock status with the highest sensitivity (Heyndrickx *et al.*, 2002). The European Commission (EC, 2005) reported litter sampling utilizing several pairs of overshoes up to 3 weeks before slaughter as the official sampling method in the baseline survey on the prevalence of *Salmonella* in broiler flocks of *Gallus gallus*, in the EU, 2005-2006. Previous studies reported that litter sampling with sock swabs provided the highest sensitivity for determining the *Salmonella* status of the flock (Heyndrickx *et al.*, 2002; Buhr *et al.*, 2007). However, our results revealed that the highest *Salmonella* isolation from faeces samples collected with sock swabs was on the 14$^{th}$ day of rearing. The highest *Salmonella* detection rate coincided with the highest *Salmonella* excretion, which occurs at around 2 weeks of rearing (Berndt *et al.*, 2004; Van Immerseel *et al.*, 2004). Later, the *Salmonella*

detection decreased and became intermittent until day of slaughter. The detection pattern occurs independently of whether day-old flocks arrived infected from the hatchery or became infected at the farm. For these reasons, evaluating the farm status in the 3 weeks before slaughter age could underestimate the detection from faeces. Probably around 2 weeks of rearing should be considered the best moment to determine the *Salmonella* status of the flock. Moreover, the sooner the flock can be detected positive for the bacteria, the fewer expenses will be involved in carrying out control and eradication measures such as flock slaughter.

Nevertheless, *Salmonella* surveillance and control programmes do not end at farm level. Control should be considered until the end of the line, in processing plant and markets. The importance of transport in *Salmonella* spreading is frequently ruled out (Belles, 2007). Transport of poultry is a complicated multifactorial stressful and traumatic event that had been reported to induce carrier flocks to shed the bacteria at a higher rate (Mulder, 1995; Corry *et al.*, 2002). Birds are caught by the legs (in groups of 4-5) and inverted, then carried to crates, loaded and transported under variable climatic conditions (low temperatures, rain, wind, etc). This study revealed that there is an increase of *Salmonella* detection in faeces from 15.4 % to 41.2 %, before and after transport, respectively. However, the most significant result showed that transport period induced that 50.0 % of the flocks determined negative at farm level shed the bacteria at the slaughterhouse. Consequently, poultry determined negative at farm level, entered the processing plant shedding the bacteria. Birds that arrive at the processing plant carrying the bacteria, internally or externally, are considered a major source of contamination in poultry end products (Ramesh *et al.*, 2002).

In accordance with the above consideration, if *Salmonella* status of the flock is evaluated before transport, the number of *Salmonella* infected flocks could be underestimated. However, the main problem of slaughterhouse analyses is the time necessary to determine a positive sample according to the official ISO method 6579:2002 (Annex D). It is well known that after 24 hours of slaughtering, carcasses are in markets prepared for human consumption. Consequently, modern techniques, practical, cost-effective and more suitable for routine diagnosis should be developed to determine the status of the flocks in a short period of time and with the highest

sensitivity. As a result of the above considerations, probably around 2 weeks of rearing and at arrival for slaughter should be considered the best times to determine the *Salmonella* status of a broiler flock.

Regarding the *Salmonella* serotypes isolated, the results of this study coincide with those reported by the EFSA in 2007, with *S.* Enteritidis, *S.* Virchow, *S.* Hadar and *S.* Ohio being the most prevalent serotypes isolated from broiler production in Spain. However, it is important to stress that after transport no cases of *S.* Virchow and *S.* Ohio were isolated from faeces samples. Even though the *Salmonella* serotypes most frequently isolated from poultry are zoonotic and could infect human through poultry meat, each serotype has its own pattern during rearing and after transport. For this reason, in the future it may be interesting to find out which factors affect the appearance or disappearance of the different serotypes in broiler production.

*Salmonella* has been the subject of many studies worldwide since first isolated. Nowadays, much is known about its epidemiology. However, why can *Salmonella* not be removed from the sheds? Why are current control measures ineffective against the bacteria? Our results showed that cleaning and disinfection procedures applied in poultry houses are insufficient to remove *Salmonella*. These results coincide with those of Rose *et al.* (2000, France) in broilers and Davies and Breslin (2003, United Kingdom) in layers, who found high rates of *Salmonella* in poultry houses after cleaning and disinfection. Several hypotheses are related with this high persistence of *Salmonella*, such as absence of standardized cleaning and disinfection guidelines (Gradel *et al.*, 2004), lack of scientific literature related with disinfection in the agricultural sector (Ramesh *et al.*, 2002), absence of official methods for testing disinfectants (Lasa, 2007), inaccurate use of disinfectants (Davies and Breslin, 2003), incorrect hardness and temperature of cleaning water (Leriche *et al.*, 1995; Taylor *et al.*, 1996), high contents of protective compounds in poultry houses (fats, carbohydrates and proteins, Gradel *et al.*, 2004) and life in a biofilm state that protects the bacteria against environmental insults (Alvarez *et al.*, 1997). Our results showed that irrespective of their origin, around 50.0 % of the *Salmonella* strains isolated from each risk factor were able to produce biofilm. Carr *et al.* (1999) found that most disinfectants were ineffective against the bacteria in a field situation, because of persistently adherent and

protected bacteria, probably in a biofilm state. Disinfectants against bacterial cells in suspension may not be as effective when treating bacterial cells embedded in a Biofilm (Holland *et al.*, 1990). Biofilm presence, as well as organic load, will increase the disinfecting compound (Ramesh *et al.*, 2002). In our study, the use of glutaraldehyde, formaldehyde and hydrogen peroxide (1.0 % vol/vol) were insufficient to remove *Salmonella* from the poultry houses in field conditions, irrespective of the strain capacity of biofilm development, *Salmonella* serotype and the disinfectant contact time. Currently, poultry industry used cleaning and disinfection protocols with a mix of disinfectant active ingredients that boost their efficacy against *Salmonella*. Therefore, the main question is: why are some farmers able to remove *Salmonella* from broiler houses but there are a high percentage that are unable to eradicate it? In future studies it is important to find an answer to this question, assessing standardized cleaning and disinfection guidelines, evaluating methods for testing disinfectants in field conditions and providing effective farmer training in cleaning and disinfection management and self-control procedures.

In conclusion, the most contaminated samples related with poultry production during the rearing period were delivery-box liners, faeces, dust, farming boots and feed from feeders. Moreover, the main risk factors for *Salmonella* contamination of broiler flocks at the end of the rearing period were feed from feeders, *Salmonella* status of the house after C&D, and *Salmonella* status of day-old chicks. For these reasons, suitable measures should be implemented to control *Salmonella* at these three points during broiler production. Moreover, the 14$^{th}$ day of rearing and after transport to the slaughterhouse have been reported as the best moment to determine the *Salmonella* status of the broiler flocks. In further studies it will be necessary to analyse cleaning and disinfection in depth, in order to eradicate *Salmonella* presence from broiler production.

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# CHAPTER 5. CONCLUSIONS

1. Most contaminated samples related with poultry production during the rearing period are delivery-box liners, faeces, dust, farming boots and feed from feeders. Moreover, the most prevalent serotype isolated from broiler production is *S.* Enteritidis, followed by *S.* Hadar, *S.* Virchow and *S.* Ohio.

2. The main risk factors for *Salmonella* contamination of broiler flocks at the end of the rearing period are feed from feeders, *Salmonella* status of the house after cleaning and disinfection, and *Salmonella* status of day-old chick flocks.

3. Regardless of whether broiler flocks reached the farm already shedding the bacteria in faeces or were infected during rearing, the maximum rate of *Salmonella* detection is around 14$^{th}$ day of rearing. Moreover, the pattern of most prevalent serotypes varies throughout the rearing period.

4. Loading and transport to the slaughterhouse induce a significant increase in *Salmonella* detection rates. Moreover, the patterns of most prevalent serotypes during rearing change after transport to the slaughterhouse.

5. Nearly 50.0 % of the strains isolated from poultry risk factors are able to develop biofilm.

6. The use of glutaraldehyde, formaldehyde and peroxygen at a concentration of 1.0 % in field conditions is insufficient to eradicate *Salmonella*. However, more studies are necessary to find the correct concentrations and application of disinfectants in field conditions and compare this effect with biofilm or non-biofilm strains.