



# Protection of broilers against *Salmonella* using colonization-inhibition

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## List of abbreviations

| | |
|---|---|
| AXOS | Arabinoxylooligosaccharides |
| BGA | Brilliant Green Agar |
| BPW | Buffered Peptone Water |
| CE | Competitive Exclusion |
| CFU | Colony-Forming Units |
| CI | Colonization-Inhibition |
| CXC | Chemokine |
| DNA | Deoxyribonucleic Acid |
| DT | Phage Type |
| EFSA | European Food Safety Authority |
| FOS | Fructo-Oligosaccharides |
| GIT | Gastrointestinal Tract |
| GMO | Genetically Modified Organism |
| GOS | Galacto-Oligosaccharides |
| GRAS | Generally Recognized As Safe |
| HBSS | Hank's Balanced Salt Solution |
| IFN-γ | Interferon-gamma |
| IL | Interleukin |
| LB | Luria-Bertani |
| LPS | Lipopolysaccharide |
| MCFA | Medium Chain Fatty Acids |
| mRNA | messenger Ribonucleic Acid |

| | |
|---|---|
| MSRV | Modified Semi-solid Rappaport-Vassiliadis |
| NAP | Nucleoid Associated Protein |
| NF-κB | Nuclear Factor Kappa Bèta |
| OMP | Outer Membrane Proteins |
| PAMP | Pathogen-Associated Molecular Patterns |
| SCFA | Short Chain Fatty Acids |
| SCV | *Salmonella* Containing Vacuole |
| SIF | *Salmonella* Induced Filaments |
| Sip | *Salmonella* invasion proteins |
| Sop | *Salmonella* outer proteins |
| SPI-1 | *Salmonella* Pathogenicity Island 1 |
| SPI-2 | *Salmonella* Pathogenicity Island 2 |
| T3SS | Type Three Secretion System |
| TLR | Toll-Like Receptor |
| TNF-α | Tumor Necrosis Factor Alpha |
| VAP | Vacuole-associated Actin Polymerization |
| XLD agar | Xylose Lysine Deoxycholate agar |
| YOPI | Young, Old, Pregnant and Immunosuppressed |

# Chapter 1: General Introduction

# 1 General Introduction

## 1.1 *Salmonella* taxonomy and characteristics

*Salmonella* is a genus of rod-shaped, Gram-negative bacteria belonging to the family Enterobacteriaceae. They are non-spore forming, facultative anaerobes and often possess peritrichous flagellae for motility. Salmonellae are commonly found pathogens because most have a broad spectrum of hosts (both warm- and cold blooded organisms) and transmission vectors (food, feed, water sources, farm material and animals such as rodents). Additionally, they are capable of surviving in the environment for prolonged periods of time. They are able to multiply when temperatures range between 7 - 45 °C and when pH is ranging between 4.0 and 9.5. Additionally, they are able to survive freezing temperatures and dehydration. Therefore, these zoonotic bacteria are frequently found in the environment and in animals, and are of importance because they can cause severe illnesses depending on the serotype and host.

The genus *Salmonella* consists of only two species, namely *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* is further sub-divided in six subspecies: *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae* and *indica* [1]. Together, these six subspecies of *S. enterica* and *S. bongori* comprise more than 2600 characterized serotypes or serovars [2]. *Salmonella* serovars are divided according to the White-Kauffmann-Le Minor scheme, which is based on the presence and structure of different surface antigens. The antigens that define the serotype are the 'O' antigen, which is determined by the oligosaccharide type of lipopolysaccharide (LPS), the 'H' antigen, determined by flagellar proteins and the capsular

'Vi' antigen. The *Salmonella* serovars can be further divided based on their susceptibility to antimicrobials or bacteriophages. With the latter method, different phage types (DT) can be distinguished within certain serotypes [3].

Most *Salmonella* serovars of zoonotic importance belong to *Salmonella enterica* subsp. e*nterica* [1, 4] and are capable of causing disease in a broad range of hosts, such as *Salmonella* Enteritidis and *Salmonella* Typhimurium. Other *Salmonella* serotypes, like *Salmonella* Typhi in humans and *Salmonella* Gallinarum in chickens, are adapted to a single host species and only cause disease in their respective hosts.

## 1.2 *Salmonella* in humans

In 2012, 91 034 confirmed cases of human salmonellosis were reported in the European Union, with a notification rate of 22.2 cases per 100 000 population [5, 6]. The estimated case-fatality rate for *Salmonella* was reported to be 0.14 %, as 61 deaths occurred due to non-typhoidal salmonellosis. This is however an underestimation of the real number of human salmonellosis cases as not all infected people develop symptoms, not all infected persons are sampled and because it is not obliged to report positive samples to public health authorities. While the number of human salmonellosis cases has been decreasing since 2008 (with 32 % fewer cases in 2012), *Salmonella* remains the second most frequently reported zoonotic agent in humans in the European Union (Figure 1) [5]. The decrease in human salmonellosis cases is often attributed to the successful *Salmonella* control programs in laying hens, which resulted in a lower occurrence of *Salmonella* in eggs and consequently in a reduced introduction of *Salmonella* in the food chain. Campylobacteriosis was however the

most frequently reported human zoonosis in the European Union in 2012 with more than double the number of reported *Salmonella* cases, illustrating the importance of *Campylobacter* for human health as well [5].

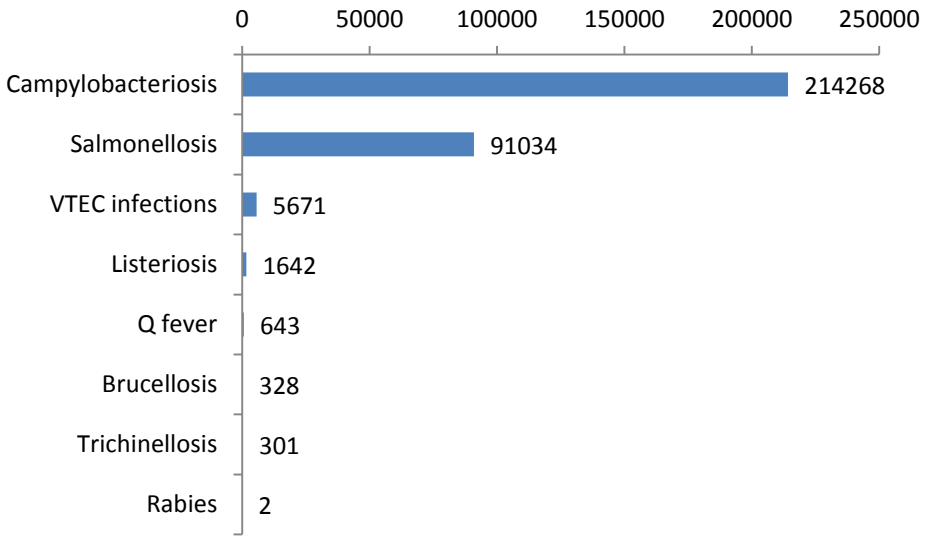


**Figure 1: The number of confirmed human cases for different zoonoses in the EU in 2012 (adapted from [5]).**

*Salmonella enterica* subsp. *enterica* serovar Enteritidis (more commonly known as *Salmonella* Enteritidis) was the most prevalent serovar isolated from humans in 2012, with 41.3% of all human *Salmonella* isolates belonging to this serovar [5]. The second most isolated serovar was *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*Salmonella* Typhimurium), to which 22.1 % of all human isolates belonged. Monophasic *Salmonella* Typhimurium 1,4,[5],12:i:- was reported to be the third most prevalent serovar due to several large outbreaks, followed by *Salmonella* Infantis (Figure 2).

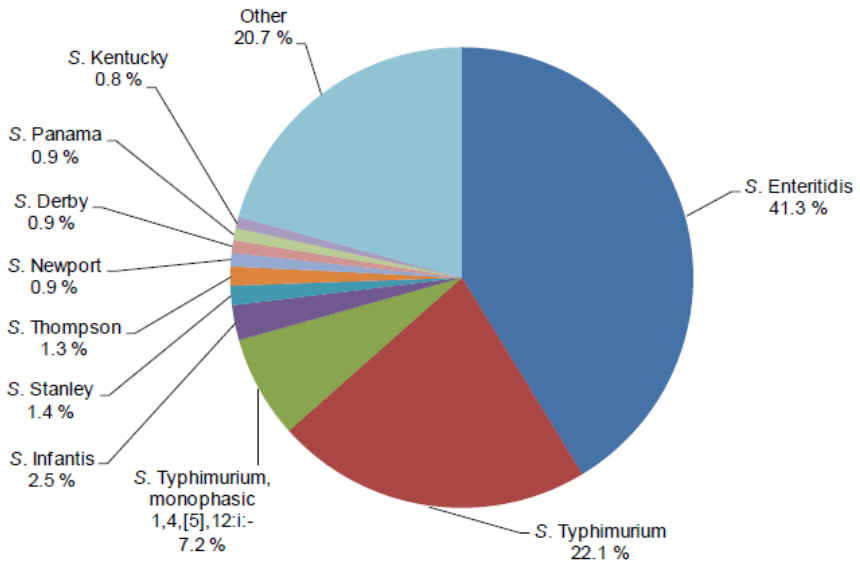


**Figure 2: Distribution of the *Salmonella* serovars found in humans in the EU in 2012 [5].**

Human salmonellosis, when not asymptomatically, is usually characterized by acute onset of fever, abdominal pain, nausea and sometimes vomiting, after an incubation period of 12-36 hours. These symptoms are often mild and infections are frequently self-limiting, lasting only a few days. However, this is not always the case, and in some patients (especially the YOPI group; Young, Old, Pregnant and Immunosuppressed) this infection can become more serious and the associated dehydration can become life-threatening. When this is the case, and when *Salmonella* causes systemic infections, antimicrobials are necessary for treatment. However, rehydration and electrolyte supplementation therapy may be more appropriate when suffering from severe diarrhea, as excessive use of antimicrobials has resulted in an increase in microbial resistance [7, 8], limiting therapeutic alternatives. Additionally, antimicrobial treatment does not shorten the symptomatic period and might even prolong the duration of carriage [9]. Consequently, antibiotics should be reserved for patients at increased risk or suffering from severe disease.

## 1.3 *Salmonella* in food and food products

Despite the decreasing number of human salmonellosis cases, *Salmonella* remains the zoonotic agent responsible for most food borne outbreaks in the European Union in 2012. It caused 28.6 % of all food-borne outbreaks, while bacterial toxins, viruses and *Campylobacter* (the other major causative agents) caused only 14.5 %, 14.1 % and 9.3 % of all foodborne outbreaks respectively (Figure 3). Humans can also become infected with *Salmonella* through direct or indirect contact with animals [10], but most human salmonellosis cases are caused by the consumption of contaminated food. In order to reduce the prevalence of *Salmonella* in food and food products, the European Union laid down Regulation (EC) No 2073/2005, which was modified by Regulation (EC) No 1441/2007. These regulations describe rules for sampling and testing food and food products and set limits for the presence of *Salmonella* in different food categories and in samples derived from food processing. More specifically, they describe that *Salmonella* must be absent from several food categories, such as minced meat and meat preparations intended to be eaten raw, milk and whey powder, ice cream and egg products. Absence of *Salmonella* is determined by testing five or 30 samples of 25 g per batch, depending on the food category.

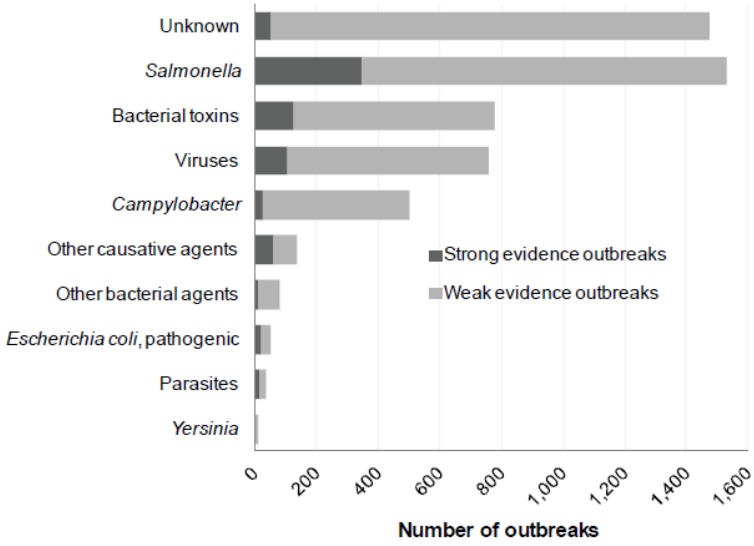


**Figure 3: Distribution of food-borne outbreaks per causative agent in the EU in 2012 [5].**

*Salmonella* can be found in different types of food, ranging from poultry, pig and bovine products and meat to vegetables, fish or other fishery products. It is most frequently found in fresh broiler meat and turkey meat, less often in pig or bovine meat and rarely in table eggs [5]. However, because table eggs are consumed in large quantities, this low prevalence also remains relevant for consumer food safety [5]. Similarly, *Salmonella* contaminated pig meat and derived products are an important source of food-borne salmonellosis as well. *Salmonella* could be found in 0.7 % of the tested pig meat in the European Union in 2012, and pig meat and products thereof are believed to be responsible for 5.8 % of all strong-evidence *Salmonella* outbreaks [5]. Minced meat and meat preparations from poultry however have the highest level of non-compliance with EU *Salmonella* criteria, with 8.7 % of single samples and 5.7 % of batches being positive for *Salmonella* in 2012 [5]. Additionally, the highest proportions of *Salmonella*-positive single samples were reported for fresh broiler meat at an average level of 5.5 % [5]. Consequently, broiler meat is an important source of foodborne *Salmonella* outbreaks and efforts should be made to reduce the amount of

*Salmonella* in broiler meat and derived products. These efforts should focus on the whole broiler meat production chain and the subsequent storage and handling of meat. A key part in this 'farm-to-fork' strategy would be to reduce *Salmonella* prevalence in poultry flocks, which in turn would result in a reduced prevalence of *Salmonella* in poultry products. This could possibly be done by exploiting a colonization-inhibition (CI) phenomenon, which is currently not used in practice but represents a potentially powerful method to reduce *Salmonella* prevalence in poultry flocks. Therefore, several aspects of this CI phenomenon as a possible novel control method to reduce *Salmonella* colonization of poultry will be investigated in this work.

## 1.4 *Salmonella* in animals other than chickens

In addition to chickens, pigs represent an important reservoir for *Salmonella* as well. In 2012, 5.5 % of all tested pigs and 17.5 % of all tested herds in Europe were positive for *Salmonella* [5]. In Belgium, 10.8 % of all tested pig carcasses in the slaughterhouse were positive for *Salmonella* [5]. *Salmonella* Typhimurium was the serovar most commonly isolated from pigs and pig meat in Europe in 2011, followed by *Salmonella* Derby and monophasic *Salmonella* Typhimurium [11]. Similarly, in Belgium, *Salmonella* Typhimurium was the *Salmonella* serovar most commonly associated with pigs, with 67.5 % of tested strains being *Salmonella* Typhimurium isolates in 2010 [12]. *Salmonella* Derby was the second most prevalent serotype in pigs in Belgium in 2010, with 7.3 % of tested strains belonging to this serotype [12].

*Salmonella* can also be found in other animals, such as cattle, turkeys, ducks and geese. In 2012 in Europe, 2.3 % of all tested cattle and 3.8 % of all tested herds were positive for *Salmonella* [5]. The overall prevalence for *Salmonella* in fattening turkey flocks in the European Union was 0.4 %, a prevalence similar to that in 2011 (0.5 %) [5]. Consequently, these animals represent a reservoir for *Salmonella* as well and contribute to the introduction of *Salmonella* in the food chain. However, as *Salmonella* contaminated eggs, pig meat, broiler meat and derived products are far more important for human health, efforts to control *Salmonella* in the food chain should mainly focus on chickens and pigs.

## 1.5 *Salmonella* in chickens

### 1.5.1 Epidemiology

In 2012, 0.4 % of *Gallus gallus* breeding flocks were positive for either *Salmonella* Enteritidis, *Salmonella* Typhimurium, *Salmonella* Hadar, *Salmonella* Infantis or *Salmonella* Virchow, the serovars covered by European legislation in breeders [5]. Overall, 2.0 % of the breeding flocks of *Gallus gallus* in the European Union were positive for *Salmonella* spp in 2012, which is a small increase since 2011 when 1.9 % were found positive [5]. In Belgium, 2.5 % of *Gallus gallus* breeding flocks were positive for *Salmonella* spp in 2012 and 0.2 % were positive for the target serovars covered by European legislation [5]. In the case of laying hens, the prevalence of *Salmonella* Enteritidis and *Salmonella* Typhimurium reduced from 1.5 % in 2011 to 1.3 % in 2012 in Europe [5]. Altogether, 3.2 % and 4.7 % of the laying hen flocks were positive for *Salmonella* spp in Europe and Belgium respectively [5]. A similar prevalence was observed in broilers in Europe, where 3.1 % of all flocks were positive for *Salmonella* spp

in 2012. This was a decrease of 0.1 % since 2011. In Belgium, 3.4 % of all broiler flocks were positive for *Salmonella* spp in 2012. The prevalence for the serovars covered by European legislation in broilers, *Salmonella* Enteritidis and *Salmonella* Typhimurium, was 0.3 % in Europe and 0.6 % in Belgium [5]. The prevalence of these two serotypes in broilers is thus quite low, but *Salmonella* Enteritidis and Typhimurium remain relevant in broilers due to their importance for human health. The prevalence of *Salmonella* in poultry has, in general, been decreasing the past few years, and this decrease is mainly due to implemented control programs. As of 2009, European Union member states are obliged to implement national programs to control *Salmonella* in broiler flocks in accordance with Regulation (EC) No 2160/2003. This Regulation states that measures should be taken to prevent, detect and control *Salmonella* at all relevant stages of the production, processing and distribution chain in order to reduce *Salmonella* prevalence and thus the risk to public health. Legislation in the European Union dictates that the maximum percentage of broiler flocks positive for the serovars Enteritidis and Typhimurium should be 1 % or less, because of their importance for human health. Sampling of broiler flocks takes place 3 weeks before the chickens are moved to the slaughterhouse, by taking at least two pairs of overshoe samples per flock.

In addition to the staggering evidence linking consumption of contaminated poultry products to salmonellosis cases, the presence of different serotypes of importance for public health in poultry flocks indicates that poultry is an important reservoir for human infections. Vaccinating layers, which is mandatory in Belgium since 2007 [13], has resulted in a lower *Salmonella* Enteritidis prevalence in egg and egg products, which in turn has resulted in a significantly decreased introduction of *Salmonella* Enteritidis in the food chain. Similar efforts should be made to lower *Salmonella* prevalence in broiler meat, not only by focusing

on the later stages of production (transport to slaughterhouse, slaughter) which have been proven of major importance [14], but also by controlling *Salmonella* prevalence in broiler flocks.

Poultry can become infected with *Salmonella* through both vertical and horizontal transmission of the bacterium [15]. Vertical transmission from the parent to the day-old chicken has often been reported, and both *Salmonella* Enteritidis and Typhimurium can be transmitted in this manner. Controlling this transmission route has been the main factor in several eradication programs [16-18]. Nevertheless, horizontal transmission in hatcheries and farms is often of greater importance, and serovars other than Enteritidis and Typhimurium only spread in this way. Risk factors for horizontal transmission include inadequate cleaning and disinfection of broiler rearing houses leading to contamination of the next flock upon introduction in the rearing house [19-23], a poor level of hygiene in general [24], and contamination of the feed [25-27]. Other risk factors for horizontal transmission are the size of the farm [27, 28], the season in which the chickens are reared [28], the presence of insects and rodents on the farm [29], and contamination of *Salmonella* negative flocks during transport to the slaughterhouse due to contaminated crates and contamination in the slaughterhouse [30-32]. Chickens are thus usually infected by oral uptake of *Salmonella* bacteria from the environment (oral-fecal route) and the infection will spread rapidly through bird to bird contact [33]. Chickens can, however, also become infected with *Salmonella* after inhaling even low numbers of the bacteria, and thus is the respiratory route an additional portal of entry for *Salmonella* [34, 35].

### 1.5.2 *Salmonella* pathogenesis

#### *1.5.2.1 Initial colonization of the host*

Chickens primarily become infected with *Salmonella* after oral ingestion of the bacterium. Once ingested, the bacterium needs to pass through the alimentary system and survive the acidic environment of the stomach. It is able to do so because of the so-called acid-tolerance response, a complex adaptive system which allows the bacteria to synthesize a large number of acid shock proteins, including the RpoS σ-factor, Ada and Fur, which ensure bacterial survival in acidic environments [36, 37]. The *Salmonella* bacteria that survive the acidic environment of the stomach proceed through the gastrointestinal tract (GIT), the primary site of attachment for *Salmonella* in the chicken. Here, epithelial and immune cells lining the GIT are the initial protective barrier against *Salmonella*. Additionally, *Salmonella* has to compete with the gut microbiota to make contact with enterocytes in order to colonize the GIT.

#### *1.5.2.2 Invasion and epithelial inflammation*

Once contact has been made with the intestinal enterocytes, *Salmonella* adheres to the epithelial cells lining the GIT, a process facilitated by flagellae and fimbriae present on the bacterial cell wall [38, 39]. When attached to the intestinal epithelium, *Salmonella* expresses a type three secretion system (T3SS), a multiprotein effector complex which facilitates uptake and invasion in epithelial cells [40, 41]. This “molecular needle” is encoded on *Salmonella* Pathogenicity Island 1 (SPI-1), a genomic region which contains virulence genes

involved in *Salmonella* adhesion, invasion and toxicity. The structure consists of more than 20 proteins, forms a structure spanning both the inner and outer membrane of the bacterial cell wall and ending in a needle-like structure that extends outward from the bacterial cell surface (Figure 4).

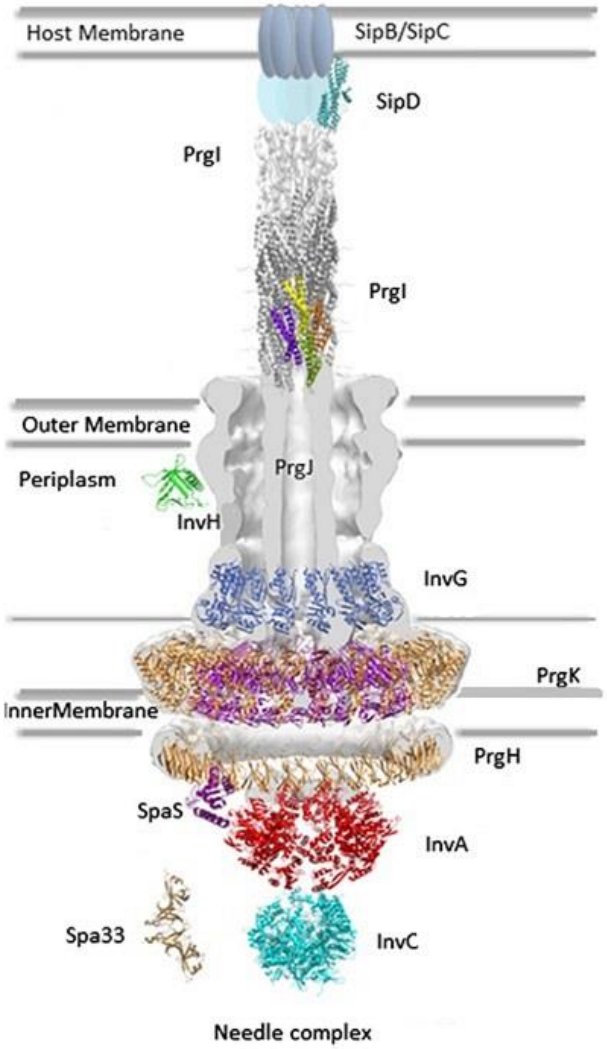


**Figure 4: Structural overview of the Type Three Secretion System (adapted from [42]).**

The main function of the T3SS is to inject effector proteins in the intestinal epithelial cells to which *Salmonella* is attached. The effector proteins injected in epithelial cells by the T3SS are often also encoded on the SPI-1, and include proteins like Sip (*Salmonella* invasion proteins),

Sop (*Salmonella* outer proteins) and several others [43]. These proteins have different functions, and can contribute to *Salmonella* pathogenicity in various ways. For instance, SopB, one of the proteins translocated through the T3SS, activates secretory pathways in the epithelial cells, facilitating inflammation and disturbing the ion balance within the cell [44-46]. This results in an increased secretion of fluids in the GIT and consequently diarrhea, which contributes to the further spread of the *Salmonella* bacterium [43]. Other effector proteins, such as SopA and SopE2 also play a role in *Salmonella* gastroenteritis [45]. SopE2 does this by reprogramming host gene expression through the transcriptional factor NF-κB, which results in the induction of pro-inflammatory cytokines such as IL-8 and TNF-α, eliciting mucosal inflammation [47]. Other factors than those associated with SPI-1 contribute to the gastroenteritis associated with *Salmonella* infection as well. The *Salmonella* genome encodes several molecules that are also present in other bacteria, like lipopolysaccharides (LPS), flagella and curli fimbriae, which are recognized by the host as pathogen-associated molecular patterns (PAMP's). These PAMP's interact with Toll-like receptors (TLR's) and are thus capable of stimulating the pro-inflammatory pathways of the immune system, inducing IL-8 production and neutrophil influx [48-50]. For instance, flagellin, the monomer which forms the filaments in the bacterial flagellum, interacts with TLR5, hereby activating NF- κB and IL-8 secretion [51]. The developed pro-inflammatory response eventually leads to epithelial damage, allowing essential nutrients to become available for the *Salmonella* bacteria [51]. Overactivation of these pathways will however disturb host cell homeostasis in various ways, which might affect the ability of *Salmonella* to survive, replicate and disseminate in the host [51]. To avoid this, *Salmonella* has developed mechanisms to downregulate the pro-inflammatory response by delivering antagonic and thus anti-inflammatory effectors (like SptP and SpvC) into the host cell. One of these anti-

inflammatory effectors, SptP, which is encoded on SPI-1, disrupts the actin cytoskeleton and thus antagonizes the cytoskeleton rearrangements induced by SopE [52, 53], limiting *Salmonella* invasion and thus reducing the severity of the inflammatory response.

Invasion of *Salmonella* in GIT epithelial cells is a complex process, induced by effector proteins injected through the T3SS which interact with the actin cytoskeleton of the epithelial cell (Figure 5). Injection of SopE, SopE2 and SopB effector proteins activates the RhoGTPases Rac, Cdc42 and RhoG, which results in actin cytoskeleton remodeling via cellular proteins [54, 55]. SipA and SipC induce rearrangements in the cytoskeleton by binding directly to actin, localizing actin polymerization at the bacterial entry site [54, 55]. SipC is able to nucleate filamentous actin (F-actin) and promote F-actin bundling [54]. SipA stimulates these SipC activities and can stabilize F-actin by directly antagonizing the action of depolymerizing factors in the cell, thus inhibiting actin depolymerisation at early stages of infection [54, 56]. These interactions with the host cell machinery cause the host cell membrane to extend outwards and to form filopodia [51], a process known as membrane ruffling which facilitates engulfment of *Salmonella* by the host cell membrane through pinocytosis. Another T3SS effector protein, SopD, is able to directly promote this process due to its role in membrane fission and macropinosome formation, and increases inflammation and fluid secretion as well [57]. After the bacteria are taken up through pinocytosis, they becomes internalized in a vesicle known as the *Salmonella* containing vacuole (SCV) [58].

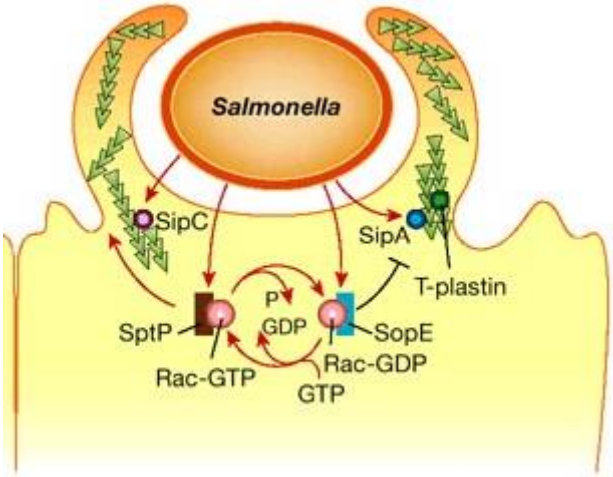


**Figure 5: The role of different *Salmonella* Pathogenicity Island 1 (SPI-1) effector proteins in *Salmonella* invasion (adapted from [59]).** After adhesion to the epithelial cell, *Salmonella* injects several effector proteins in the host cell through a Type Three Secretion System (T3SS). One of these effector proteins, SipA, binds directly to actin and indirectly to the actin-bundling protein T-plastin. This results in localized actin polymerization at the bacterial entry site and the formation of filopodia, facilitating cellular entry for *Salmonella*. SipC, another effector protein, induces actin bundling as well and contributes to the formation of filopodia. The SopE effector proteins binds and activates host cell proteins Rac and Cdc42, which results in the formation of membrane ruffles and facilitates entry of *Salmonella* in the host cell as well. SptP, on the other hand, binds and inactivates Rac and Cdc42, antagonizing the effects of SopE and limiting the host pro-inflammatory response.

Recent research has however suggested that *Salmonella* is able to invade host cells in ways independent of SPI-1 T3SS [54, 60, 61]. It is known that the association of *Salmonella* Outer Membrane Proteins (OMP) with host cells triggers a variety of biological events, like the induction of innate and adaptive immune responses [62]. However, it has been shown that the *Salmonella* OMP Rck and PagN are able to induce cell invasion through an alternate mechanism, which is suggested to play a role in systemic infection as well [54, 60, 63]. This so-called "Zipper" mechanism allows *Salmonella* to enter the host cell in a receptor-mediated way, by tightly binding the bacteria to the host cell membrane and initiating only minor cytoskeletal rearrangements through contact between bacterial ligands and host cell surface receptors (Figure 6) [54, 61].

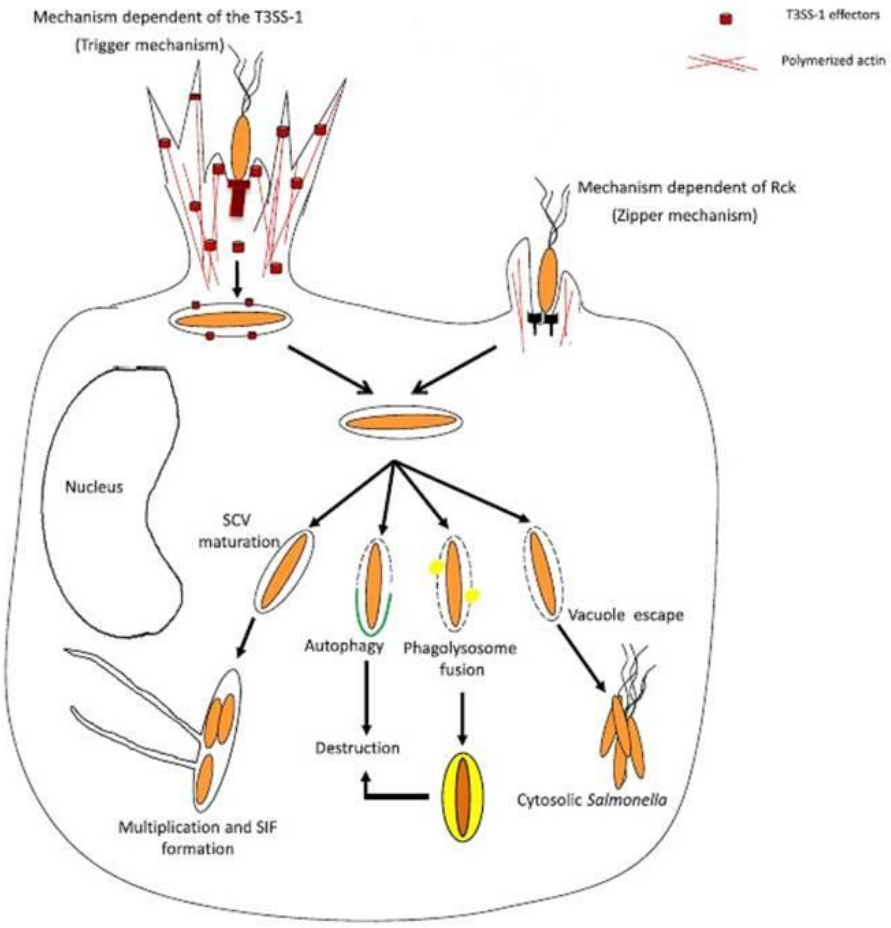


**Figure 6: Different strategies used by *Salmonella* for invasion and proliferation in eukaryotic cells (adapted from [61]).** *Salmonella* is able to invade host cells through a Trigger mechanism or a Zipper mechanism. The Trigger mechanism is mediated by the T3SS and induces major cytoskeletal rearrangements. Following entry, *Salmonella* is taken up in the SCV, which undergoes different stages of maturation while *Salmonella* replicates inside. This replication is associated with the formation of SIF's, which facilitate delivery of nutrients to the SCV. A portion of the bacteria are able to escape the SCV and multiply in the cytosol of the epithelial cell. The Zipper mechanism, mediated by Rck, induces minor cytoskeletal rearrangements and leads to internalization of the bacteria into a vacuole [61].

In either way, internalization in the SCV plays a key role in *Salmonella* survival and proliferation in intestinal epithelial cells and macrophages, as the SCV is the only cellular compartment in which *Salmonella* cells survive and replicate [51, 64]. This SCV is a membrane-bound compartment initially integrated in the early endocytic pathway (Figure 7). In order for *Salmonella* to avoid destruction by phagolysosomal pathways in the cell and survive here, the delivery of host lysosomal enzymes to the SCV needs to be circumvented

[51, 65, 66]. To do so, the *Salmonella* bacterium expresses a second T3SS encoded on *Salmonella* Pathogenicity Island 2 (SPI-2) once it becomes incorporated in the SCV, which allows for the delivery of a wide variety of effector proteins in the SCV. One of these effector proteins, SsaB, inhibits intracellular trafficking by blocking the fusion of the SCV with the lysosomes, and thus allows *Salmonella* to avoid destruction by phagolysosomal pathways [67, 68]. Other SPI-2 T3SS effectors seem to mediate generally later steps in SCV biogenesis, including movement of the SCV to the juxtanuclear region and the subsequent maintenance of this position, formation of an actin meshwork around the SCV and anterograde extension of *Salmonella* induced filaments (SIF) along microtubules [66]. The formation of an F-actin network around the SCV is termed vacuole-associated actin polymerization (VAP), a process which allows the SCV to migrate to a perinuclear position in the host cell close to the Golgi apparatus, presumably to facilitate interception of transport vesicles containing nutrients and membrane fragments, essential for bacterial replication within the SCV [51, 69]. This movement is mediated by two SPI-2 T3SS effectors, SseF and SseG, which supposedly recruit the motor protein dynein to the SCV via a Rab7-RILP interaction [70, 71]. In addition, it has been observed that intracellular *Salmonella* can induce the formation of lysosomal SIF's through the expression of the SifA effector protein [72]. Although the role of these long filamentous membrane structures is not completely clear, they facilitate fusion of the SCV with other vesicles in the cell, and are suggested to play a role in *Salmonella* replication [40]. The SifA protein has another role also, as it contributes to maintaining the membrane integrity of the SCV, together with PipB2, SseJ and SopD2 SPI-2 T3SS effector proteins [73]. Other SPI-2 T3SS effectors are involved in ubiquitin modification, immune signaling and target the host cytoskeleton [66, 73], and contribute to the survival and replication of

*Salmonella* in the SCV. *Salmonella* is thus able to survive and replicate in epithelial cells and even spread to underlying tissues, resulting in a systemic infection.

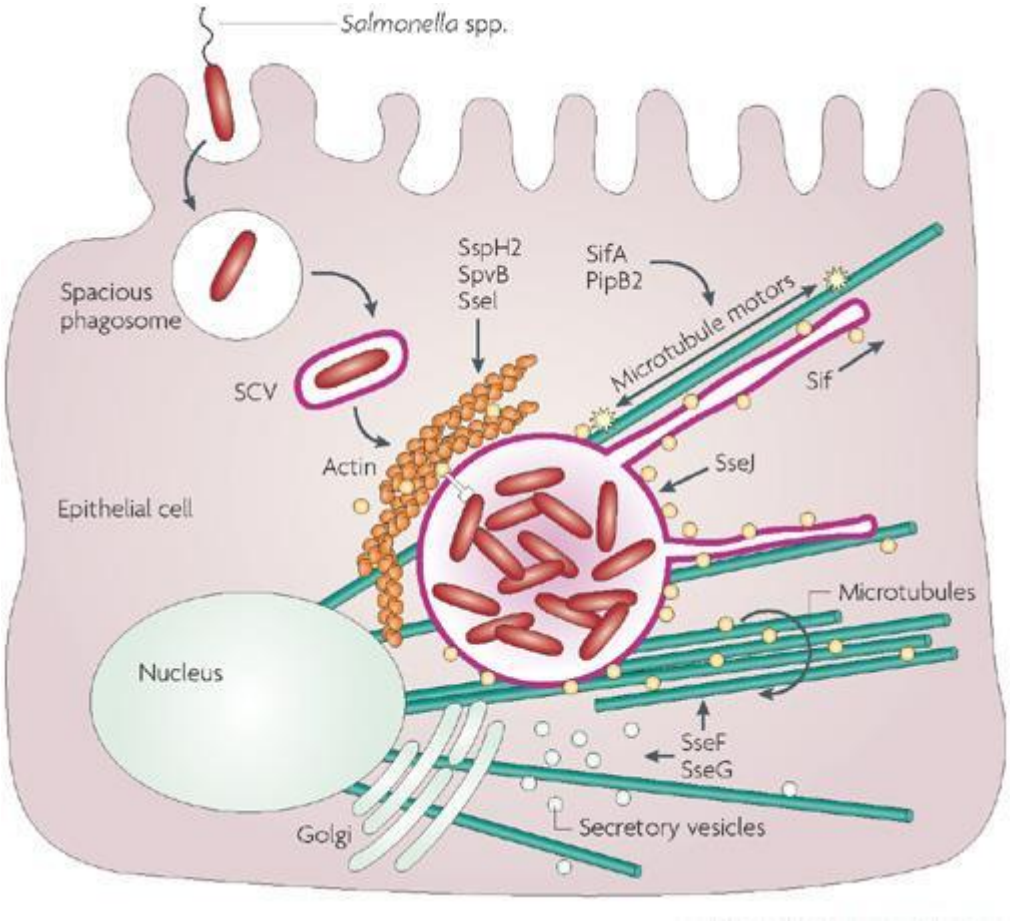


**Figure 7: The *Salmonella* containing vacuole (SCV) and the role of different *Salmonella* Pathogenicity Island 2 (SPI-2) effector proteins during *Salmonella*'s intracellular phase (adapted from [74]).** After internalization in the epithelial cell, *Salmonella* becomes enclosed in a phagosome that fuses with lysosomes, acidifies and shrinks to become the *Salmonella* containing vacuole (SCV). The SPI-2 Type Three Secretion System (T3SS) is induced within the SCV and translocates different effector proteins (yellow spheres) across the SCV membrane. SPI-2 effector proteins such as SifA and PipB2 contribute to the formation of *Salmonella*-induced filaments (Sif) along microtubules (green) and regulate the accumulation of microtubule-motor proteins (yellow stars) on the Sif and the SCV. Another effector protein, SseJ, is active on the phagosome membrane and contributes to maintaining membrane integrity of the SCV. Effector proteins SseF and SseG direct Golgi-derived vesicle traffic toward the SCV, presumably to provide nutrients and membrane fragments to the SCV. Other SPI-2 T3SS effector proteins, such as SspH2, SpvB and SseI are believed to play a role in the actin accumulation around the SCV.

#### *1.5.2.3 Survival in macrophages and dendritic cells*

*Salmonella* is able to develop invasive and systemic infections [43]. When this is the case, a fraction of the SCV's transcytose to the basolateral membrane of the epithelial cells, where the *Salmonella* bacteria are engulfed by phagocytes (neutrophils, inflammatory monocytes which differentiate into macrophages or dendritic cells) which interact with these invading bacteria [51]. In addition, dendritic cells have also been reported to directly take up bacteria from the intestinal lumen by opening the tight junctions and sending dendrites to the lumen [75]. It has been shown that *Salmonella* is able to replicate within macrophages [76, 77], while they do not appear to replicate within dendritic cells even though they remain viable [64]. After engulfment by these phagocytes, the *Salmonella* bacterium is once again internalized in a SCV, triggering events similar to those when internalized in epithelial cells that allow survival and replication within the infected cells. Migration of these infected phagocytes leads to systemic dissemination of the bacteria to several other tissues, such as the liver and spleen, where *Salmonella* preferentially replicates [78]. When this is the case, *Salmonella* is rapidly distributed in lymphoid and non-lymphoid tissues and eventually transported to different internal organs, resulting in life-threatening septicemia.

#### *1.5.2.4 Regulation of* Salmonella *pathogenicity*

*Salmonella* Pathogenicity Island 1 (SPI-1), is a genomic region which contains virulence genes involved in *Salmonella* adhesion, invasion and toxicity. Expression of these SPI-1 genes is however tightly regulated by both environmental conditions and a variety of regulatory genes [79, 80]. The main regulatory gene for SPI-1 is HilA, which is necessary for controlling

expression of the invasive phenotype and is essential for colonization of the chicken caecum [81]. HilA activates other SPI-1 genes, like *invF*, by interacting with their promoters [79, 80, 82]. Transcription of HilA itself is governed by several other regulatory proteins, like HilC and HilD (also encoded on SPI-1) and Lon, Fis, FliZ, Hha and Png (encoded outside the SPI-1) [80]. Transcription of HilC and HilD is in turn governed by regulatory cascades, in which the two component regulators BarA/SirA and PhoP/Q, and the Csr post-translational control system play an important role [79]. Several proteins which are part of these regulatory cascades are able to sense environmental changes, resulting in a system in which SPI-1 genes are only expressed under the right conditions, namely when invading host cells. Optimal expression of SPI-1 occurs under conditions present in the gut lumen, like low oxygen, high osmolarity, at neutral to slightly basic pH and during exponential growth phase [80, 83]. Additionally, the short chain fatty acid acetate stimulates the expression of SPI-1 genes, while propionate and butyrate repress the expression [84]. This is because *Salmonella* has adapted in a way that the genes involved in invasion are only expressed when necessary, that is, when present in the GIT of a susceptible host.

*Salmonella* Pathogenicity Island 2 (SPI-2), on the other hand, is a genomic region containing virulence genes involved in intracellular *Salmonella* survival and replication. The *ssrA* gene plays an important role in this process, as the corresponding protein is an essential component of the SsrAB regulatory system for the SPI-2 [85]. Therefore, the s*srA* gene is crucial for survival of *Salmonella* in host cells [86]. Expression of *ssrA* itself is modulated by global regulatory proteins [87] such as PhoPQ [88], OmpR/EnzV [89-91] and SlyA [92, 93]. Additionally, nucleoid associated proteins (NAP's) like IHF and Fis and remarkably, HilD also have a role in SPI-2 transcription regulation [85, 94, 95]. Similarly to regulation of SPI-1, the

regulatory proteins form part of a cascade which allows *Salmonella* to sense its environment and allows expression of SPI-2 genes when residing in the SCV. SPI-2 gene expression is induced by low concentrations of $Ca^{2+}$, $Mg^{2+}$, phosphate starvation and a decrease in osmolarity [85, 89, 96]. SPI-2 is also expressed under low pH and repressed under alkaline pH conditions [85, 89, 97]. At the same time, expression of SPI-2 is repressed when SPI-1 is upregulated and vice versa, a mechanism in which HilD plays a central role [94, 95]. This complex regulation for both SPI-1 and SPI-2 allows for a carefully regulated temporal and spatial expression of *Salmonella* virulence genes, which is essential for *Salmonella enterica* pathogenesis [85].

## 1.6 *Salmonella* control in broilers

Poultry meat is consumed in large quantities worldwide and because its consumption can be linked to foodborne *Salmonella* outbreaks, efforts have been made to reduce *Salmonella* contamination in broiler meat and poultry products in general. These efforts often focus on the whole broiler meat production chain and form part of a 'farm-to-fork' strategy [98]. Safety measures can be taken at different levels, namely pre-harvest (i.e. on-farm), on the harvest level (i.e. during transport to the slaughterhouse and at the slaughter line) and post-harvest (i.e. during processing and during preparation). It is however most probable that concurrent control measures applied at every level of the broiler production chain will lead to the largest reduction in poultry meat contamination with *Salmonella*.

### 1.6.1 Biosecurity measures

Biosecurity is defined as a collection of rules and procedures that minimize exposure of a susceptible population to an infectious (biological) agent [99, 100]. In practice, this comprises a wide range of hygienic and management measures, taken on different levels of the production chain. An important measure is the exclusion of enteropathogens such as *Salmonella* at the top of the production chain by testing and culling infected flocks, which would otherwise result a widespread dissemination of *Salmonella* from the purelines to their progeny through vertical transmission [15, 100]. This remains also important for the lower stages in the broiler production chain, as vertical transmission of *Salmonella* Enteritidis and Typhimurium from a parent flock to day-old chickens leaving the hatchery has often been reported as well [14, 17, 18]. In addition, eggs generate large amounts of dust and airborne fluff during the hatching process which, in the case of contaminated eggs, can contribute to the spread of *Salmonella* to other eggs and hatched chicks [100-102]. To avoid this, numerous methods are employed to disinfect eggs, such as disinfection with ultraviolet light, ozone or chemicals [100, 103, 104]. Consequently, reducing the vertical transmission of *Salmonella* has been a main controlling factor in many eradication programs.

In addition to vertical transmission, horizontal transmission of *Salmonella* in hatcheries and on the farm during the rearing period is of major importance for the spread of *Salmonella*. It is known that inadequate cleaning and disinfection of the broiler rearing houses can lead to contamination of the following flock [14]. Therefore, broiler rearing houses should be cleaned and disinfected thoroughly between production cycles. Introduction of possible carriers such as birds, rodents and insects in the farm house should obviously be avoided

[105]. To do so, housing must be designed in a way that it prevents entry of any carrier, and incorporate pest-control measures such as traps and baits [100]. In addition, not only pests can be responsible for the introduction of *Salmonella*, but also humans can be a source of enteric pathogens [106]. Human access to the rearing house is however necessary, and sanitation and hygiene measures such as hygiene gates should thus be employed [100].

Another critical point in control of *Salmonella* in poultry production is the poultry feed, as this has frequently been found to be a major vehicle of transmission of *Salmonella* [100, 107]. While feed components are typically heat-treated to avoid this, the feed often becomes contaminated during processing [107, 108]. This can however be overcome by thermal processing or chemical treatment of the complete feed [109]. Similarly, drinking water provided to the chickens should be free of enteropathogens and of potable quality. In practice, on-farm water is frequently drawn from natural sources and should be treated with chemicals or filtered before being presented to the animals [100]. Additionally, the drinking systems used in broiler production are often susceptible to biofilm formation, which makes sanitation and regular cleaning essential [100].

Movement of the animals should be done on all-in-all-out basis, even on farms with different flocks and houses, as multi-age stocking increases the risk of the infection spreading from one flock to another. All-in-all-out movement thus minimizes the likelihood of cross-contamination between flocks [100, 110]. Transport of poultry from the farm to the slaughterhouse can result in their exposure to *Salmonella* through the use of contaminated dirty crates, trucks and catching or pickup crews, resulting in contamination and subsequent cross-contamination [100]. Transport can also contribute to the horizontal spreading of

*Salmonella* from farm to farm, and should be managed through washing of truck tires and transport crates [14].

During processing in the slaughterhouse, there are several critical points that can impact the contamination rate of the broiler carcasses by *Salmonella*. A first critical point is scalding [100]. During this process the carcasses pass through a tank filled with hot water, which opens the follicles of the skin and facilitates removal of the feathers. However, when the water in the tank is stagnant, temperature of the water is too low and there are excessive excreta in the tank, pathogens are able to proliferate and spread to all carcasses entering the tank [100]. Time spent by the carcass in the tank, temperature and pH of the tank and the use of antimicrobial chemicals (which is not allowed in Europe) thus need to be adjusted in a way that enteric pathogen prevalence is minimized [100]. Another critical point is head pulling and evisceration, as removal of the head and viscera can lead to carcass contamination with *Salmonella* via crop leakage and intestinal rupture [100, 111]. Hygienic measures, such as frequent washing of hands and the use of automated systems with high pressure sprays that minimize contact between soil or viscera and carcass can help reduce cross-contamination during this process [100]. Chilling, the process in which the carcass temperature is reduced, can be exploited to reduce carcass pathogen load, by cooling the carcasses in a chlorinated liquid. This is however prohibited in the European Union where, due to consumer demand for more chemical free products, air-chilling is used as the final chilling stage [100]. Finally, rapid freezing of the chicken carcasses (which is most frequently applied in the USA) may offer additional control for enteric pathogens [100].

### 1.6.2 Feed and drinking water: composition and supplementations

It is clear that, in order to avoid the introduction of *Salmonella* on the farm, poultry feed and drinking water should be *Salmonella*-free. However, the poultry feed can also play a more prominent role in *Salmonella* control, as several features of the feed can influence *Salmonella* colonization. It has been shown that the feed structure affects susceptibility of poultry to *Salmonella* infection [112] and that the cereal type used in broiler feed can affect *Salmonella* colonization, probably due to changes in intestinal health of the birds [113]. Feed particle size and feed form also have an effect on *Salmonella* colonization, with pelleted feed resulting in higher caecal *Salmonella* concentrations than a mashed diet [114]. In addition, it has been shown that fermented feed can also reduce caecal *Salmonella* colonization [115]. Similarly, protein content and protein source may help to protect the intestine from pathogen colonization [112]. Plant-protein based feeds often contain various non-starch polysaccharides that are fermented in the caecum to organic acids, which are disadvantageous to pathogens and thus lower their colonization [112]. Adding exogenous enzymes to the feed can improve the nutritional value of the feed and alter the microbial population of the GIT indirectly, reducing pathogen load [112]. For instance, by adding endoxylanase and alpha-amylase to a corn-based diet, it is possible to lower *Salmonella* prevalence and reduce caecal colonization [112]. As such, poultry feed can have an effect on *Salmonella* infections and play a role in control programs.

Adding certain supplements to feed and drinking water can also help lower *Salmonella* colonization and shedding. Obvious examples of such additives are antibiotics, but as these antimicrobials are no longer allowed as animal growth promoters and their usage should be

avoided due to microbial resistance, alternatives have been investigated and developed. One type of such alternatives are organic acids, whose addition to feed or drinking water can decrease *Salmonella* colonization and possibly other pathogenic bacteria [116]. These compounds comprise short chain fatty acids (SCFA) (such as acetic acid, propionic acid, butyrate), medium chain fatty acids (MCFA) (contain 6 to 12 carbons, such as caproic, caprylic, capric and lauric acids) and other organic acids, with SCFA's and MCFA's being mostly used as additives in poultry feed. Originally, organic acids were added to the feed in order to decontaminate it, but later it was shown that this could also help reduce *Salmonella* colonization and invasion of chicken tissues [116]. In high concentrations (> 1%), such as in the drinking water or in the crop after ingestion, these acids exert bacteriostatic or bactericidal effects against Gram-negative bacteria by entering the cell in undissociated form [112, 116]. Once inside the bacterial cell, the acid dissociates and as a result the intracellular pH decreases, disrupting cellular processes and delaying growth [112, 116]. In the case of *Salmonella*, low concentrations of organic acids can also negatively influence epithelial invasion by interacting with genes that play a role in invasion. It has been shown that butyrate and propionate decrease *hilA*, *invF* and *sipC* expression, while butyrate also downregulates *hilD* and as a consequence, SPI-1 expression [84, 116, 117]. SCFA's can thus regulate the invasive phenotype of *Salmonella*. MCFA's have also been shown to decrease *Salmonella* invasion in intestinal epithelial cells [118]. Organic acids have however more pronounced antimicrobial effects in the upper part of the GIT because they are absorbed before they can reach the lower GIT [98]. As *Salmonella* mainly colonizes the lower GIT [119], coating or micro-encapsulating organic acids might prevent early absorption and allow organic acids to be released further down the GIT, where *Salmonella* resides [98, 116]. Overall, SCFA's and MCFA's can thus be used to reduce *Salmonella* colonization and invasion

in broilers by adding them to the feed or drinking water, which can help to reduce infection pressure on the farm [112].

Other important feed and drinking water additives are probiotics and prebiotics. By administering probiotics to poultry, it is possible to induce changes in the chicken intestinal community structure [120], and this can be done by adding probiotics to the feed or drinking water. Administration of probiotics can also stimulate the immune system, elicit crosstalk with other beneficial bacteria and induce the production of host enzymes, which in turn may lead to a beneficial nutritional and growth-promoting effect [112, 121, 122]. Other potential mechanisms allowing probiotic cultures to exclude enteric pathogens such as *Salmonella* include competition for receptor sites, competition for nutrients and the production of antimicrobial metabolites (such as bacteriocins, fatty acids and hydrogen peroxide) [98, 112, 121]. Microorganisms used as probiotics in animal feed are mainly strains belonging to the genera *Lactobacillus*, *Enterococcus*, *Pediococcus* and *Bacillus*, while yeasts such as *Saccharomyces* also have been used [112, 123]. It has been shown that certain probiotic strains are able to reduce *Salmonella* colonization [122, 124-126], although the observed effects are often of minor magnitude [98].

Prebiotics are non-digestible feed ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or more bacterial species already present in the GIT of the host [98, 127]. Prebiotics are oligo- and polysaccharides such as lactose, lactulose, fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS) and arabinoxylooligosaccharides (AXOS), which confer beneficial effects on poultry health by indirect means, as the fermentable prebiotics provide a substrate for metabolism and

growth of the normal intestinal microbiota [98, 112, 128]. They thus inhibit pathogen colonization by competitive exclusion (see below) and may stimulate production of antibacterial metabolites such as lactic acid, fatty acids or bacteriocins [112]. It is also possible that prebiotics (such as mannanoligosaccharides) can bind to pathogens in the intestinal lumen, and therefore block adhesion of these pathogens to epithelial cells [112, 129]. However, studies on the ability of prebiotics to control colonization by enteropathogens such as *Salmonella* have produced inconsistent results [112, 130]. Lactose for instance, was shown to reduce *Salmonella* organ invasion when added to the feed [131], but when administered through the drinking water, it failed to reduce *Salmonella* colonization in crop and caecum [132]. Therefore, to achieve more unambiguous results, it might be more advisable to simply directly administer beneficial micro-organisms to poultry.

Newly hatched chicks have little opportunity to develop a normal intestinal flora due to the clean housing conditions in which they are reared [98, 112, 133]. This absence of a natural microbiota makes young chickens highly vulnerable for enteric pathogen infection, and has been linked with a pronounced susceptibility for *Salmonella* infections [98, 112, 134]. It is however possible to administer an alimentary tract suspension derived from adult birds to young ones, which confers them an adult-type microflora and decreases their susceptibility not only for *Salmonella*, but for enteric pathogen infection in general [135-137]. This is called competitive exclusion (CE), and several hypothetical mechanisms have been proposed to explain the efficacy of CE products. One is that the introduced microbiota physically obstruct attachment sites lining the intestine, preventing pathogen attachment [109]. Other possible mechanisms include modulation of the immune system, competition for essential nutrients (limiting pathogen growth) and the production of volatile fatty acids or bacteriocins [109].

The true mode of action of CE products however remains enigmatic. In practice, CE products can be added to the drinking water, but uptake of drinking water in the first 24h after hatching is variable and viability of anaerobic microbiota can be variable in water [98]. To overcome these problems, spray application in the hatchery can be applied [98, 109, 138]. The efficacy of these CE products has been clearly documented in both experimental studies and in the field [98, 137], and it has been shown that treatment with a CE product can reduce *Salmonella* prevalence in flocks. A drawback of these CE cultures is that they are undefined mixtures containing a wide array of micro-organisms and thus may contain pathogenic microorganisms [98]. However, this problem can be overcome by thoroughly testing the CE culture for the presence of pathogens. Attempts to use simpler defined CE mixtures, which would not comprise a pathogen risk, have however been less successful [109].

Another possible alternative to combat *Salmonella* is bacteriophage therapy. Bacteriophages are natural predators of their bacterial hosts and can cause lysis of bacterial cells as part of their life cycle. Indeed, it has been shown that certain bacteriophages are able to reduce carriage of *Salmonella* in live birds [139, 140]. At the moment, a bacteriophage-containing product (SALMONELEX®, Micreos) which can be used to reduce *Salmonella* contamination during food processing has been "generally recognized as safe" (GRAS) by American authorities and is commercially available, but the consequences of its use in the poultry industry remains to be investigated.

### 1.6.3 Vaccination

Vaccination can be an effective means to protect poultry against *Salmonella* and it is generally accepted that cell-mediated immunity plays a more important role in the protection against *Salmonella* than humoral responses after vaccination [133]. In accordance with this, it has been observed that CD8+ T-cells play an important role in the immunological defense after primary infection in young chicks [141] and that clearance of *Salmonella* Typhimurium infections in chickens correlates with high cell-mediated responses [142, 143]. In addition, intraperitoneal administration of recombinant IFN-gamma decreases *Salmonella* colonization, underlining the importance of cell-mediated immune mechanisms in the systematic clearance of *Salmonella* [144]. Also, experimental infection of chickens with a *Salmonella* Enteritidis field strain causes heterophilic granulocytes to accumulate in the propria mucosae of the caeca, which is accompanied by expression of CXC chemokines and polumorphonuclear cell (heterophil, PMN) influx [145]. It has been shown that heterophils up-regulate mRNA expression of pro-inflammatory chemokines IL-6 and Il-8 and the inflammatory cytokine TGF-b4 in response to *Salmonella* Enteritidis, while expression of Il-18 and IFN-γ is down-regulated [146]. Finally, heterophil-depleted chickens are much more susceptible to *Salmonella* Enteritidis [147, 148], further illustrating the importance of cell-mediated immunity in *Salmonella* infections in poultry. On the other hand, it has been observed that B-cell-depleted chickens have increased faecal excretion and higher caecal *Salmonella* counts, indicating that humoral immune responses might also play a role in intestinal *Salmonella* colonization [149].

By vaccinating chickens it is possible to stimulate the immune system and induce an immune response against *Salmonella* which decreases the amount of *Salmonella* colonizing the animal [150, 151]. It has been used successfully against host-specific *Salmonella* serotypes (e.g. *Salmonella* Gallinarum) that cause severe systemic disease in poultry [152] and to reduce *Salmonella* Enteritidis prevalence in layer flocks and consequently the number of *Salmonella* contaminated eggs [153-155]. However, vaccinating to protect broilers against *Salmonella* is different from layers, as the life span of broiler chickens is only 5-6 weeks while the production period for laying hens is much longer [150]. Because the immune system is not yet fully developed the first weeks post-hatch, immunization in the first weeks of the life of the broiler chicken is not very effective [156-158]. This is however the period that chickens are most vulnerable for infection, and infection with *Salmonella* during this period in which the chick is highly susceptible often results in persistent infections [159-161]. Despite this, vaccination is sometimes used in broilers. The *Salmonella* vaccines used in practice for poultry are divided in two categories: live-attenuated and inactivated vaccines [150].

Live attenuated vaccines are *Salmonella* strains containing mutations or deletions in genes that are essential for metabolism, virulence or survival in the host [133, 150]. They have several advantages over inactivated vaccines, as they stimulate both cell-mediated and humoral immune responses and usually express all appropriate antigens *in vivo* [133, 162]. Moreover, they can easily be administered orally [150, 151]. A major disadvantage is that they might persist in the chickens as well as in the environment, possibly resulting in the introduction of the vaccine in the food chain and posing a threat to human health [163, 164]. On the other hand, such a persistence can be desirable, as horizontal spread of the live

vaccine would result in the protection of birds that were not originally immunized [150]. Other disadvantages of live vaccines are possible interference with *Salmonella* testing procedures and the possibility of reversion to virulence [165, 166]. This reversion to virulence has already been observed in the field [167], yet can be avoided by using vaccine strains in which the genetic alteration is introduced by deleting the target genes instead of mutating these. Several challenges are associated with administering live vaccines and vaccines in general to poultry, like the high costs of vaccine administration, a too late induction of immunity and difficulties with delivering the vaccine in a uniform way to the poultry flock [150]. However, because live vaccines can easily be administered orally, different methods to administer these on a large scale have been developed. Application through the drinking water or by spray are most widely used for *Salmonella* vaccines, allowing for a relatively uniform, early and cheap administration.

Killed vaccines are whole bacteria that are inactivated by heat, formalin, acetone or other treatments [150]. A major advantage of these vaccines is the absence of a living organism that can spread and persist in the environment and can potentially cause a health risk [150]. Killed vaccines are also able to elicit strong antibody responses, yet do not stimulate a cell mediated response like live vaccines do [150] (while cell-mediated immunity is more important than humoral responses in protection against *Salmonella*). In addition, these killed vaccines can express only a limited number of antigens (those present at harvest), while live vaccines usually express all relevant antigens [151, 152]. Consequently, killed vaccines often need an adjuvant to improve the sub-optimal immune response they elicit [150]. Another drawback is that they may provide rather limited cross-protection against salmonellae of

antigenically unrelated serotypes [151]. Despite this, killed vaccines are more widely employed as they raise fewer customer concerns [100].

Other vaccines, such as subunit vaccines and vector vaccines are also being used in poultry practice, but not to combat *Salmonella*. However, it is believed that the next generation of poultry vaccines will primarily be subunit vaccines delivering antigens from possibly multiple pathogens in viral or DNA vectors [168].

### 1.6.4 Colonization-inhibition

Immunization with live vaccines represents an important method to increase resistance of chickens against *Salmonella* infection [169]. However, in addition to the development of an adaptive immune response, oral administration of a live attenuated *Salmonella* strain to day-old chicks can confer protection against *Salmonella* infection within hours after administration [169]. This phenomenon is called colonization-inhibition (CI) and can be exploited to protect young chickens against *Salmonella* infection. Chickens are most vulnerable for infection shortly after hatching, and infection with *Salmonella* during this period in which the chick is highly susceptible often results in persistent infection [159-161]. As other treatments such as feed additives, drinking water supplementation or classical vaccination do not offer protection that early in the life of the broiler chick, CI may represent a powerful tool to protect broilers against *Salmonella* infection early after hatching.

The potential of CI was discovered in the 1980's, during a study to identify bacteria possessing colonization-inhibition activity against *Salmonella* [170]. In this study, one group

of chicks was found to be completely protected against infection by *Salmonella* Typhimurium after they were infected with a *Salmonella* Montevideo strain picked up from contaminated feed shortly after hatching. When isolating the *Salmonella* Montevideo strain from the feed and administering it to newly hatched chickens, they were again able to establish a profound protection when the animals were challenged with a *Salmonella* Typhimurium strain 24 h later. Further studies confirmed these early observations and showed that live bacteria were necessary for CI to take place, as killed preparations were not able to confer protection to the chicks [171]. In addition, it was shown that only organisms from within the *Salmonella* genus were able to inhibit *Salmonella* colonization, while other Enterobacteriaceae were not able to do so [171, 172]. Further research showed that the observed CI effect was induced quickly (6 h) after administration of the first strain and could still be observed when the chickens were challenged 7 days after administration of the first strain [173]. This CI effect could also be observed in ducks and gnotobiotic pigs, suggesting that CI is not restricted to chickens [174, 175]. In addition, the genus-specific CI effect becomes less pronounced as the normal intestinal microbiota develops further [176]. Other research concerning CI showed that not all *Salmonella* strains are equally inhibitory and that no strain is fully protective against all *Salmonella* strains [169]. The colonization inhibition effect also appears to be serovar-specific [158, 177], meaning that a better protection is established when inhibitory strain and challenge strain belong to the same serotype [173, 176]. The most profound inhibition is however observed between isogenic strains [169, 176]. These observations led to the belief that oral administration of live *Salmonella* vaccines could thus allow for an early protection of young chickens by CI, followed by the development of a long-lasting immunity when the birds reach immunological maturity [133, 178].

*Salmonella* strains that are to be used as CI strains need to be attenuated in a way that they are no longer virulent, and should thus no longer be shed or carried by the chicken at slaughter [133]. Additionally, it is preferable that a CI strain is altered in a way that it is distinguishable from wild-type strains, so that it does not interfere with detection of wild-type strains in testing procedures and control programs [165]. A CI strain should confer protection quickly after administration and this protection should last until slaughter age [133]. Preferably this CI strain would also confer protection against a broad range of *Salmonella* serotypes, but since protection is stronger when CI strain and challenge strain are more closely related, this might be a difficult criterion to achieve. Finally, like live vaccines, CI strains can possibly revert to virulence [166, 167]. This risk can however be avoided by using CI strains in which the genetic alteration is introduced by deleting the target gene instead of mutating it. Such strains are however genetically modified organisms (GMO's) and thus have to comply to the high standard of safety defined for this class of organisms. A lot of effort has already been put in developing CI strains. Most of these developed strains had mutations in genes involved in energy metabolism, anabolic pathways or virulence. Examples are *aroA*, *phoP*, *ompC*, *lon*, *cpxR* or the genes coding for adenylate cyclase, a cAMP receptor and DNA adenine methylase [179-188]. In addition to these strains, several commercially available live vaccine strains have been developed which could possibly induce a CI effect as well [150].

Earlier research has shown that a *Salmonella* Enteritidis Δ*hilA* strain is a strain that is significantly reduced in its ability to colonize orally infected one day old chicks [81]. More importantly, when this strain was used as a CI strain and administered to day old chickens, it conferred a pronounced protection against *Salmonella* Enteritidis infection by lowering

faecal shedding and caecal and internal organ colonization [189, 190]. The *Salmonella* Enteritidis Δ*hilA* strain was however still present at slaughter age in low amounts, and could thus not be used as a CI strain in practice. Therefore, it was proposed to introduce additional mutations in this strain to further attenuate it and obtain a putative *Salmonella* Enteritidis CI strain that is cleared by slaughter age. Additional research had already revealed that a *Salmonella* Enteritidis Δ*ssrA* strain maintains its invasiveness, yet is impaired in intracellular survival and thus colonizes internal organs to a much more limited extent [86]. When administering this strain as a CI strain to one day old chicks, it offered protection against experimental *Salmonella* Enteritidis challenge, but was also still present at slaughter age. Introducing both the *hilA* and *ssrA* mutations might however result in a sufficiently attenuated strain that is cleared by slaughter age and maintains the protective effect conferred by the single mutants.

A major drawback for vaccination with live vaccines is that vaccinated animals can no longer be distinguished from field-exposed animals by serological tests, as the immunized animals produce antibodies against both the vaccine strain and the wild-type strain [165]. Earlier research has however shown that non-flagellated live vaccines (due to deletion of a gene crucial for flagellin structure or assembly) are not able to induce an anti-flagellin immune response after administration, which allows easy serological differentiation between vaccinated and infected animals that do produce a detectable anti-flagellin immune response [165]. Introducing an additional mutation in a flagellin gene would thus also allow for serological differentiation between animals treated with the CI strain and animals that are actually infected with *Salmonella*. In addition, such a mutation would render the strain immotile, which allows for easy bacteriological differentiation between wild-type and

attenuated strains and ensures that the CI strain does not interfere with *Salmonella* monitoring programs (which often comprise a motility test).

It is currently thus unknown whether deletion of the *hilA*, *ssrA* and *fliG* gene in a *Salmonella* Enteritidis and *Salmonella* Typhimurium strain will result in CI strains that are safe for use in broilers and are able to protect broilers against *Salmonella* Enteritidis and Typhimurium colonization from the moment of hatch until slaughter. Additionally, it is not known how CI strains should be administered to broilers in practice.

# Chapter 2: Aims

# 2 Aims

Despite the implementation of different control measures, up to 3.4 % of Belgian broiler flocks and 4.9 % of the broiler meat available in retail were contaminated with *Salmonella* in 2012. Therefore, consumption of contaminated poultry meat remains an important cause of food-borne *Salmonella* infections in humans. Young chickens often become infected with *Salmonella* early in life, as they are highly susceptible to *Salmonella* infections due to the absence of a natural microflora and an underdeveloped immune system. Therefore, there is a need for control methods that protect broilers from day-of-hatch until slaughter age against infection with *Salmonella*. Colonization-inhibition, a concept in which a live *Salmonella* strain is orally administered to day-old chickens and protects against subsequent challenge, can potentially be used as a novel control method to achieve this goal.

The aim of this thesis was the development of a CI strain that is safe for use in broilers and is able to protect them early after hatching against *Salmonella* infection. This means that this CI strain should no longer be virulent, cannot revert to virulence, should be cleared by slaughter age and does not interfere with testing procedures. In addition, this strain should be able to offer protection against *Samonella* early in the broiler life and this protection should last until slaughter age. Finally, this CI strain or a combination of CI strains belonging to different serovars should offer protection against a broad range of *Salmonella* serovars.

In a first study, we investigate whether a *Salmonella* Enteritidis *hilAssrAfliG* deletion strain can be used as a CI strain to protect broilers against *Salmonella* Enteritidis. This was done by

investigating whether this CI strain is safe and whether this strain offers protection against experimental *Salmonella* Enteritidis challenge.

In a second study, we investigate whether a *Salmonella* Typhimurium deletion *hilAssrAfliG* mutant strain is safe for use in broilers and whether this strain is able to protect them against *Salmonella* Typhimurium infection early after hatching. In addition, we studied whether the *Salmonella* Enteritidis *hilAssrAfliG* deletion mutant strain and the *Salmonella* Typhimurium *hilAssrAfliG* deletion strain can be applied simultaneously to protect broilers early after hatching against both *Salmonella* Enteritidis and *Salmonella* Typhimurium infection.

In a third study, a practical application method which allows for early and uniform application of the *Salmonella* Enteritidis *hilAssrAfliG* strain was investigated. To do so, two application methods frequently used in practice, drinking water and spray application, were compared.

# Chapter 3: Experimental Studies

## 3.1 A *Salmonella* Enteritidis *hilAssrAfliG* deletion mutant is a safe live vaccine strain that confers protection against colonisation by *Salmonella* Enteritidis in broilers

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## Abstract

Consumption of contaminated poultry meat is an important cause of *Salmonella* infections in humans. Therefore, there is a need for control methods that protect broilers from day-of-hatch until slaughter age against infection with *Salmonella*. Colonization-inhibition, a concept in which a live *Salmonella* strain is orally administered to day-old chickens and protects against subsequent challenge, can potentially be used as control method. In this study, the safety and efficacy of a *Salmonella* Enteritidis *ΔhilAssrAfliG* strain as a colonization-inhibition strain for protection of broilers against *Salmonella* Enteritidis was evaluated. After administration of the *Salmonella* Enteritidis *ΔhilAssrAfliG* strain to day-old chickens, this strain could not be isolated from the gut, internal organs or faeces after 21 days of age. In addition, administration of this strain to one-day-old broiler chickens decreased faecal shedding and caecal and internal organ colonization of a *Salmonella* Enteritidis challenge strain administered one day later using a seeder bird model. To our knowledge, this is the first report of an attenuated *Salmonella* strain for which both the safety and efficacy has been shown in long-term experiments (until slaughter age) in broilers. Consequently, the *Salmonella* Enteritidis *ΔhilAssrAfliG* strain can potentially be used as a live colonization-inhibition strain for controlling *Salmonella* Enteritidis infections in broilers.

## Introduction

Despite the implementation of numerous monitoring and control measures in broiler production, *Salmonella* is still an important cause of poultry meat associated human infections [5]. Broilers can be infected with *Salmonella* at any time during the production period or even post-harvest. In the first days after hatching however, chicks are highly susceptible to *Salmonella* infections arising from environmental contamination [159]. Contact in this period, even with very low numbers of *Salmonella*, may result in persistent infections [160, 161]. Following early infection, broilers may thus still be colonised by the time they reach slaughter age, resulting in introduction of *Salmonella* in the slaughterhouse and contamination of broiler meat [14].

Because young chickens are immunologically immature, classical vaccination as a means to protect the animals is not an option [133]. Due to the short production cycle, there is little time to develop a protective immune response. Oral administration of a live *Salmonella* culture at day-of-hatch, however, can induce rapid protection of newly hatched chickens against a subsequent challenge. This phenomenon is called colonisation-inhibition (CI) [171, 173, 189-191]. Live attenuated *Salmonella* strains may be used as CI strains provided that they do not colonise the animal persistently and have been cleared at slaughter age. The construction of attenuated *Salmonella* strains by introducing mutations in genes that are essential for virulence, while maintaining properties essential for colonisation-inhibition and immunogenicity has been proposed as a possible approach for producing CI-strains [133]. As demonstrated earlier, *Salmonella* Enteritidis mutants with a deleted *hilA* gene, the regulator of the *Salmonella* Pathogenicity Island 1 (SPI-1), inhibit caecal colonisation of challenge strains [189, 190]. However, the *hilA* deletion mutant is insufficiently attenuated [189] and

thus additional deletions in virulence genes needed to be added. A candidate gene is *ssrA*, the key regulator of the SPI-2 virulence genes [86]. Mutations that ensure easy serological differentiation between animals that are colonised by either wild-type strains or attenuated CI strains are also of interest. Therefore, a mutation in the flagellar rotor protein gene *fliG* is a candidate as this ensures loss of flagellar assembly, and thus the inability to induce anti-flagellin antibodies [192].

In the current study, a *Salmonella* Enteritidis Δ*hilAssrAfliG* strain was evaluated as a potential safe and protective colonisation-inhibiting strain. To evaluate safety, faecal shedding, caecal and internal organ colonisation of the Δ*hilAssrAfliG* strain was analysed following oral inoculation of day-old chicks. Additionally, we evaluated the efficacy of the Δ*hilAssrAfliG* strain in decreasing colonisation after challenge with a virulent strain, using a seeder bird infection model.

## Material & methods

### Chickens

One-day-old Ross broiler chickens were obtained from a local hatchery and housed in isolation. Experimental groups were housed in separate rooms in containers on wood shavings. Commercial feed and drinking water were provided *ad libitum*. Experiments were performed with the permission of the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University, Belgium.

## Bacterial strains and *Salmonella* Enteritidis deletion mutants

*Salmonella* Enteritidis 76Sa88 $Nal^R$ is a well-characterized strain originally isolated on a poultry farm [159, 193] and was used for the production of isogenic mutants. Deletion of *hilA*, *ssrA* and *fliG* genes was done using the one-step inactivation method described by Datsenko and Wanner [81, 194]. By transducing the mutant *srrA* allele into a *Salmonella* Enteritidis 76Sa88 $Nal^R$ *ΔhilA* background the *ΔhilAssrA* double mutant was constructed. Similarly, the *ΔhilAssrAfliG* triple mutant was constructed by transducing the mutant *fliG* allele into a S*almonella* Enteritidis 76Sa88 $Nal^R$ *ΔhilAssrA* background.

*Salmonella* Enteritidis 147 $Strep^R$, originally isolated from egg white, was used as challenge strain. This strain is known to colonise the gut and internal organs of chickens to a high level [189, 195, 196].

## Experimental design

**Analysis of the colonisation pattern of *Salmonella* Enteritidis *ΔhilAssrAfliG*: evaluation of safety.** Three hundred and sixty one-day-old chicks were divided into 6 groups of 60 and each housed in a container of 1 $m^2$. Each group was given $10^8$ CFU of the parent strain or one of the mutant strains (Δ*hilA*, Δ*ssrA*, Δ*fliG*, Δ*hilAssrA* or Δ*hilAssrAfliG*) by oral gavage in the crop on the first day of life (day 1). To evaluate colonisation by the wild-type strain and the mutant strains, their numbers in caecum and spleen were determined for 10 animals at days 2, 14, 21, 28, 35 and 42. Shedding of *Salmonella* was evaluated during the experiment by bacteriological analysis of cloacal swabs taken on days 1, 2, 7, 14, 21, 28, 35 and 42.

**Evaluation of the colonisation inhibiting potential of *Salmonella* Enteritidis *ΔhilAssrAfliG***. For this experiment, 2 groups of 75 one-day-old chicks were each housed in a container of 2

m². One group was given $10^8$ CFU of the *ΔhilAssrAfliG* triple mutant strain (CI group) by oral gavage in the crop while the other group was given sterile PBS (control group) on day 1. Twenty-four hours later, 15 randomly selected chicks in each group were given $10^5$ CFU *Salmonella* Enteritidis 147 Strep$^R$ (seeder birds) and housed together with the non-infected chicks. To evaluate the colonisation inhibiting potential of the triple mutant strain, bacterial counts of both the mutant strain and the challenge strain in caecum and spleen were determined on days 7, 21 and 42 for 25 chickens (of which 5 were seeder birds at each time point). Shedding of the challenge strain and the triple mutant was followed by cloacal swabbing on days 2, 3, 9, 16, 23, 30 and 37.

## Bacteriological analysis

Cloacal swabs were directly inoculated on Brilliant Green Agar (BGA, Oxoid, Basingstoke, England) (experiment 1) or Xylose Lysine Deoxycholate agar (XLD, Oxoid, Basingstoke, England) (experiment 2) plates with 20 µg/ml nalidixic acid (for the detection of *Salmonella* Enteritidis 76Sa88 Nal$^R$ strain and the isogenic mutants derived from this strain) or 100 µg/ml streptomycin (for the detection of *Salmonella* Enteritidis 147 Strep$^R$). Samples negative after direct inoculation were pre-enriched in buffered peptone water (BPW, Oxoid, Basingstoke, England) and incubated overnight at 37 °C. One ml of this BPW suspension was further enriched by adding 9 ml tetrathionate-brilliant green broth. After overnight incubation at 37 °C, a loopful of this suspension was plated on BGA or XLD plates supplemented with the appropriate antibiotic.

Samples of caecum and spleen were homogenized in BPW and 10-fold dilutions were made in Hank's Balanced Salt Solution (HBSS, Invitrogen, Paisley, England). Six droplets of 20 µl of each dilution were plated on BGA or XLD supplemented with 20 µg/ml nalidixic acid

(experiments 1 and 2) and on plates with 100 µg/ml streptomycin (experiment 2). After overnight incubation at 37 °C, the number of CFU/g tissue was determined by counting the number of bacterial colonies for the appropriate dilution, dividing this number by 6, and multiplying this again by 50 times 10 and the dilution factor, as shown in the following formula: $\text{CFU/g tissue} = \frac{\text{CFU}}{6} * 50 * 10 * \text{dilution factor}$

Negative samples were enriched as described above.

## Statistical analysis

GraphPad Prism 5 software was used for statistical analysis. A Fisher's exact test (one-sided) was used to analyse mortality rates within differently treated groups. A Kruskal-Wallis test (one-way ANOVA, with Dunn's multiple comparison test) was used to determine statistical differences in the number of *Salmonella* positive cloaca swabs and the number of *Salmonella* positive (after enrichment) spleen and caecum samples, between groups. For statistical analysis of numbers of CFU *Salmonella* per g spleen and caeca, bacterial counts were converted into logarithmic form. Samples of caecum and spleen negative after direct plating were rated as $\log_{10}= 0$. A two way ANOVA (with Bonferroni correction) was used to determine statistical differences in organ colonisation between the group inoculated with the *Salmonella* Enteritidis 76Sa88 $\text{Nal}^{R}$ strain and those inoculated with a CI strain (experiment 1). For the second experiment, the mean CFU/g tissue was calculated for each group at every time point and differences between groups were analysed using a Mann-Whitney test. Differences with p-values lower than 0.05 were considered to be significant.

## Results

**Colonisation pattern of *Salmonella* Enteritidis *ΔhilAssrAfliG*: evaluation of safety.** Mortality in the group inoculated with the wild-type strain (6 out of 60) was significantly higher ($p = 0.0137$) as compared to the groups inoculated with any of the deletion mutants (1/60 for groups inoculated with *ΔhilA*, *ΔsrrA* and *ΔhilAssrA* strains and zero in groups inoculated with *ΔfliG* and *ΔhilAssrAfliG* strains).

As shown in Table 1, nearly all cloacal swabs taken 1 day after inoculation were positive, except those taken from chickens inoculated with the *ΔhilA*, *ΔhilAssrA* (about 90% positive) and *ΔhilAssrAfliG* strain (75% positive). In general, shedding of the wild-type strain and all mutant strains gradually declined during the experiment, but the decline was faster for the mutant strains. Starting from day 28, none of the animals was shedding the mutant strains, except for the *ΔfliG* mutant. Statistical differences are shown in Table 1.

**Table 1: The number of cloacal swabs positive for *Salmonella* Enteritidis 76Sa88 wild-type strain or its *hilA*, *ssrA*, *fliG*, *hilAssrA* and *hilAssrAfliG* deletion mutants at direct plating and after enrichment, after inoculation with one of these strains at day of hatch.**

| Strain | Days | | | | | | |
|---|---|---|---|---|---|---|---|
| | 2 | 7 | 14 | 21 | 28 | 35 | 42 |
| Wild type | 58/60[a] (59[b]) | 48/49 (48) | 40/46 (45) | 29/36 (33) | 16/28 (25) | 2/17 (5) | 0/8 (0) |
| *ΔhilA* | 53/60 (54) | 14*/49 (35) | 12*/49 (29) | 0*/39 (5*) | 0*/29 (0*) | 0/19 (0) | 0/9 (0) |
| *ΔssrA* | 59/60 (59) | 40/50 (50) | 14*/50 (37) | 2*/39 (11*) | 0*/29 (0*) | 0/19 (0) | 0/9 (0) |
| *ΔfliG* | 59/60 (59) | 48/50 (49) | 28/50 (48) | 12*/40 (22) | 2/30 (4*) | 3/20 (9) | 0/10 (0) |
| *ΔhilAssrA* | 53/60 (53) | 37/50 (50) | 19*/49 (41) | 5*/39 (12*) | 0*/29 (0*) | 0/19 (0) | 0/9 (0) |
| *ΔhilAssrAfliG* | 45/60 (45) | 37/50 (48) | 3*/50 (5*) | 0*/40 (0*) | 0*/30 (0*) | 0/20 (0) | 0/10 (0) |

[a] Number of positive samples after direct plating/total number of samples

[b] Number of positive samples after enrichment

* Significant difference in positive samples in comparison to the 76Sa88 wild type strain (p-value < 0,05)

Bacteriological analysis showed that inoculation with the wild-type strain or the mutant strains resulted in a high level of caecal colonisation on the first day after inoculation for all strains (ca. $10^8$ CFU/g caecum) (Fig. 1A). Generally, the wild-type strain colonised the caeca to higher levels at many time points as compared to the mutants. Bacteriological analysis of the spleen showed that the mutant strains colonised the spleen less extensively as compared to the wild-type strain, already from the first day post-administration (Fig. 1B). Generally, the wild-type strain was present in the highest numbers as compared to the mutant strains ($10^2$-$10^3$ CFU/g until day 21), with still half the samples positive after enrichment at day 42. Details and statistical differences are mentioned in the legend of Figure 1. The number of caeca and spleen samples positive after enrichment for the *Salmonella* Enteritidis wild-type strain or its deletion mutants are shown in Table 2.

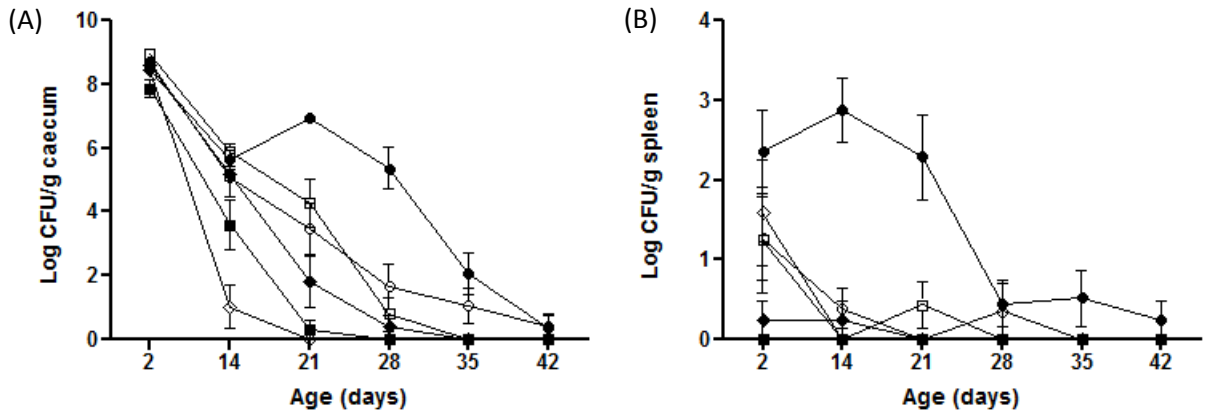


**Figure 1: Average caecal (A) and spleen (B) colonization of the *Salmonella* Enteritidis 76Sa88 Nal$^R$ wild-type strain (—●—), its *hilA* deletion mutant (—■—), *srrA* deletion mutant (—◆—), *fliG* deletion mutant (—○—), *hilAssrA* deletion mutant (—□—) or *hilAssrAfliG* deletion mutant (—◇—) after inoculation of chickens on day of hatch with $10^8$ CFU of one of these strains.** Represented values are $\log_{10}$ CFU/g sample. Samples were taken on day 2, 14, 21, 28, 35 and 42. The error bars represent the standard error of the means (SEM). Statistical significant differences in caecal colonization between the *Salmonella* Enteritidis 76Sa88 Nal$^R$ strain and the CI strains were observed on day 14,21 28 and 35 for the *ΔhilA* strain, day 21, 28 and 35 for the *ΔssrA* strain, day 21 and 28 for the *ΔfliG* strain, day 21, 28 and 35 for the *ΔhilAssrA* strain and day 14, 21, 28 and 35 for the *ΔhilAssrAfliG* strain. Statistical significant differences in spleen colonization between the *Salmonella* Enteritidis 76Sa88 Nal$^R$ strain and the CI strains were observed on day 2, 14 and 21 for the *ΔhilA* strain, day 2, 14 and 21 for the *ΔssrA* strain, day 2, 14 and 21 for the *ΔfliG* strain, 2, 14 and 21 for the *ΔhilAssrA* strain and day 14 and 21 for the *ΔhilAssrAfliG* strain.

**Table 2. The number of caeca or spleen samples positive after enrichment for *Salmonella* Enteritidis 76Sa88 wild-type strain or its *hilA*, *ssrA*, *fliG*, *hilAssrA* and *hilAssrAfliG* deletion mutants after inoculation with one of these strains at day of hatch.**

| Groups | | Days | | | | | |
|---|---|---|---|---|---|---|---|
| | | 2 | 14 | 21 | 28 | 35 | 42 |
| Caecum | Wild type | 10[a]/10[b] | 9/9 | 8/8 | 10/10 | 9/9 | 1/8 |
| | *ΔhilA* | 10/10 | 8/10 | 3/10 | 0*/10 | 1/10 | 0/9 |
| | *ΔssrA* | 10/10 | 10/10 | 10/10 | 4/10 | 0*/10 | 0/9 |
| | *ΔfliG* | 10/10 | 10/10 | 7/10 | 4/10 | 9/10 | 1/10 |
| | *ΔhilAssrA* | 10/10 | 10/10 | 10/10 | 3/10 | 0*/10 | 0/9 |
| | *ΔhilAssrAfliG* | 10/10 | 2/10 | 0*/10 | 0*/10 | 0*/10 | 0/10 |
| Spleen | Wild type | 9/10 | 9/9 | 8/8 | 9/10 | 5/9 | 4/8 |
| | *ΔhilA* | 7/10 | 3/10 | 0*/10 | 0*/10 | 0/10 | 0/9 |
| | *ΔssrA* | 8/10 | 6/10 | 1*/10 | 0*/10 | 1/10 | 0/9 |
| | *ΔfliG* | 10/10 | 9/10 | 1*/10 | 1*/10 | 0/10 | 0/10 |
| | *ΔhilAssrA* | 10/10 | 4/10 | 3/10 | 2/10 | 2/10 | 0/9 |
| | *ΔhilAssrAfliG* | 10/10 | 1*/10 | 0*/10 | 0*/10 | 0/10 | 0/10 |

[a] Number of positive samples after enrichment

[b] Total number of samples

* Significant difference in positive samples in comparison to the 76Sa88 wild type strain (p-value < 0,05)

**Evaluation of the colonisation inhibiting potential of *Salmonella* Enteritidis *ΔhilAssrAfliG*.**

Because the *ΔhilAssrAfliG* triple mutant strain was cleared after 21 days and was thus regarded as safe, this strain was tested for its colonisation inhibition potential. During the experiment, 6 chickens out of 75 died in the group inoculated with the *Salmonella* Enteritidis 147 challenge strain only (control group), while in the group inoculated with both the *Salmonella* Enteritidis *ΔhilAssrAfliG* colonisation-inhibition strain and the challenge strain (CI group) only 2 chickens out of 75 died. Birds in the control group had severe diarrhoea with pasting of the vent, reduced appetite and showed lethargy with drooping of head and wings, in contrast to the birds in the CI group.

As shown in Table 3, shedding of the *ΔhilAssrAfliG* strain in the CI group declined rapidly after inoculation. Shedding of the challenge strain was higher in the control group as compared to the CI group throughout the entire experiment. The number of swabs positive for the challenge strain after direct plating remained low in the CI group throughout the entire experiment. Furthermore, the number of swabs positive for the challenge strain after enrichment was lower for the CI group during the whole experiment. Statistical differences are shown in Table 3.

**Table 3. The number of cloacal swabs positive for the *Salmonella* Enteritidis 76Sa88 *hilAssrAfliG* deletion mutant and the *Salmonella* Enteritidis challenge strain SE147.**

| Strains | | Days | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 2 | 3 | 9 | 16 | 23 | 30 | 37 |
| Δ*hilAssrAfliG* mutant | Control | 0/75[a] (0[b]) | 0/75 (0) | 0/49 (0) | 0/46 (0) | 0/22 (0) | 0/22 (0) | 0/22 (0) |
| | CI group | 12/75 (70) | 6/75 (25) | 0/50 (0) | 0/48 (0) | 0/24 (0) | 0/24 (0) | 0/24 (0) |
| SE147 | Control | N/A | 11/75 (21) | 18*/47 (39) | 18*/46 (39) | 3/22 (11) | 4/22 (8) | 2/22 (7) |
| | CI group | N/A | 4/75 (13) | 5*/50 (33) | 2*/48 (25) | 1/24 (4) | 0/24 (1) | 1/24 (2) |

The number of cloacal swabs positive for the *Salmonella* Enteritidis 76Sa88 *hilAssrAfliG* deletion mutant after inoculation with PBS (Control group) or $10^8$ CFU of the *Salmonella* Enteritidis *ΔhilAssrAfliG* strain (CI group) at day of hatch is shown in the first 2 rows. The third and fourth row show the number of cloacal swabs positive for the *Salmonella* Enteritidis challenge strain SE147 after inoculation with $10^5$ CFU (both groups) on day 2 of the experiment.

[a] Number of positive samples after direct plating/total number of samples

[b] Number of positive samples after enrichment

* Significant difference in positive samples between control and CI group (p-value < 0,05)

Bacteriological analysis shows that by day 7, the *ΔhilAssrAfliG* strain was no longer present in the caecum of any of the chickens, not even after enrichment (data not shown). In the group treated with the CI strain, bacterial loads of the challenge strain in the caecum were significantly lower than in the control group throughout the entire study ($p<0.0001$ on day 7, $p=0.0003$ on day 21 and $p=0.0069$ on day 42) (Figure 2). Analysis of the spleen samples

showed that the *ΔhilAssrAfliG* strain was no longer present on day 7, not even after enrichment (data not shown). The difference in spleen colonisation by the challenge strain between both groups was significant (p=0.0035) on day 7, but not on day 21 (Figure 2). The challenge strain could only be detected after enrichment on day 42 (data not shown).

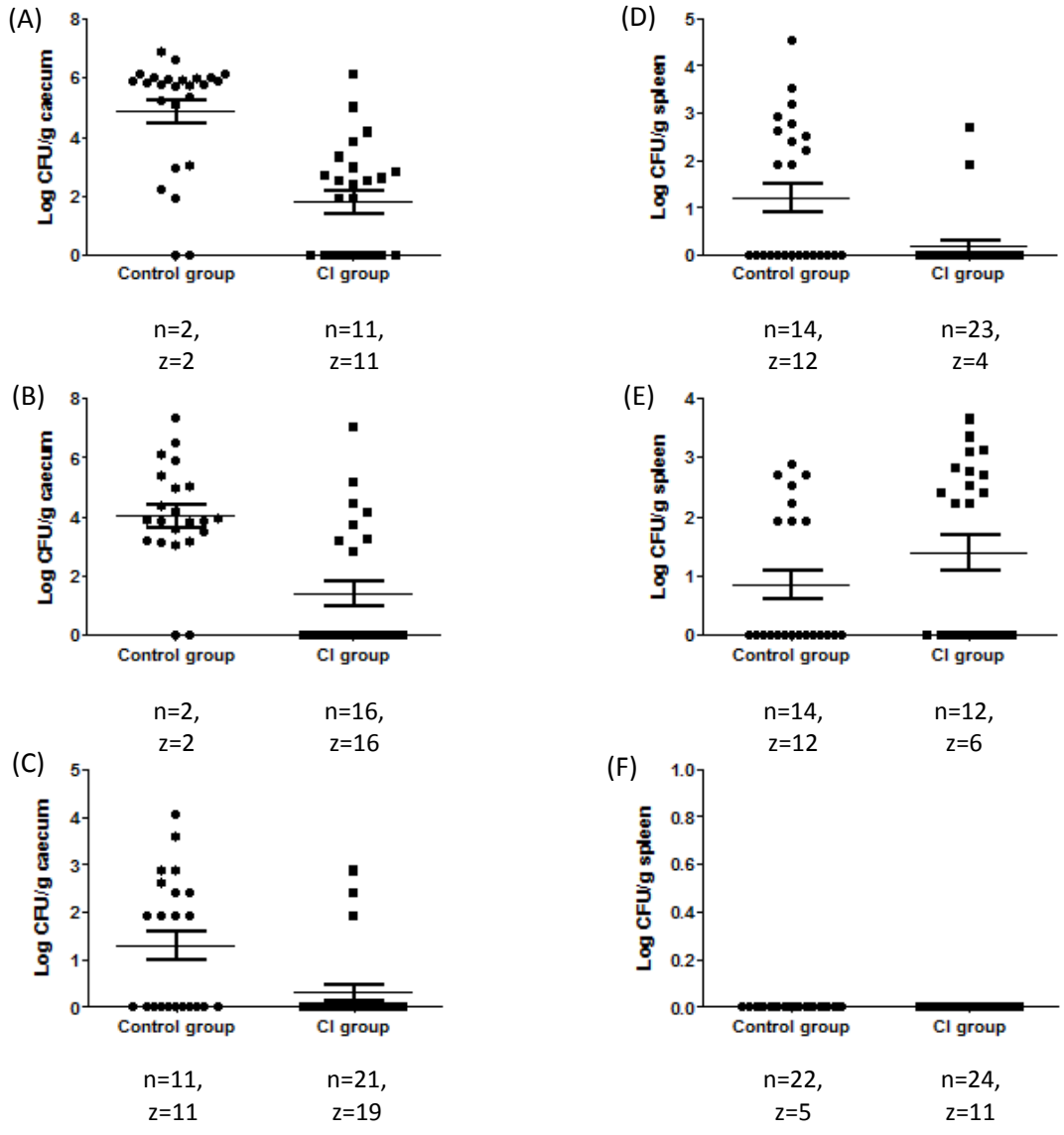


**Figure 2. Caecal (A, B, C) and spleen (D, E, F) colonization of the *Salmonella* Enteritidis challenge strain SE147.** Animals were orally challenged at day 2 with $10^5$ CFU, either (CI group) or not (control group) after administration of $10^8$ CFU of a *Salmonella* Enteritidis *ΔhilAssrAfliG* deletion mutant strain. Subfigures A and D represent colonization on day 7, B and E on day 21 and C and F on day 42. Represented values are $\log_{10}$ of CFU/g sample. The middle horizontal line represents the mean, the error bars represent the standard error of mean (SEM). The number of chickens equals 25, 22 and 22 in the control group and 25, 24 and 24 in the CI group on day 7, 21 and 42 respectively. The number of samples negative after direct plating (n) and the number of samples negative after direct plating but positive after enrichment (z) are displayed below the group name.

## Discussion

Newly hatched chicks are highly susceptible to *Salmonella* infections during the first days of life and inoculation with very low doses can result in persistent infections [159-161]. This can be explained by the absence of a normal gut microbiota [197, 198] and immaturity of the immune system of young chickens [156, 157, 199]. Because the immune system of young chickens is still immature, classical vaccination is not an effective means to achieve protection against *Salmonella* infection during the first days of life [133]. However, administration of a live *Salmonella* vaccine can induce a rapid colonisation-inhibiting effect that protects the bird against subsequent challenge with another *Salmonella* strain.

Colonisation-inhibition is induced by orally administering live *Salmonella* organisms that subsequently protect against challenge with another *Salmonella* strain belonging to the same serotype [158, 173, 200]. By administering the live *Salmonella* strain at day-of-hatch, it is possible to achieve protection against *Salmonella* very early in life [133]. A lot of effort has been put in developing strains that are appropriate for use as CI strains [170, 171]. In addition to being protective against *Salmonella* infection, it is essential that the CI strains do not induce clinical signs, have no adverse effects on performance and are cleared from the host at slaughter age to avoid transmission to the food chain. In practice, colonisation of the host and shedding should be zero at even earlier ages, i.e. the age at which sampling is performed in national or international monitoring schemes. The *ΔhilAssrAfliG* mutant strain used in the current study did not induce clinical symptoms and was cleared at 21 days of age in the first experiment and at 7 days of age in the second experiment, showing a very good safety profile. This observed difference in clearance could be due to the difference in

experimental design (stocking density) between both experiments, or due to a different intestinal microflora acquired by the chicks prior to inoculation. In any way, this rapid clearance is in contrast with several other tested colonisation-inhibiting strains, including *Salmonella* Enteritidis *hilA*, *ssrA* and *sipA* deletion mutant strains, as these are not cleared by 3 weeks of age or even slaughter age, which would interfere with implemented *Salmonella* monitoring measures or even result in introduction of *Salmonella* in the slaughterhouse [189, 190, 201]. A *Salmonella* Typhimurium *ΔrpoS* deletion mutant displayed an unaffected potential to inhibit caecal colonisation of a challenge strain but was still fully virulent to chickens and thus cannot be used as a CI strain in practice [183]. Several other colonisation-inhibiting strains, including *ompC*, *lon*, *cpxR*, *rfc*, *rfaH* and *crp* deletion mutants have been used only in short-term studies [182-184]. It is thus unknown whether they would interfere with implemented monitoring measures.

An efficacious live vaccine for broilers is a strain that induces a high degree of protection against intestinal colonisation and shedding, and a high degree of protection against systemic infection. The *ΔhilAssrAfliG* mutant strain inhibited caecal and spleen colonisation and shedding of a virulent *Salmonella* Enteritidis strain throughout the production period. Because the young chickens are immunologically immature and the virulent strain is rapidly cleared from the chicken caecum and internal organs, it is unlikely that an immune response against *Salmonella* is responsible for this early effect. Because the CI strain has to fulfil certain criteria concerning safety, of which rapid clearance is one, and it is unlikely that an immune response against *Salmonella* has been elicited before the CI strain is cleared from the host, it is possible that the protection offered by the CI strain will disappear along with the strain as it is cleared from the host. However, by this time point, the chickens are much

less susceptible to *Salmonella* infection due to maturation of the immune system and acquisition of gut microbiota.

The risk of *Salmonella* infection is very high the first days after introduction of the chicks on the farm due to environmental contamination [133]. To obtain protection induced by colonisation-inhibition as early as possible, the colonisation-inhibiting strain should be administered as soon as possible after hatching, to minimize the period in which the chicks are not protected. In practice, this could be done by spraying a solution containing the *Salmonella* Enteritidis *ΔhilAssrAfliG* strain on the newly hatched chicks in the hatchery. Therefore, the colonisation-inhibiting strain would need to be able to survive on the newborn chick's body. Other methods used for controlling *Salmonella* in poultry, such as acidification of the drinking water or the use of feed additives, do not offer similar protection levels compared to CI strains soon after hatching, mostly because the young birds have a delay in consumption of feed and water after introduction in a poultry house. The use of organic acids, essential oils, pro- and prebiotics as feed supplement can be an effective control measure used to protect broiler chickens against caecal colonisation and faecal shedding by *Salmonella* but the protective responses elicited by these compounds only start several days post-hatch [116, 202, 203]. Therefore, administration of CI strains that protect chicks early post-hatch might be a valuable addition to these strategies.

Broiler chickens can be colonised by several *Salmonella* serotypes other than *Salmonella* Enteritidis. Earlier observations suggest that the colonisation-inhibiting effect is stronger if the CI strain and the challenge strain belong to the same serovar [169]. Generally, a higher degree of protection is observed if the CI strain and the challenge strain are more closely

related [133]. It is therefore highly probable that the *Salmonella* Enteritidis *ΔhilAssrAfliG* mutant strain does not offer a high degree of protection against serotypes other than *Salmonella* Enteritidis. It is however possible to generate deletion mutants similar to the *ΔhilAssrAfliG* mutant strain for other serovars, which would allow to quickly and efficiently respond to *Salmonella* serovars that are emerging or are of greater importance for broilers. In 2012, only 0.2 % of all broiler flocks in the European Union were positive for *Salmonella* Enteritidis while 2.9 % were positive for other serovars [5]. It is therefore important to develop a means of protection against not only *Salmonella* Enteritidis, but other *Salmonella* serovars as well. Because colonisation-inhibition is limited between serovars, it is theoretically possible to administer a mixture of *Salmonella* strains belonging to different serovars that do not inhibit each other but do inhibit virulent wild-type strains. This has already been shown for a mixture of wild-type *Salmonella* strains and similar levels of protection should be obtainable using CI stains [169]. Ideally, *Salmonella* vaccines should have markers enabling the differentiation from *Salmonella* wild-type strains. As the *ΔhilAssrAfliG* mutant strain is non-motile, it is distinguishable from wild-type strains by bacteriological means. Additionally, it has been shown that different flagellin deficient strains do not generate anti-flagellin antibodies in laying hens, allowing serological detection of anti-flagellin antibodies to discriminate between a vaccinated and infected flock [165, 188]. However, because the strain is rapidly eliminated from the host and its reduced ability to colonize systemic sites, it is unlikely that the *ΔhilAssrAfliG* mutant strain induces an immune response in broilers that would permit differentiation between a vaccinated and infected flock.

In conclusion, a significant reduction in faecal shedding and caecal and internal organ colonisation by a virulent *Salmonella* Enteritidis challenge strain could be obtained by administering a *Salmonella* Enteritidis *ΔhilAssrAfliG* mutant strain to one day old broiler chickens. Consequently, this strain can potentially be used as a live colonisation-inhibition strain for controlling *Salmonella* Enteritidis in broilers.

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## 3.2 A *Salmonella* Typhimurium Δ*hilAssrAfliG* strains protects broilers against *Salmonella* Typhimurium colonisation and a colonisation-inhibition culture consisting of *Salmonella* Enteritidis and Typhimurium Δ*hilAssrAfliG* strains protects against infection by strains of both serotypes in broilers

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## Abstract

Consumption of contaminated poultry meat is still an important cause of *Salmonella* infections in humans and there is a need for control methods that protect broilers from day-of-hatch until slaughter age against infection with *Salmonella*. Colonisation-inhibition, a concept in which a live *Salmonella* strain is orally administered to day-old chickens and protects against subsequent challenge, can potentially be used as control method. In this study, the efficacy of a *Salmonella* Typhimurium *ΔhilAssrAfliG* strain as a colonisation-inhibition strain for protection of broilers against *Salmonella* Typhimurium was evaluated. Administration of a *Salmonella* Typhimurium *ΔhilAssrAfliG* strain to day-old broiler chickens decreased faecal shedding and strongly reduced caecal and internal organ colonisation of a *Salmonella* Typhimurium challenge strain administered one day later using a seeder bird model. In addition, it was verified whether a colonisation-inhibition culture could be developed that protects against both *Salmonella* Enteritidis and Typhimurium. Therefore, the *Salmonella* Typhimurium *ΔhilAssrAfliG* strain was orally administered simultaneously with a *Salmonella* Enteritidis *ΔhilAssrAfliG* strain to day-old broiler chickens, which resulted in a decreased caecal and internal organ colonisation for both a *Salmonella* Enteritidis and a *Salmonella* Typhimurium challenge strain short after hatching, using a seeder bird model. The combined culture was not protective against *Salmonella* Paratyphi B varietas Java challenge, indicating serotype-specific protection mechanisms. The data suggest that colonisation-inhibition can potentially be used as a versatile control method to protect poultry against several *Salmonella* serotypes.

## Introduction

Despite the implementation of monitoring and control measures in broiler production, *Salmonella* is still an important cause of poultry meat associated human infections [5]. Broilers often become infected with *Salmonella* early after hatching as they are highly susceptible to infection during these first days of life [159]. This is mainly due to the absence of normal gut microbiota in young chickens and the immaturity of their immune system [156, 157, 197-199]. Infection during this period, even with low numbers of *Salmonella*, can lead to persistent carriers [160, 161]. These broilers are often still infected at slaughter age, which may result in introduction of *Salmonella* in the slaughterhouse and food chain [14]. Consequently, prevention of infection in this period in which the chick is highly susceptible to infection could strongly reduce the introduction of *Salmonella* in the food chain.

Colonisation-inhibition (CI) is a phenomenon in which chickens are administered a live *Salmonella* strain that protects against subsequent challenge with another *Salmonella* strain [170]. By administering a CI strain that colonises the gut rapidly and extensively, it is possible to increase resistance to *Salmonella* strains quickly after hatching [189]. This concept has been recognized for a long time, and a great deal of effort has been put in developing strains that are appropriate for use as CI strains [133, 171]. Earlier research demonstrated that deletion of the *hilA*, *ssrA* and *fliG* genes in a *Salmonella* Enteritidis strain resulted in a CI strain that was safe and effective in protecting broilers against challenge with a *Salmonella* Enteritidis wild-type strain [204]. Because there is greater inhibition within a serovar than between serovars [169], the *Salmonella* Enteritidis Δ*hilAssrAfliG* strain can be expected to mainly protect against *Salmonella* Enteritidis infection, and not or to a lesser extent against e.g. *Salmonella* Typhimurium infection. In 2012, 0.3 % of all broiler flocks were positive for

*Salmonella* Enteritidis and *Salmonella* Typhimurium in Europe, while 2.8 % were positive for other *Salmonella* serotypes [5]. Consequently, if CI strains are needed that protect against these other serovars, new CI strains need to be developed. It is however unknown whether introduction of the *hilA*, *ssrA* and *fliG* mutations in a *Salmonella* strain belonging to another serovar yields a CI strain displaying the same degree of attenuation and similar protective properties as the *Salmonella* Enteritidis Δ*hilAssrAfliG* strain. Additionally, it is not known whether a combination of two or more CI strains, belonging to different serovars, is able to protect against infection by different *Salmonella* serovars.

In the present study, the efficacy of a *Salmonella* Typhimurium *hilAssrAfliG* deletion mutant as a CI strain was evaluated. Secondly, the protective effect of a CI culture consisting of both a *Salmonella* Enteritidis and Typhimurium *hilAssrAfliG* deletion mutant against *Salmonella* Enteritidis, Typhimurium and Paratyphi B var. Java infection was evaluated.

## Material & methods

### Chickens

One-day-old Ross 308 broiler chickens were obtained from a local hatchery and housed in separate rooms in containers on wood shavings. Commercial feed and drinking water were provided *ad libitum*. Experiments were performed with the permission of the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University, Belgium (experiment authorisation number: EC2012/96).

## Bacterial strains and deletion mutants

A spontaneous nalidixic acid-resistant mutant of *Salmonella* Typhimurium strain 112910a, originally isolated from a pig stool sample [205], was used for the production of isogenic mutants. This resistance has previously been shown to have no impact on the *in vivo* results [171]. Deletion of *hilA*, *ssrA* and *fliG* genes in this strain was done using the one-step inactivation method described by Datsenko and Wanner [194]. *Salmonella* Typhimurium MB2136, a streptomycin resistant wild-type strain originally isolated from swine was used as a challenge strain. A nalidixic acid-resistant *Salmonella* Enteritidis *hilAssrAfliG* deletion mutant, which has been described earlier [204], was also used in this study. The original *Salmonella* Enteritidis 76Sa88 strain, from which the *Salmonella* Enteritidis *hilAssrAfliG* deletion mutant is derived, was originally isolated on a poultry farm [159, 193]. *Salmonella* Enteritidis 147 (streptomycin resistant), a strain originally isolated from chicken egg white and which is known to colonise the gut and internal organs of chickens to a high level [189, 195, 196], was used as a challenge strain. Additionally, a wild-type *Salmonella* Paratyphi B. var. Java strain (carbenicillin resistant) originally isolated from poultry, was also used as a challenge strain.

## Experimental design

### Experiment 1: Efficacy of a *Salmonella* Typhimurium *hilAssrAfliG* deletion mutant against experimental *Salmonella* Typhimurium infection

In order to evaluate the persistence of a *Salmonella* Typhimurium Δ*hilAssrAfliG* strain in chickens and its efficacy against colonisation by a wild type *Salmonella* Typhimurium strain, 225 one-day-old chicks were divided into three groups of 75 animals and each group was

housed in a container of 2 m². Two groups (Group V and C) were given $10^9$ CFU of the *Salmonella* Typhimurium *ΔhilAssrAfliG* strain by oral gavage while the third group was given sterile HBSS (Hank's Balanced Salt Solution, Invitrogen, Paisley, England) as a control (Group I). Twenty-four hours later, 25 randomly selected chickens in Group I and Group C were given $10^5$ CFU *Salmonella* Typhimurium MB2136 by oral gavage. These seeder birds were housed together with the other chickens of their group. Bacterial counts in caecum and spleen were determined for one third of the original number of chickens by bacteriological analysis at 7, 21 and 42 days old. At each time point, one in three sampled animals were seeder birds. Shedding of both strains was monitored by cloacal swabbing on days 2, 3, 9, 16, 23, 30 and 37.

**Experiment 2: Efficacy of simultaneous administration of a *Salmonella* Typhimurium *hilAssrAfliG* and a *Salmonella* Enteritidis *hilAssrAfliG* deletion mutant against infection with several *Salmonella* serotypes**

In order to evaluate the efficacy of a CI culture containing a *Salmonella* Enteritidis Δ*hilAssrAfliG* and *Salmonella* Typhimurium Δ*hilAssrAfliG* strain against infection by several *Salmonella* serotypes, 60 one-day-old chickens were divided into 6 groups of 10 animals. Three of these groups (Group VSE, VST and VSJ) were given 0.5 ml of a mixture containing 2 x $10^8$ CFU/ml of the *Salmonella* Enteritidis Δ*hilAssrAfliG* strain and 2 x $10^8$ CFU/ml of the *Salmonella* Typhimurium Δ*hilAssrAfliG* strain by oral gavage. The three remaining groups (CSE, CST and CSJ) were given sterile HBSS as a control. Twenty-four hours later, 2 randomly selected chickens in each group were given $10^5$ CFU of a challenge strain by oral gavage. These seeder birds were then housed together again with the other chickens of their group. Groups VSE and CSE were challenged with *Salmonella* Enteritidis strain 147, Groups VST and

CST with *Salmonella* Typhimurium strain MB2136 and Groups VSJ and CSJ with the wild-type *Salmonella* Paratyphi B var. Java strain. Bacterial counts of CI strains and challenge strains in caecum and spleen were determined by bacteriological analysis on day 7.

## Bacteriological analysis

Cloacal swabs were directly inoculated on Xylose Lysine Deoxycholate agar (XLD, Oxoid, Basingstoke, England) plates with 20 µg/ml nalidixic acid (Sigma-Aldrich, St. Louis, MO, USA) or 100 µg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Samples negative after direct inoculation were pre-enriched in buffered peptone water (BPW, Oxoid, Basingstoke, England) and incubated overnight at 37 °C. One ml of this suspension was further enriched by adding 9 ml tetrathionate-brilliant green broth (Merck, Darmstadt, Germany). After overnight incubation at 37 °C, this suspension was plated on XLD plates supplemented with the appropriate antibiotic. Samples of caecum and spleen were homogenized in BPW and 10-fold dilutions were made in HBSS. Six droplets of 20 µl of each dilution were plated on XLD plates supplemented with 20 µg/ml nalidixic acid, 100 µg/ml streptomycin or 100 µg/ml carbenicillin (Sigma-Aldrich, St. Louis, MO, USA). After overnight incubation at 37 °C, the number of CFU/g tissue was determined by counting the number of bacterial colonies on the plates.

## Statistical analysis

GraphPad Prism 5 software was used for statistical analysis. A Fisher's exact test (one-sided) was used to analyse differences in mortality between groups. A Kruskal-Wallis test (one-way ANOVA, with Dunn's multiple comparison test) was used to determine statistical differences of the number of *Salmonella* positive cloaca swabs among groups. Bacterial counts in

caecum and spleen were converted into logarithmic form for statistical analysis. Samples of caecum and spleen negative after direct plating were rated as $\log_{10}$= 0. The mean CFU/g tissue was calculated for each group on every time point and differences between groups were analysed using a Kruskal-Wallis test (one-way ANOVA, with Dunn's multiple comparison test) (experiment 1) or a Mann-Whitney test (experiment 2). Differences with p-values lower than 0.05 were considered to be significant.

## Results

**Experiment 1: Efficacy of a *Salmonella* Typhimurium *hilAssrAfliG* deletion mutant against experimental *Salmonella* Typhimurium infection**

During the experiment, four chickens died in Group V, five in Group I and four in Group C. The observed differences in mortality were not statistically significant.

Shedding of the *Salmonella* Typhimurium Δ*hilAssrAfliG* strain remained high during the entire experiment in Group V (Table 1). Shedding of this strain decreased quickly in Group C. The *Salmonella* Typhimurium Δ*hilAssrAfliG* strain was excreted until day 16 by a limited number of chickens, after which it could no longer be detected. Shedding of the *Salmonella* Typhimurium challenge strain was lower in the group treated with a *Salmonella* Typhimurium Δ*hilAssrAfliG* strain during the entire experiment when compared to the sham-treated control. Statistical differences are shown in Table 1. Data on shedding of the *Salmonella* Typhimurium Δ*hilAssrAfliG* strain on days 3, 9 and 16 of the experiment are not available due to overgrowth of other bacteria on the culture media.

**Table 1. The number of cloacal swabs positive for a *Salmonella* Typhimurium *ΔhilAssrAfliG* strain or a *Salmonella* Typhimurium challenge strain.** Groups V and C were orally inoculated with $10^9$ CFU of a *Salmonella* Typhimurium *ΔhilAssrAfliG* strain at day 1 of the experiment (day-of-hatch). Groups I and C were challenged with $10^5$ CFU of a *Salmonella* Typhimurium challenge strain on day 2 of the experiment. Samples were taken at days 2, 3, 9, 16, 23, 30 and 37 of the experiment.

| Strain | Group | Day 2 | Day 3 | Day 9 | Day 16 | Day 23 | Day 30 | Day 37 |
|---|---|---|---|---|---|---|---|---|
| *Salmonella* Typhimurium *ΔhilAssrAfliG* strain | V | 75/75[a] (75[b]) | 75/75 (72*) | 49/49 (47) | 47/47 (30*) | 24/24 (20*) | 24*/24 (20*) | 22*/23 (18*) |
| | C | 75/75 (67) | NA/75 (17*) | NA/50 (NA) | NA/48 (10*) | 0/23 (0*) | 0*/22 (0*) | 0*/22 (0*) |
| *Salmonella* Typhimurium challenge strain | I | NA | 18/75 (8) | 45*/50 (41*) | 35*/49 (12) | 5/25 (5) | 10/25 (7) | 10/25 (7) |
| | C | NA | 12/75 (0) | 10*/50 (0*) | 0*/48 (0) | 0/23 (0) | 0/22 (0) | 0/22 (0) |

[a] Number of positive samples after enrichment/total number of samples

[b] Number of positive samples after direct plating

* Significant difference in positive samples between both groups (p-value < 0.05)

NA = Not available

Bacteriological analysis of the caecum samples showed that the *Salmonella* Typhimurium Δ*hilAssrAfliG* strain colonised the caecum to similar high levels in Group V and Group C on day 7 (Figure 1). The bacterial load of this strain was mean $\log_{10}$= 7.48 CFU/g in Group V and mean $\log_{10}$= 6.12 CFU/g in Group C. This reduced to mean $\log_{10}$= 6.01 CFU/g on day 21 and mean $\log_{10}$= 4.23 CFU/g on day 42 in Group V. The strain could no longer be detected on day 42 in Group C. No data were available for the caecum of Group C on day 21 due to overgrowth of other bacteria on the culture media. In the spleen, the bacterial load of the *Salmonella* Typhimurium Δ*hilAssrAfliG* strain amounted on day 7 to mean $\log_{10}$= 2.05 CFU/g in Group V and $\log_{10}$= 1.61 CFU/g in Group C. Bacterial numbers reduced as the experiment proceeded, as the load amounted to mean $\log_{10}$= 0.337 CFU/g in Group V and mean $\log_{10}$= 0.341 CFU/g in Group C on day 21. This reduced further to mean $\log_{10}$= 0.097 CFU/g in Group V on day 42. By then, the *Salmonella* Typhimurium Δ*hilAssrAfliG* strain could no longer be detected in Group C.

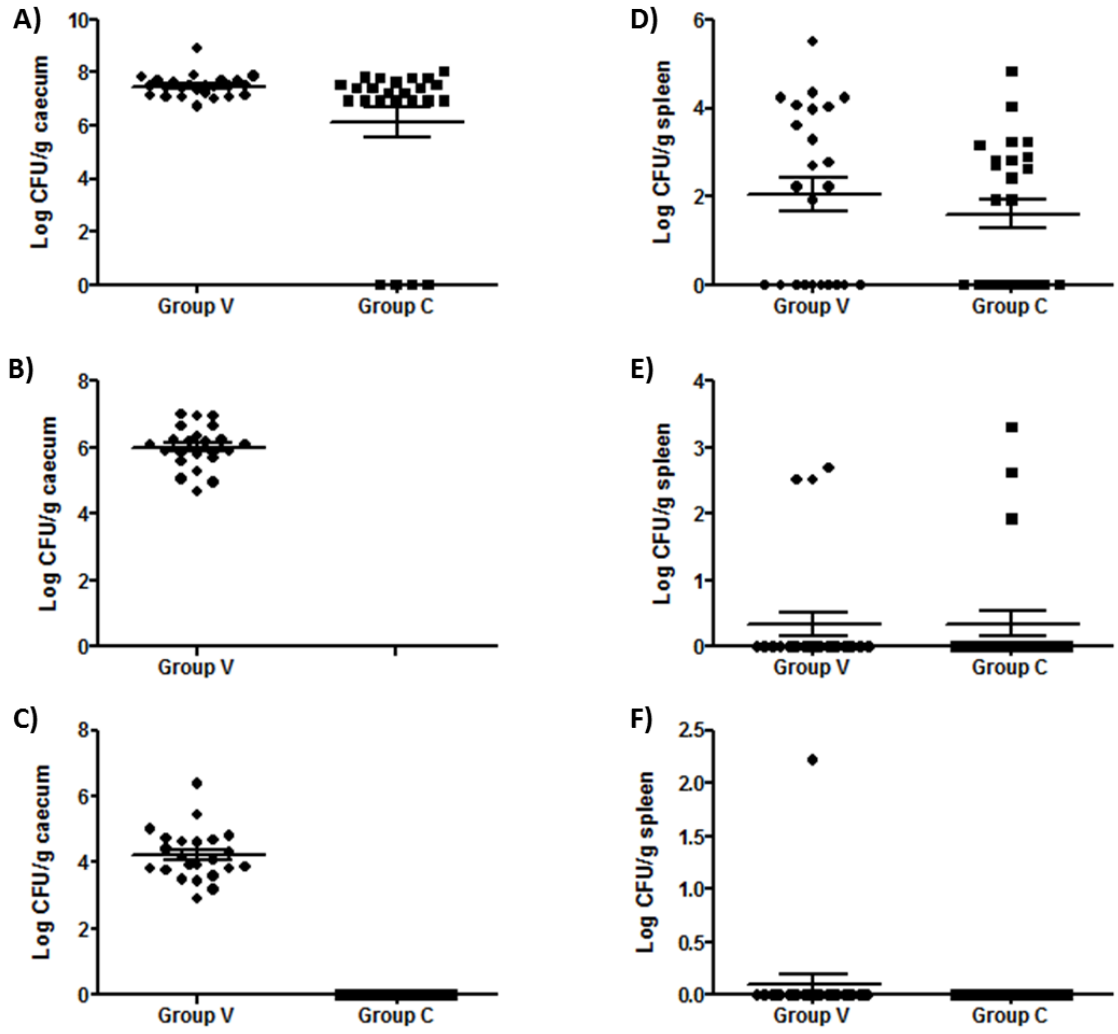


**Figure 1. Caecal (A, B, C) and spleen (D, E, F) colonisation by a *Salmonella* Typhimurium *hilAssrAfliG* deletion mutant strain.** Chickens in Group V and C were given $10^9$ CFU of a *Salmonella* Typhimurium *ΔhilAssrAfliG* deletion mutant strain at day-of-hatch. Additionally, chickens in Group C were given $10^5$ CFU of a *Salmonella* Typhimurium challenge strain on day 2 of the experiment. Subfigures A and D show colonization on day 7, B and E on day 21 and C and F on day 42. Values shown are $\log_{10}$ of CFU/g sample. The horizontal lines represent the mean, the error bars represent the standard error of mean (SEM). The number of samples equals 25, 23 and 23 in Group V and 25, 23 and 22 in Group C on day 7, 21 and 42 respectively. No data are available for the caeca of Group C on day 21.

Bacteriological analysis of the caecum and spleen showed that the *Salmonella* Typhimurium challenge strain colonised the caecum of the chickens in Group I to high levels, while it could not be detected in any of the caeca of the chickens in Group C at any time point (Figure 2). The bacterial load and number of spleens positive for the *Salmonella* Typhimurium challenge

strain in Group I was initially high, but declined as the experiment proceeded. The *Salmonella* Typhimurium challenge strain could not be detected in any of the spleens on day 42 in Group I, and in any of the spleens of the chickens belonging to Group C at any time point.

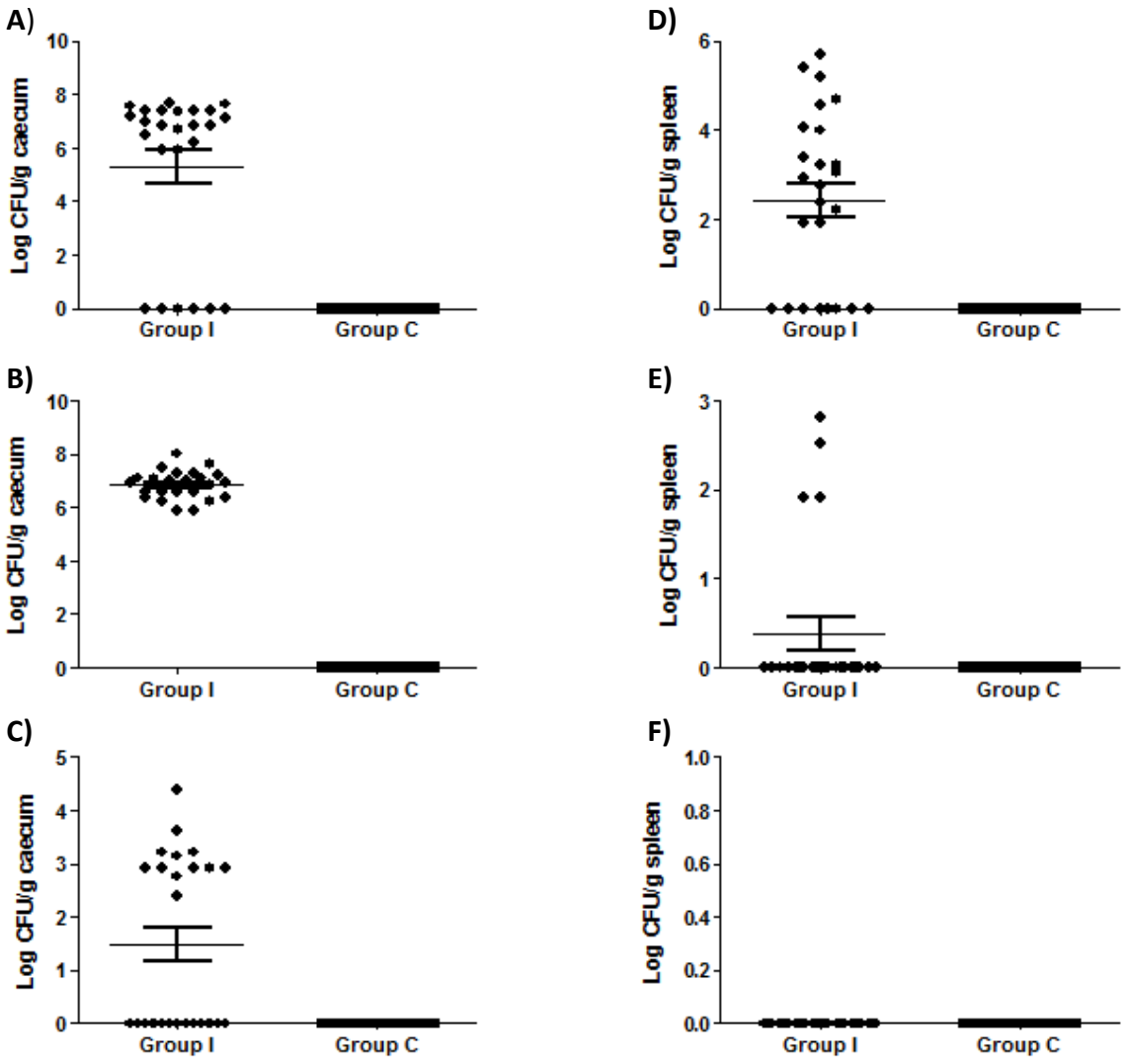


**Figure 2. Caecal (A, B, C) and spleen (D, E, F) colonisation by a *Salmonella* Typhimurium challenge strain.** Chickens in Group C were given $10^9$ CFU of a *Salmonella* Typhimurium *hilAssrAfliG* deletion mutant strain at day-of-hatch. Chickens in Group I and C were given $10^5$ CFU of a *Salmonella* Typhimurium challenge strain on day 2 of the experiment. Subfigures A and D show colonization on day 7, B and E on day 21 and C and F on day 42. Values shown are $\log_{10}$ of CFU/g sample. The horizontal lines represent the mean, the error bars represent

the standard error of mean (SEM). The number of samples equals 25, 24 and 25 in Group I and 25, 23 and 22 in Group C on day 7, 21 and 42 respectively.

**Experiment 2: Efficacy of simultaneous administration of a *Salmonella* Typhimurium *hilAssrAfliG* and a *Salmonella* Enteritidis *hilAssrAfliG* deletion mutant against infection with several *Salmonella* serotypes**

None of the chickens died during the experiment. Bacteriological analysis of the samples showed that the *Salmonella* Enteritidis and Typhimurium Δ*hilAssrAfliG* strains colonised the caecum and spleen to a similar level in all treated groups. Mean colonisation was $\log_{10}$= 6.87 ± 0.12 and 6.44 ± 0.76 CFU/g in the caeca, and $\log_{10}$= 0.74 ± 0.50 and 2.02 ± 0.77 CFU/g in the spleens of Groups VSE and VST, respectively. Data on colonisation by the CI culture in Group VSJ is not available because the *Salmonella* Paratyphi B var. Java strain is, like the CI strains, nalidixic acid resistant. Consequently, the CI strains could not be distinguished from the challenge strain. Additionally, bacteriological analysis of caecum and spleen showed that colonisation by the *Salmonella* Enteritidis challenge strain was significantly lower in the caecum of the group treated with CI culture (Figure 3). No differences could be observed in spleen colonisation. Similarly, colonisation by the *Salmonella* Typhimurium strain was significantly lower in the caecum of the treated group, while no significant difference could be observed in the spleen. Colonisation by the *Salmonella* Paratyphi B var. Java strain did not differ significantly between the treated and untreated group.

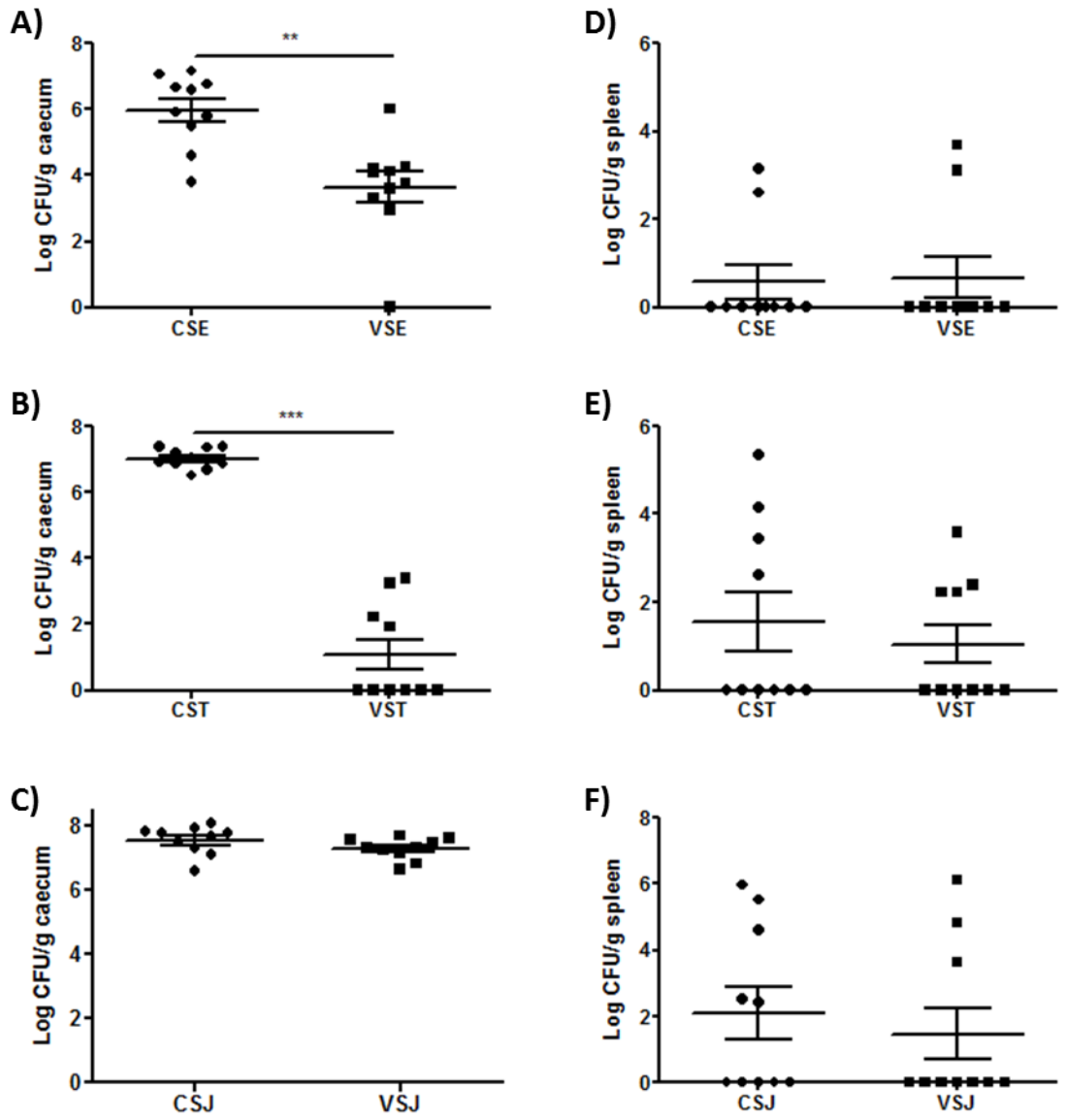


**Figure 3. Caecal (A, B, C) and spleen (D, E, F) colonisation by a *Salmonella* Enteritidis, *Salmonella* Typhimurium or *Salmonella* Paratyphi B var. Java challenge strain.** Animals from groups VSE, VST and VSJ were orally inoculated with a combination of $10^8$ CFU of a *Salmonella* Enteritidis *ΔhilAssrAfliG* strain and $10^8$ CFU of a *Salmonella* Typhimurium *ΔhilAssrAfliG* strain at day 1 of the experiment (day-of-hatch). All groups were infected with $10^5$ CFU of a challenge strain on day 2 of the experiment. Groups CSE and VSE were infected with a *Salmonella* Enteritidis challenge strain (A, D), groups CST and VST with a *Salmonella* Typhimurium strain (B, E) and groups CSJ and VSJ with a *Salmonella* Paratyphi B var. Java strain (C, F). Samples were taken at day 7 of the experiment. Represented values are $\log_{10}$ of CFU/g sample. The horizontal lines represent the mean, the error bars represent the standard error of mean (SEM). The number of samples equals 10 in every group.

## Discussion

Newly hatched chicks are highly susceptible to *Salmonella* infections during the first days of life and inoculation with very low doses can result in persistent infections [159-161]. This high susceptibility has been associated with the absence of normal gut microbiota [197, 198] and the immature immune system of young chickens [156, 157, 199]. As a consequence, classical vaccination is not an effective means to achieve protection against *Salmonella* infection during the first days of life [133]. Alternatively, the use of organic acids, essential oils, pro- and prebiotics as feed supplements can help to control *Salmonella* infections in broiler chickens, but the protective responses elicited by these compounds only start several days post-hatch [116, 202, 203]. Since a rapid colonisation-inhibiting effect has been described in birds inoculated with a live *Salmonella* strain that protected the animals against subsequent challenge with another *Salmonella* strain, administration of CI strains to chickens early post-hatch might be a valuable addition to these strategies.

The *Salmonella* Typhimurium Δ*hilAssrAfliG* strain used in the present study was very effective at protecting against *Salmonella* Typhimurium challenge. Unfortunately, the *Salmonella* Typhimurium Δ*hilAssrAfliG* strain was highly colonising and persisted in the caecum until slaughter age when the chickens were not challenged with a wild-type strain. However, when challenging the chickens with a wild-type strain, the *Salmonella* Typhimurium Δ*hilAssrAfliG* strain was cleared rapidly from the chickens. This suggests an interaction between both strains that influences persistence and clearance of the CI strain from the chickens. Still, because this might result in the introduction of the deletion mutant strain in the food chain when applied in the field, the developed CI strain might not be an

appropriate candidate for use in broiler production. As the ability of *Salmonella* strains to colonise represents an important prerequisite for effective colonisation inhibition of wild-type strains, persistence of a CI strain and protection offered by a CI strain are probably related to each other. Furthermore, it has been suggested that a CI strain is more protective against challenge when it is highly colonising [169, 173]. It is therefore not improbable that the observed strong protective effect of the *Salmonella* Typhimurium Δ*hilAssrAfliG* strain is due to its high colonising capacity. A *Salmonella* Enteritidis *hilAssrAfliG* deletion mutant has been shown to colonise spleen and caecum to a lesser extent and was cleared rapidly from poultry, but also offered relatively less protection against *Salmonella* Enteritidis infection [204]. These and earlier observations suggest thus that there will be a trade-off between persistence and protection, as a highly colonising and thus protective strain will probably not be eliminated by slaughter age [190]. In contrast, a strain that is poorly colonising will be eliminated by slaughter age, but will probably not offer a long lasting protection. Obviously, these aspects should be taken into account when developing a CI strain.

Earlier research showed that the colonisation-inhibition effect is more pronounced between isogenic strains and that there is greater inhibition within a serovar than between serovars [133, 169, 183]. Consequently, it is likely that the *Salmonella* Typhimurium Δ*hilAssrAfliG* strain is not able to inhibit strains belonging to other serovars than Typhimurium. It has however been suggested that a mixture of *Salmonella* strains belonging to several serovars would be able to inhibit a broad spectrum of virulent wild-type strains [169]. Therefore, we investigated the protective properties of a mixed culture consisting of both the *Salmonella* Enteritidis and Typhimurium Δ*hilAssrAfliG* strain against infection by 3 different *Salmonella* serovars. The results obtained in this study showed that the combined CI culture confers

protection against a non-isogenic *Salmonella* Enteritidis and *Salmonella* Typhimurium challenge strain quickly after hatching. This suggests that both CI strains do not inhibit each other, or if they do it is to such a limited extent that they do not impede each other's protective properties. Earlier research showed that administering a mixture consisting of a wild-type *Salmonella* Enteritidis and *Salmonella* Typhimurium strain resulted in a pronounced protection against their isogenic challenge strains, but also against *Salmonella* Hadar and *Salmonella* Infantis challenge strains [169]. This suggests a synergistic protective effect when administering multiple CI strains simultaneously. In the present study, the CI mixture consisting of the *Salmonella* Enteritidis Δ*hilAssrAfliG* strain and *Salmonella* Typhimurium Δ*hilAssrAfliG* strain did, however, not offer protection against challenge with a *Salmonella* Paratyphi B. var. Java strain. Consequently, this suggests that a *Salmonella* Paratyphi B. var. Java CI strain needs to be developed and added to the CI culture in order to achieve simultaneous protection against *Salmonella* Enteritidis, Typhimurium and Paratyphi B. var. Java infection.

In conclusion, a significant reduction in faecal shedding and caecal and internal organ colonisation by a virulent *Salmonella* Typhimurium challenge strain could be obtained by administering a *Salmonella* Typhimurium *ΔhilAssrAfliG* mutant strain to one day old broiler chickens. Additionally, when this strain was administered simultaneously with a *Salmonella* Enteritidis *hilAssrAfliG* deletion mutant protection against infection by both a *Salmonella* Enteritidis and Typhimurium challenge strain could be obtained. These data demonstrate that colonisation-inhibition represents a promising tool to protect broilers early after hatching against multiple *Salmonella* serotypes. They pave the way for developing new CI

strains and CI cultures that are cleared at slaughter age and protect against a wide variety of *Salmonella* serovars that are of importance for broiler production.

## Acknowledgements

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## 3.3 Administration of a *Salmonella* Enteritidis Δ*hilAssrAfliG* strain by coarse spray to newly hatched broilers reduces colonization and shedding of a *Salmonella* Enteritidis challenge strain

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## Abstract

Consumption of contaminated poultry meat is still an important cause of *Salmonella* infections in humans. Colonization-inhibition (**CI**) occurs when a live *Salmonella* strain is administered to chickens and subsequently protects against challenge with another *Salmonella* strain belonging to the same serovar. A *Salmonella* Enteritidis *hilAssrAfliG* deletion mutant has previously been proven to reduce colonization and shedding of a wild-type *Salmonella* Enteritidis strain in newly hatched broilers after experimental infection. In this study, we compared two administration routes for this strain. Administering the *Salmonella* Enteritidis Δ*hilAssrAfliG* strain through drinking water on the first day of life resulted in a decreased faecal shedding and caecal colonization of a wild-type *Salmonella* Enteritidis challenge strain administered 24 h later using a seeder-bird model. When administering the CI strain by coarse spray on newly hatched broiler chicks, an even more pronounced reduction of caecal colonisation was observed and faecal shedding of the *Salmonella* Enteritidis challenge strain ceased during the course of the experiment. These data suggest that administering a *Salmonella* Enteritidis Δ*hilAssrAfliG* strain to newly hatched chicks using a coarse spray is a useful and effective method which reduces colonization and shedding of a wild-type *Salmonella* Enteritidis strain after early challenge.

## Introduction

Despite the implementation of monitoring and control measures in broiler production, poultry meat is still an important carrier of *Salmonella* causing human infections [5]. Chickens are highly susceptible to *Salmonella* infection during their first days of life [159] and contact with *Salmonella*, even in very low numbers, can lead to persistent infections [160, 161]. These broilers often remain infected until slaughter age, which leads to introduction of *Salmonella* in the slaughterhouse and food chain [14]. Consequently, prevention of infection during the early post-hatch period is of utmost importance. Colonization-inhibition (**CI**) occurs when a live *Salmonella* strain is orally administered to day-old chickens: it protects very rapidly against subsequent challenge with another *Salmonella* strain belonging to the same serotype [171, 173, 189, 191]. CI can thus be used as control method to prevent infection during the period in which the chick is highly susceptible to *Salmonella* infection [189]. Earlier research demonstrated that deletion of the *hilA*, *ssrA* and *fliG* genes in a *Salmonella enterica* subspecies *enterica* serovar Enteritidis (*Salmonella* Enteritidis) strain resulted in a CI strain that was safe and effective in protecting broilers against challenge with a *Salmonella* Enteritidis wild-type strain [204]. This strain is considered safe because it is cleared by slaughter age and effective, as it lowers faecal shedding and caecal colonization of a wild-type *Salmonella* Enteritidis challenge strain. In this earlier study, the CI strain was administered by oral gavage in the crop, which is an unrealistic administration method in practice on a large scale. Because the level of protection offered by a CI strain is dependent on the administration route [133], the efficacy of the *Salmonella* Enteritidis Δ*hilAssrAfliG* strain might be different when this strain is administered by other routes than the oral gavage. Therefore, two practically relevant administration routes for the *Salmonella*

Enteritidis *hilAssrAfliG* deletion mutant, through drinking water and by coarse spray, were investigated and compared in this study.

## Materials and methods

### Chickens

One-day-old Ross 308 broiler chicks were obtained from a local hatchery and housed in isolation. Experimental groups were housed in separate rooms in containers on wood shavings. Commercial feed and drinking water were provided *ad libitum*. Cloacal swabs of all chicks were taken at the beginning of the experiment and cultured for *Salmonella* as described below to verify *Salmonella*-free status of the chickens prior to the experiment. Experiments were performed with the permission of the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University, Belgium (experiment authorization number: EC2013/136).

### *Salmonella* Enteritidis strains

*Salmonella* Enteritidis 76Sa88 is a nalidixic acid resistant, well-characterized strain originally isolated on a poultry farm [159, 193]. It was used for the production of isogenic mutants. Deletion of *hilA*, *ssrA* and *fliG* genes was done using the one-step inactivation method described by Datsenko and Wanner [194]. *Salmonella* Enteritidis 147, a streptomycin resistant strain originally isolated from egg white, was used as a challenge strain. This strain is known to colonize the gut and internal organs of chickens to a high level [189, 195, 196].

**Experimental design**

One hundred and eighty one-day-old chicks were divided into 3 groups of 60 and each housed in a container of two $m^2$. The first group (Group C) was given sterile Hank's Balanced Salt Solution (HBSS, 14175053, Invitrogen, Paisley, England) by oral gavage as a control on the first day of the experiment. Group D was given access to drinking water that contained initially 7.03 x $10^8$ CFU/ml of the *Salmonella* Enteritidis Δ*hilAssrAfliG* strain for the first 24 h of the experiment. The chicks in Group S were transferred to a box (measuring 40 cm on 60 cm) and sprayed with 125 ml of a 7.28 x $10^8$ CFU/ml solution of the *Salmonella* Enteritidis Δ*hilAssrAfliG* strain using a coarse spray. They were transferred to their containers 10 minutes after treatment. On the second day of the experiment, 12 chickens in each group (1 out of 5) were randomly selected and given $10^5$ CFU of the *Salmonella* Enteritidis challenge strain (seeder birds) by oral gavage and housed together again with the other animals of their group. To evaluate colonization by the *Salmonella* Enteritidis Δ*hilAssrAfliG* strain and the wild-type challenge strain, their numbers in caecum and spleen were determined on days 7, 21 and 42 for 20 chickens. At each time point, one in five sampled animals were seeder birds. Shedding of both strains was evaluated during the experiment by bacteriological analysis of cloacal swabs taken on days 2, 3, 9, 16, 23, 30 and 37.

**Bacteriological analysis**

Cloacal swabs were directly inoculated on Xylose Lysine Deoxycholate agar (XLD, CM0469, Oxoid, Basingstoke, England) plates supplemented with 20 µg/ml nalidixic acid (N8878, Sigma-Aldrich, St. Louis, MO, USA) or 100 µg/ml streptomycin (S6501, Sigma-Aldrich, St. Louis, MO, USA). Samples negative after direct inoculation were pre-enriched in Buffered Peptone Water (BPW, CM0509, Oxoid, Basingstoke, England) and incubated overnight at 37

°C. One ml of this suspension was further enriched by adding 9 ml tetrathionate-brilliant green broth (1.05178.0500, Merck, Darmstadt, Germany). After overnight incubation at 37 °C, this suspension was plated on XLD plates with the appropriate antibiotic.

Samples of caecum and spleen were homogenized in BPW and 10-fold dilutions were made in HBSS. Six droplets of 20 µl of each dilution were plated on XLD plates supplemented with the appropriate antibiotic. After overnight incubation at 37 °C, the number of CFU/g tissue was determined by counting the number of bacterial colonies. Negative samples were enriched as described above. Samples of the litter were taken after termination of the experiment and enriched as described above.

**Statistical analysis**

GraphPad Prism 5 software was used for statistical analysis. A Fisher's exact test (one-sided) was used to analyze differences in mortality between groups. A Kruskal-Wallis test (one-way ANOVA, with Dunn's multiple comparison test) was used to determine statistical differences of the number of *Salmonella* positive cloaca swabs and (after enrichment) spleen and caecum samples, between groups. Bacterial counts in caecum and spleen were converted into logarithmic form for statistical analysis. Samples of caecum and spleen negative after direct plating were rated as $\log_{10}= 0$. Differences between groups were analyzed using a Kruskal-Wallis test (one-way ANOVA, with Dunn's multiple comparison test). Differences with p-values lower than 0.05 were considered to be significant.

## Results

In every group 5 chickens died during the course of the experiment. Consequently, there is no statistical difference between groups in mortality. As shown in Table 1, shedding of the *Salmonella* Enteritidis Δ*hilAssrAfliG* strain declined in both groups after inoculation, and the strain was no longer shed from day 23 of age onwards. Shedding of the *Salmonella* Enteritidis challenge strain was lower in Group S than in Group C for the entire duration of the experiment, while there was only initially a difference between Group C and D.

**Table 1. The number of cloacal swabs positive for a *Salmonella* Enteritidis *ΔhilAssrAfliG* strain or a *Salmonella* Enteritidis challenge strain.** Group C was given sterile HBSS as a control on day 1. Group S was sprayed with 125 ml of a suspension containing ± $10^9$ CFU/ml *Salmonella* Enteritidis Δ*hilAssrAfliG* strain on day 1. Group D was given access to drinking water containing ± $10^9$ CFU/ml of a *Salmonella* Enteritidis Δ*hilAssrAfliG* strain for the first 24 h of the experiment. Twelve chickens in each group (1 out of 5) were challenged with $10^5$ CFU of a *Salmonella* Enteritidis challenge strain on day 2 of the experiment (seeder birds). Samples were taken at days 2, 3, 9, 16, 23, 30 and 37 of the experiment.

| Strain | Group | Day 2 | Day 3 | Day 9 | Day 16 | Day 23 | Day 30 | Day 37 |
|---|---|---|---|---|---|---|---|---|
| *ΔhilAssrAfliG* | S | 60/60[a] (54[b]*) | 57/59 (43*) | 22/38 (4) | 2/37 (0) | 0/19 (0) | 0/19 (0) | 0/18 (0) |
| | D | 55/60 (29*) | 57/59 (22*) | 17/38 (7) | 12/38 (1) | 0/18 (0) | 0/18 (0) | 0/17 (0) |
| Challenge | C | NA | 12/60 (6) | 39/39* (28*) | 27/37* (8) | 3/18 (0) | 4/16 (2) | 6/16 (3) |
| | S | NA | 6/59 (3) | 8/38* (0*) | 1/37* (0) | 0/19 (0) | 0/19 (0) | 0/18 (0) |
| | D | NA | 8/59 (2) | 8/38* (1*) | 22/38 (7) | 4/18 (2) | 7/18 (2) | 5/17 (2) |

[a] Number of positive samples after enrichment/total number of samples

[b] Number of positive samples after direct plating

* Significant difference between groups (p-value < 0.05)

NA = Not available

Colonization by the *Salmonella* Enteritidis Δ*hilAssrAfliG* strain was initially high in the caecum, where the bacterial load amounted to 6.67 ± 0.029 log CFU/g and 6.82 ± 0.115 log CFU/g after direct plating, respectively in Groups S and D on day 7 of the experiment. On day

21, the CI strain could not be detected after direct plating in any sample belonging to Group S and only one sample was positive in Group D for which the bacterial load amounted to 4.28 log CFU/g. The CI strain could not be detected after direct plating in any of the caecum samples on day 42 or spleen samples for the entire duration of the experiment. Colonization by the *Salmonella* Enteritidis challenge strain is shown in Figure 1. Colonization of the caecum was significantly lower in Group S than in Group C for the entire duration of the experiment. Colonization of the caecum in Group D was significantly lower than the control group on day 7 and 42. There was no difference between the mean log CFU/g spleen of the different groups at any time point.

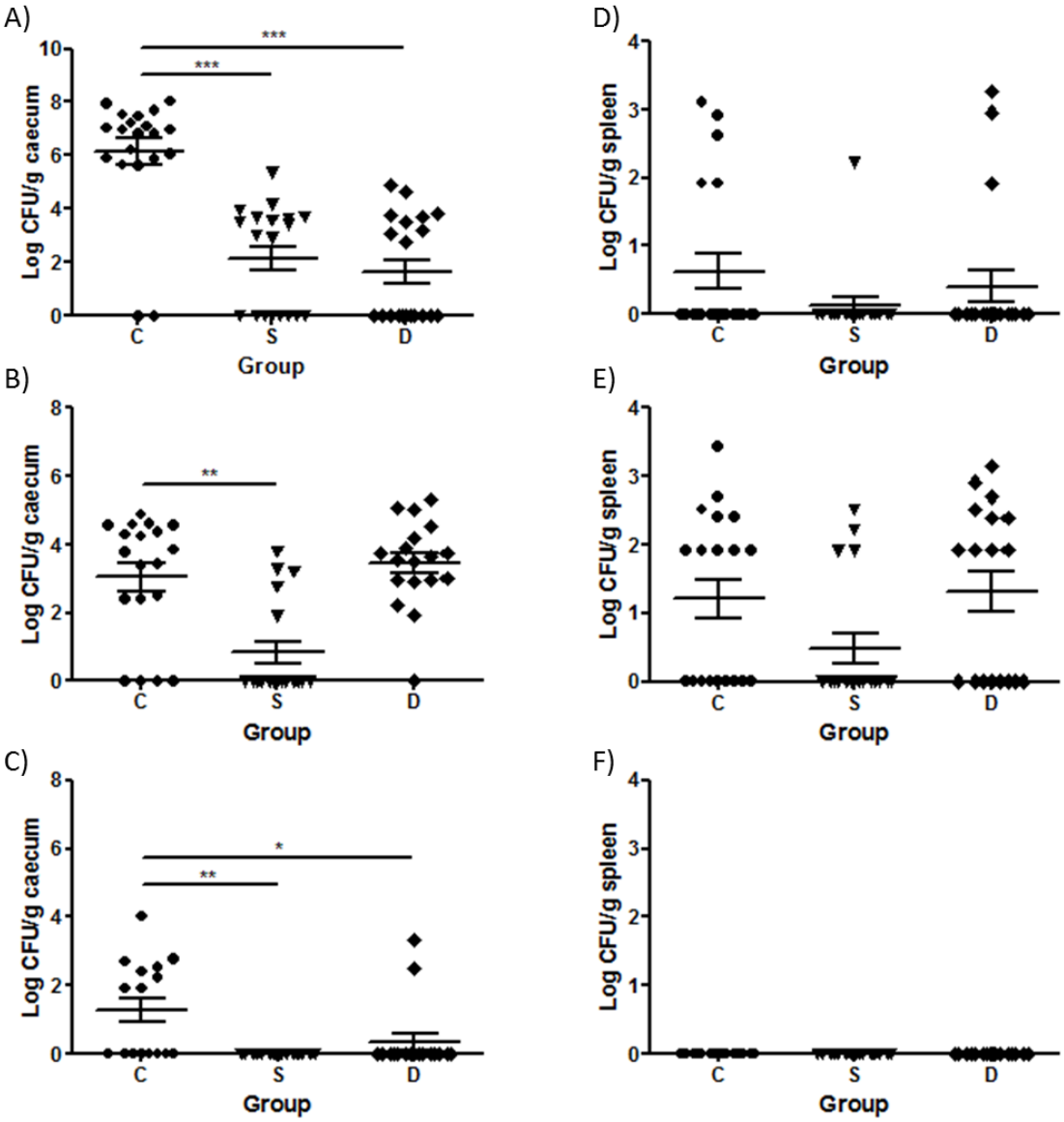


**Figure 1. Caecal (A, B, C) and spleen (D, E, F) colonization by a *Salmonella* Enteritidis challenge strain.** Group C was given sterile HBSS as a control on day 1. Group S was sprayed with a suspension containing a *Salmonella* Enteritidis Δ*hilAssrAfliG* strain on day 1. Group D was given access to drinking water containing a *Salmonella* Enteritidis Δ*hilAssrAfliG* strain for the first 24 h of the experiment. Twelve chickens in each group (1 out of 5) were challenged with a *Salmonella* Enteritidis challenge strain on day 2 of the experiment (seeder birds). Subfigures A and D show colonization on day 7, B and E on day 21 and C and F on day 42. Values shown are $\log_{10}$ of CFU/g sample. The horizontal lines represent the mean, the error bars represent the standard error of mean (SEM). Significant differences between groups are indicated with * (p-value < 0.05).

Enrichment of caecum and spleen samples showed that the *Salmonella* Enteritidis Δ*hilAssrAfliG* strain was present in only a few samples at day 21 of the experiment, and could no

longer be detected at day 42 (Table 2). The number of caecum and spleen samples positive for the *Salmonella* Enteritidis challenge strain was lower in both Group S and D than in Group C. However, this difference was only statistically significant on day 42 for the caecum when comparing Group S to the control group. The *Salmonella* Enteritidis Δ*hilAssrAfliG* strain could not be detected after enrichment of the litter samples. The *Salmonella* Enteritidis challenge strain could be detected in the litter of Group C, but was not present in the litter of the treated groups.

**Table 2. The number of caeca and spleen samples positive for a *Salmonella* Enteritidis *ΔhilAssrAfliG* strain or a *Salmonella* Enteritidis challenge strain after enrichment.** Group C was given sterile HBSS as a control on day 1. Group S was sprayed with 125 ml of a suspension containing ± $10^9$ CFU/ml *Salmonella* Enteritidis Δ*hilAssrAfliG* strain on day 1. Group D was given access to drinking water containing ± $10^9$ CFU/ml of a *Salmonella* Enteritidis Δ*hilAssrAfliG* strain for the first 24 h of the experiment. Twelve chickens in each group (1 out of 5) were challenged with $10^5$ CFU of a *Salmonella* Enteritidis challenge strain on day 2 of the experiment (seeder birds). Samples were taken at days 7, 21 and 42 of the experiment.

| Day | *Salmonella* Enteritidis Δ*hilAssrAfliG* strain | | | | *Salmonella* Enteritidis challenge strain | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Caecum | | Spleen | | Caecum | | | Spleen | | |
| | Group S | Group D | Group S | Group D | Group C | Group S | Group D | Group C | Group S | Group D |
| 7 | 16[a]/19[b] | 16/20 | 16/18 | 9/20 | 20/20 | 14/19 | 14/20 | 11/20 | 5/18 | 6/20 |
| 21 | 3/18 | 1/18 | 0/18 | 0/18 | 19/19 | 12/18 | 16/18 | 19/19* | 5/18* | 11/18 |
| 42 | 0/18 | 0/17 | 0/18 | 0/17 | 16/16* | 1/18* | 15/17 | 7/16 | 3/18 | 3/17 |

[a] Number of positive samples after enrichment

[b] Total number of samples

* Significant difference between groups (p-value < 0.05)

## Discussion

Recent research demonstrated that a *Salmonella* Enteritidis *hilAssrAfliG* deletion mutant is a CI strain that lowers colonization of a *Salmonella* Enteritidis wild-type strain after experimental infection. In addition, this deletion mutant could not be detected anymore at slaughter age [204]. Because the level of protection offered by live vaccine strains is

dependent on the administration route [133], two practically relevant administration methods for the *Salmonella* Enteritidis Δ*hilAssrAfliG* strain were investigated in this study. Only one *Salmonella* Enteritidis challenge strain was used in this study, as protection offered by colonization-inhibition is often similar for heterologous strains within the same serovar [169]. Neither of both administration methods investigated in this study offered protection against mortality caused by the *Salmonella* Enteritidis challenge strain, as there was no significant difference in mortality between the untreated group and treated groups. However, by adding the *Salmonella* Enteritidis Δ*hilAssrAfliG* strain to the drinking water, a significant reduction in colonization of the caecum could be obtained by slaughter age and shedding of the wild-type strain was reduced during the course of the experiment. An even more distinct reduction of caecal colonization was obtained when administering the strain by coarse spray and this also resulted in a significantly higher number of caeca negative for the wild-type strain. Additionally, shedding of the challenge strain ceased during the course of the experiment in the spray-treated group. This may be because spraying allows a more uniform and simultaneous distribution of the CI strain amongst the chickens, as spraying results in the formation of droplets on the birds which are taken up orally quickly after administration during preening [206, 207]. Recent research has however suggested the respiratory route as a viable route of entry for *Salmonella* in poultry [34]. Consequently, it is possible that the CI strain is also taken up through the respiratory route when spraying it. Additionally, spraying newly hatched chicks ensures better uptake of the CI strain quickly after hatching. In contrast, drinking water consumption may vary during the first days of life [208], which might result in a more variable uptake of the CI strain and consequently a more variable level of protection. However, both administration methods resulted in oral uptake of the strain and ultimately in the presence of the strain in the caeca of the chickens. This is

essential for colonization-inhibition, as the presence of the CI strain is required to inhibit growth of the wild-type strain [209, 210].

We conclude that the *Salmonella* Enteritidis Δ*hilAssrAfliG* strain should ideally be administered to newly hatched chicks using a coarse spray for the purpose of colonization-inhibition, as this resulted in the most profound reduction in caecal colonization and faecal shedding of a wild-type *Salmonella* Enteritidis strain in an early challenge model.

# Chapter 4: General Discussion

# 4 General discussion and conclusions

*Salmonella* contamination rates in poultry have declined drastically the past decade in the European Union thanks to the implication of member state control programs. One of the most important measures in the control programs of many member states was the obligatory vaccination of laying hens, which has reduced the incidence of *Salmonella* contaminated eggs and consequently the introduction of *Salmonella* in the food chain. Poultry meat is however often still contaminated with *Salmonella* [5], thus forming a possible source of *Salmonella* infections. Poultry meat contamination is often the consequence of an infection early in the chicken life, as young broiler chicks are highly susceptible to *Salmonella* infections [159-161]. Infection with *Salmonella* during this period can result in persistent infections which, despite currently implied control measures, can ultimately result in the contamination of poultry meat. In addition, it is difficult to establish protection early in the chicken life. Classical vaccination of one day old chicks does not induce production of significant amounts of specific antibodies against *Salmonella* until the animals are 10 days old [211-213]. Feed or drinking water additives have shown to decrease *Salmonella* colonization levels but not to eliminate the bacterium from the host [133]. CI, offering protection early in the chicken life, could thus be a potentially powerful tool for the poultry industry that can be used to decrease colonization levels and shedding of *Salmonella* in the environment.

## 4.1 Consequences of *hilA*, *ssrA* and *fliG* mutations in *Salmonella* Enteritidis

By introducing the *hilA*, *ssrA* and *fliG* mutations in a *Salmonella* Enteritidis strain we obtained a strain that combines several of the characteristics of the single mutants. Due to the mutation in *hilA*, the *Salmonella* Enteritidis *hilAssrAfliG* deletion mutant is a strain that is severely impaired in the invasion of epithelial cells, since *hilA* is a crucial regulator for SPI-1 and the genes associated with invasion [81]. As *hilA* is involved in the long-term shedding and colonization by *Salmonella* Enteritidis, deletion of this gene contributes strongly to the rapid clearing of the CI strain [81]. A possible explanation for this is that *Salmonella* bacteria multiply intracellular in the epithelial cells of the caeca, and are subsequently released into the lumen after death of the infected epithelial cells [81]. As the *hilA* deletion mutants are no longer able to invade the epithelial cells in a SPI-1 mediated way, they will not be able to proliferate in the GIT. The *hilA* strain is however not fully impaired for invasion, as other mechanisms are available for *Salmonella* to enter epithelial GIT cells [54, 60, 61, 63, 214]. This is confirmed by earlier observations, as the *Salmonella* Enteritidis Δ*hilA* strain can still be isolated from internal organs after oral inoculation, indicating that this strain is still able to invade epithelial cells, although to a significantly lower extent than its wild-type counterpart [81, 189, 190]. This will also be the case for the *Salmonella* Enteritidis Δ*hilAssrAfliG* strain, but as other mutations attenuate this strain further, its remaining virulence will be diminished as well.

Mutation of the *ssrA* gene in *Salmonella* Enteritidis results in a strain that is fully invasive in phagocytic and non-phagocytic cells, but fails to persist within chicken macrophages [86]. When introducing the *ssrA* mutation in a *Salmonella* Enteritidis Δ*hilA* strain, the resulting

strain is severely impaired in cellular invasion due to the *hilA* mutation, but also no longer able to persist within chicken macrophages due to the *ssrA* mutation [86]. Because of the *ssrA* mutation, these bacteria lack a functional SPI-2 T3SS, are therefore unable to translocate any effectors in the SCV and remain within an immature SCV that does not form membrane tubules and is defective at juxtanuclear positioning [215, 216]. In addition, these mutants show a replication defect in both macrophages and epithelial cells [215, 217, 218], severely reducing intestinal *Salmonella* propagation and systemic spread. However, recent evidence has shown that a proportion of intracellular *Salmonella* bacteria can escape from the SCV and replicate efficiently in the cytosol of epithelial cells, contributing to *Salmonella* propagation [215]. These cytosolic and vacuolar *Salmonella* populations are transcriptionally distinct as well, as the intravacuolar bacteria are SPI-2 induced while the cytosolic bacteria are SPI-1 induced and flagellated [215]. Epithelial cells containing hyper-replicating SPI-1 induced *Salmonella* bacteria undergo caspase-induced inflammatory cell death and are extruded from monolayers, a process in which the invasion-primed *Salmonella* are released in the extracellular milieu and are able to spread and infect additional cells [215, 218]. Due to the mutation in *hilA*, the *Salmonella* Enteritidis *hilAssrAfliG* deletion mutant's capability to invade epithelial cells is severely impaired and as a consequence, this process in which cytosolic *Salmonella* invade additional cells upon release is severely impaired as well. This could also, at least partially, explain the rapid elimination of the *Salmonella* Enteritidis Δ*hilAssrAfliG* strain from the host and contributes to the strongly reduced virulence compared to the wild-type parent strain.

In addition to the *hilA* and *ssrA* mutations, deletion of the *fliG* gene also contributes to the reduced virulence of the *Salmonella* Enteritidis triple deletion mutant, as invasion requires

not only the SPI-1 T3SS but flagellar-based motility as well [219, 220]. On the other hand, it has been shown that an aflagellar *Salmonella* Typhimurium *fliM* mutant exhibited an enhanced ability to establish systemic infection, and that it induced less IL-1β mRNA production and polymorphonuclear cell infiltration of the gut when compared to a wild-type strain during the initial stages of infection [221]. This enhanced ability to establish systemic infection was however only short-lived, and was probably related to an ability to evade early host recognition [222]. Flagellin is in fact an important and well-known TLR5 agonist and, as TLR5 is believed to play a key role in the initiation of inflammatory responses, the absence of flagella has an effect on the chicken immune response [221, 223]. The developed *Salmonella* Enteritidis Δ*hilAssrAfliG* strain does however not display an early, short-lived enhanced ability to establish systemic infection in broilers, presumably because the deletion of other important virulence genes renders the *Salmonella* Enteritidis Δ*hilAssrAfliG* strain severely attenuated. Deletion of the *fliG* gene thus results in a CI strain that no longer produces functional flagella and renders the strain immotile. This aspect of the CI strain has important consequences, as this allows for the distinction between vaccinated and infected animals when administering the strain to animals [165]. Firstly, because the strain is no longer motile, it can easily be differentiated from wild-type *Salmonella* Enteritidis strains using a motility test (such as Modified Semi-solid Rappaport-Vassiliadis (MSRV)). As such, it will not interfere with most *Salmonella* testing procedures, as these often comprise a motility test (ISO 6579:2002/Amd1:2007 Annex D). In addition, the deletion mutant strains can easily be differentiated from wild-type *Salmonella* strains using PCR, as the deleted genes can be used as targets to differentiate between both. Secondly, as the strain no longer produces functional flagellin, administering this strain to an animal would not induce an anti-flagellin immune response. A wild-type *Salmonella* strain on the other hand, expressing flagellar

antigens, does induce an anti-flagellin immune response after infection, which can be used to distinguish animals that are treated with the CI strain from those that are infected with a wild-type strain. It is even possible that the *Salmonella* Enteritidis Δ*hilAssrAfliG* strain does not induce an immune response at all, as it is cleared early in the chickens life and thus cleared before an effective immune response can be mounted. On the other hand, because it is possible that the *Salmonella* Enteritidis Δ*hilAssrAfliG* strain does not induce an immune response when administered to recently hatched chickens, it might not offer additional protection against *Salmonella* infection other than its colonization-inhibition effect. However, if this strain is administered to more mature animals with a more mature and fully functioning immune system (e.g. using a triple dose vaccination in layers), a protective immune response against *Salmonella* infections could possibly be induced by the CI strain. Combination of the *hilAssrAfliG* mutations in *Salmonella* Enteritidis results thus in a strongly attenuated strain that is strongly impaired for invasion and intracellular survival, yet maintains its CI potential.

Most research concerning the role of *Salmonella hilA* and *ssrA* genes in poultry infections haven been investigated for *Salmonella* Enteritidis [81, 86, 189, 190]. The results obtained in Chapter 3.2 have shown that introducing these mutations in a *Salmonella* Typhimurium strain does not result in an equally attenuated strain, as the *Salmonella* Typhimurium *hilAssrAfliG* deletion mutant is still shed at slaughter age and colonizes the GIT persistently, illustrating the importance of the genetic background in which the mutations are introduced.

## 4.2 Safety aspects for CI and live vaccine strains

The prolonged persistence of the *Salmonella* Typhimurium *hilAssrAfliG* deletion mutant strain, described in the third chapter (3.2), has consequences for possible practical application, as an important prerequisite for CI strains and live vaccine strains in general, is that these strains should be cleared by slaughter age to avoid the introduction of these strains in the food chain. While this is the case for the *Salmonella* Entertidis *hilAssrAfliG* deletion mutant, it is not for the *Salmonella* Typhimurium *hilAssrAfliG* deletion mutant strain. As the latter strain can still be detected by slaughter age and is still present at high levels in the caecum at 42 weeks of age, its use in broiler practice should be avoided as this could result in the introduction of this strain in the food chain. It might however be possible to administer this strain in a lower dose, which could result in an earlier clearance, or introduce additional mutations to reduce persistence and colonization rate even further. One could argue that, due to the deletions in *hilA* and *ssrA*, this strain should be safe for humans and introduction of this strain in the food chain does not imply a safety risk for human health. Additionally, the introduced mutations might reduce the ability of the *Salmonella* Typhimurium Δ*hilAssrAfliG* strain to survive on the boiler carcass after slaughter, meaning that it is possible that the strain would not be introduced in the food chain even if it is still present in the animal at slaughter age. This has however not been investigated yet and use of this CI strain in its current form in broiler practice might thus comprise a safety risk for human health. As such, public and government would not accept the use of this live *Salmonella* vaccine strain that could possibly become introduced in the food chain.

One major advantage of both the *Salmonella* Enteritidis *hilAssrAfliG* deletion mutant strain and the *Salmonella* Typhimurium *hilAssrAfliG* deletion mutant strain, and in general, live vaccines that are deletion mutants, is that these strains cannot revert back to their virulent wild-type parent strain. The live vaccines that are currently available to protect poultry against *Salmonella* were often developed by chemical mutagenesis or are metabolic drift mutants [150]. The latter are produced by isolating strains that have a longer generation time (thus producing smaller colonies) and a corresponding reduced virulence. These mutations are however unfixed, and there is a small chance of reversion to the original virulent wild-type. In addition, it is often not known where these mutations are situated in the genome or how attenuation is achieved. A *Salmonella* Gallinarum mutant (SG9R), which was originally used to protect poultry against fowl typhoid, offers protection against *Salmonella* Enteritidis as well and consequently, was sometimes used in practice to protect poultry against *Salmonella* Enteritidis infection. It is however strongly suspected that the SG9R strain has been the cause of fowl typhoid outbreaks in poultry, due to reversion of the strain to a virulent phenotype [167, 224]. Research has shown that a single nucleotide nonsense mutation of *rfaJ* can again confer virulence to the SG9R strain [224] and that different *Salmonella* Gallinarum field strain isolates are closely related to the SG9R vaccine strain, suggesting that the *Salmonella* Gallinarum field strain is a SG9R strain that regained its virulence [167]. Such mutations associated with reversion arise at a low frequency, yet may become quickly fixed in the population if there is a strong selection pressure for virulence and eventually lead to sporadic outbreaks of the virulent strain [167]. This risk of reversion can be outweighed by the benefits of decreasing the number of outbreaks in regions with a high infection pressure from field strains. However, the genetic tools to delete whole and multiple genes are readily available, and can be used to limit the chance of

reversion to virulence by developing well-defined genetically modified live vaccine strains. Despite that this allows for the development of safer strains, these well-defined deletion mutants might raise consumer concerns as they are genetically modified organisms.

In addition to the reduced chance of reverting back to their virulent phenotype, the developed CI strains also possess other advantages when compared to the live vaccines that are currently available for use in poultry practice. The CI strains developed in these studies are resistant to the antibiotic nalidixic acid, which facilitated their isolation and enumeration during the *in vivo* trials. It is however also possible to generate CI strains that share the same characteristics of the developed CI strains, yet are not resistant to any antibiotic. As there are concerns that antibiotic resistant live vaccine strains could contribute to the spread of antibiotic resistance markers in the microbial community, the absence of such antibiotic resistance markers in CI strains or live vaccine strains confers them another advantage over the currently available live vaccines (which often are resistant to one or more antibiotics). Finally, it is not unlikely that the currently commercially available live vaccine strains are able to induce a CI effect as well (see Chapter 1), and could thus also be used to protect broilers against *Salmonella* early in life. However, because most of these commercially available live vaccine strains were developed for use in layers, their safety and short-term efficacy when applied to young broilers is often unknown. The developed CI strains were, in contrast, designed for use in broilers and as such, were tested for both their safety and efficacy in broilers. As a consequence, applicable CI strains are thus more suited to protect broilers quickly post-hatch against early *Salmonella* challenge while being cleared by slaughter age.

## 4.3 Protection against a broad spectrum of *Salmonella* serotypes using CI

Earlier research showed that colonisation-inhibition is more pronounced between isogenic strains and that there is greater inhibition within a serovar than between serovars [133, 169, 183]. In accordance with these observations, we found that that the *Salmonella* Enteritidis and Typhimurium deletion mutant strains are able to inhibit strains belonging to their respective serovar, but were not able to inhibit a *Salmonella* Paratyphi B var. Java strain. This also explains why the *Salmonella* Enteritidis *hilAssrAfliG* deletion mutant strain and the *Salmonella* Typhimurium *hilAssrAfliG* deletion mutant strain do not inhibit each other (Chapter 3.2), and are able to still offer protection against both *Salmonella* Entertidis and Typhimurium after simultaneous administration. This also has practical implications, because this means that multiple serotype preparations can be produced that are protective against multiple *Salmonella* serovars. It has been suggested that a mixture of *Salmonella* CI strains belonging to several serovars could be able to inhibit a broad spectrum of virulent wild-type strains, as a mixture consisting of a wild-type *Salmonella* Enteritidis and *Salmonella* Typhimurium strain not only protects against their isogenic challenge strains, but against *Salmonella* Hadar and *Salmonella* Infantis challenge strains as well [169]. This suggests a synergistic protective effect when administering multiple CI strains simultaneously, yet this is not observed in our studies. The combined CI culture consisting of the *Salmonella* Enteritidis Δ*hilAssrAfliG* strain and *Salmonella* Typhimurium Δ*hilAssrAfliG* strain does not offer protection against infection with *Salmonella* Paratyphi B var. Java, suggesting a *Salmonella* Paratyphi B var. Java deletion mutant needs to be developed in order to obtain a CI strain protecting against this serotype.

Because methods have been developed to easily and quickly develop *Salmonella* deletion mutants [194], it is possible to quickly generate new mutants belonging to other serovars of importance for broiler production. Such an approach might however be more difficult than expected, as the introduced mutations might not always result in the same level of attenuation in the different *Salmonella* serovars. In Chapter 3.2, we observed a difference between the *Salmonella* Enteritidis *hilAssrAfliG* deletion mutant strain and the *Salmonella* Typhimurium *hilAssrAfliG* deletion mutant strain in persistence and colonization rate, while both strains were anticipated to be attenuated to the same extent as the same genes were deleted in both strains. This difference in persistence and colonization rate for the CI strains could be explained by the different genetic background in which the mutations are introduced, and might thus be dependent on the *Salmonella* serovar or even strains in which the mutations are introduced [169]. These observations underline the importance of the genetic background in which the deletions are introduced, and that, in order to obtain the same level of attenuation, additional or even different mutations might need to be introduced in different CI strains.

## 4.4 Practical relevance and compatibility with implemented *Salmonella* control measures

When considering the practical relevance of the developed CI strains, an important aspect for the *Salmonella* Enteritidis *hilAssrAfliG* deletion mutant strain is the in Chapter 3.3 suggested reduced environmental persistence. The deletion mutant strain could not be found again in the litter on which the treated birds were housed after the end of the experiment, which might indicate that this strain has reduced capabilities to survive and

persist in the environment. As a consequence, the risk of introducing a live CI strain in the environment would be avoided when using this strain in practice.

Our research has also shown that it is possible to administer a *Salmonella* Enteritidis Δh*ilAssrAfliG* strain early after hatching using a coarse spray, which results in an early and sufficiently long lasting protection against *Salmonella* Enteritidis challenge. In practice, it would thus be possible to administer the CI strain before leaving the hatchery, conferring the chicks protection against *Salmonella* Enteritidis by the time they arrive on the farm (whilst avoiding the use of a live *Salmonella* vaccine on the farm itself). As young chicks often become infected upon introduction on the farm due to environmental contamination [19-21, 23-28], *Salmonella* contamination could be further reduced if the chicks are protected before they are exposed to possible risk factors for *Salmonella* infection. Adding the *Salmonella* Enteritidis Δh*ilAssrAfliG* strain to the drinking water resulted only initially in a decrease in *Salmonella* Enteritidis shedding and colonization. In order to obtain a lasting protection, this could be combined with other control measures such as competitive exclusion treatment or by short chain fatty acid (SCFA) administration. This would however raise production cost and as drinking water is administered only after the chickens are already introduced on the farm, could imply exposure of the chickens to *Salmonella* before feeding and drinking water additives are administered. As application by coarse spray is an easy and cheap method offering early and lasting protection, administering CI strains in this manner is recommended.

The observed difference in efficacy between the spray and drinking water treatment can possibly be explained by differences in uptake of the CI preparation. When administering the

CI strain using a spray, all chicks take up the CI strain within a very short period of time, that is, during the treatment itself and during preening, when chickens preen themselves [206, 207]. As such, the CI strain can be administered quickly after hatching and there is time for the CI strain to colonize the gut before the chickens are exposed to *Salmonella* contamination on the farm. When administering the strain through the drinking water, it is possible that some chicks take up the CI strain later after hatching (as onset of thirst and thus drinking water uptake is variable) and there is less time for the CI strain to colonize the gut before chickens are infected, reducing the CI potential of the *Salmonella* Enteritidis Δ*hilAssrAfliG* strain. These birds will thus take up the CI strain, yet possibly too late to offer protection against the experimental *Salmonella* challenge.

The implementation of other control measures on the farm might hamper the possible use of CI as an additional control method. Treating the chickens with a competitive exclusion product before administering the CI strain will drastically reduce colonization by the CI strain and consequently its CI potential [191]. On the other hand, when administering the CI strain prior to or simultaneously with a competitive exclusion product, they work synergistically and strongly reduce *Salmonella* colonization [191, 225]. Antibiotics can strongly reduce viability and consequently efficacy of a CI strain, but these compounds are prohibited in the European Union for control of *Salmonella* in broiler practice. Similarly, SCFA's that are used as feed additives reduce *Salmonella* invasion [202], and are bacteriostatic or bactericidal for *Salmonella* in high concentrations. Administering these might lower CI strain colonization as well, and thus reduce CI potential of the strain. On the other hand, these control measures could contribute to faster clearing of CI strains that are not cleared by slaughter age under normal conditions, avoiding possible introduction of these CI strains in the food chain. In

addition, as the protective effect offered by CI is most important early after hatching and CI strains such as the *Salmonella* Enteritidis Δ*hilAssrAfliG* strain are cleared quickly after administration, it might be possible to effectively combine these. CI strains would then confer early protection against *Salmonella* challenge and after they are cleared or protection can be conferred by other means, administration of these other measures could commence.

Another major advantage for CI is that the protective effect offered is, at least initially, of much larger magnitude than most other control measures available for *Salmonella* control in broilers. Administration of a CI strain can reduce caecal *Salmonella* colonization with several log units or even almost fully prevent colonization, as observed in Chapter 3.2. Other control measures, such as SCFA's, MCFA's, altered feed composition or pre- and probiotics only reduce *Salmonella* colonization and shedding to a limited extent. As such, they can help contribute to *Salmonella* reduction in poultry, but CI might have a bigger impact on *Salmonella* prevalence if implemented in practice. Resent research has shown that bacteriophages can also be used to control *Salmonella* in poultry [139, 140, 226-230]. Not only can these lytic phages be used to reduce intestinal *Salmonella* levels, they can also be used to reduce *Salmonella* contamination on carcasses and eggs [226-230]. In addition, bacteriophages can cause a significant reduction in *Salmonella* levels, up to several log units or even eliminate *Salmonella* completely. As such, bacteriophages might represent a useful novel tool for *Salmonella* control in poultry and are a possible alternative for CI as a novel control measure. Competitive exclusion products offer a significant level of protection as well, reducing *Salmonella* colonization levels several log units and are thus a viable alternative to CI too [98, 135-137]. In addition, CE products can be sprayed on the chickens allowing for early and significant protection, much like CI [98, 109, 138]. The only drawback

for these products is that they might contain pathogenic bacteria, as they are undefined mixtures of bacteria [98]. Thoroughly testing and analyzing these products for the presence of pathogenic bacteria before application can however avoid this potential threat, allowing for a treatment which not only protects against *Salmonella* but other enteropathogenic bacteria as well. Other measures, such as bacteriocins could possibly be a viable alternative to the currently applied control measures as well [231-233]. Long-term studies on the effect of bacteriocins on *Salmonella* colonization in poultry have however not been performed yet, but research indicates that at least one bacteriocin is capable of reducing *Salmonella* colonization by several log units in the caecum of experimentally infected broilers [233]. As such, bacteriocins might represent another possible alternative to protect broilers against *Salmonella*.

Due to implemented control measures and control programs, *Salmonella* Enteritids and *Salmonella* Typhimurium prevalence in broilers has decreased considerably in Europe to the point that many member states have reached the target set forward by the European Union (< 1 % of broiler flocks positive for *Salmonella* Enteritidis and Typhimurium). Therefore, CI might be especially useful for countries where there are little control measures available or no control programs established and where *Salmonella* represents an even bigger threat for public health. In such countries, infection pressure on farms is often very high, which would reduce efficacy of some control measures. The used seeder-bird model shows however that CI can be used to protect broilers against *Salmonella* under a high infection pressure, as in the model a significant number of animals are inoculated with a high dose of *Salmonella*. Additionally, this seeder bird model mimics infections in the field, as the infection is allowed to spread within the flock after initial infection of a part of the birds, illustrating CI also can

be used under field conditions. In addition, all birds were treated with the CI strain prior to infection in the performed experiments, even the seeder birds, which resembles field conditions more closely if CI would be integrated in a control program.

Finally, because the *Salmonella* Enteritidis Δ*hilAssrAfliG* strain and *Salmonella* Typhimurium Δ*hilAssrAfliG* strain are GMO's, administration procedures preceding marketing authorization would be more comprehensive as compared to non-GMO strains. In the European Union, veterinary medicinal products containing GMO's are subject to both pharmaceutical and GMO regulations before authorization is granted [234]. Nevertheless, there are several veterinary medicinal products containing or consisting of GMO's available on the European market [234]. As such, the developed CI strains can be considered for use in Europe and other parts of the world as well, where administration procedures are often less difficult for live GMO vaccines.

## 4.5 Future prospects

The developed *Salmonella* Typhimurium Δ*hilAssrAfliG* strain cannot be used in practice due to safety concerns, as it is not cleared by slaughter age and its use would comprise a risk for food chain safety. It would however be possible to further attenuate this strain by introducing one or more mutations, resulting in a strain that is cleared by slaughter age. However, before additional genes are deleted, their role should be comprehensively studied in order to be able to generate a safe CI strain while maintaining its CI potential. Another possibility might be administering lower amounts of the strain, reducing intestinal colonization and expediting clearance.

Due to implemented control measures and control programs, *Salmonella* Enteritids and *Salmonella* Typhimurium prevalence in broilers has decreased in Europe for the past decade. Coinciding with this decrease, other serotypes such as *Salmonella* Paratyphi B var. Java have become increasingly important for broiler production and rearing. CI strains that protect against these increasingly more important serotypes could be developed as well, in order to be able to quickly respond to these possible treats for public health. This might however be more difficult than anticipated, as introducing the same mutations in different serovars not always results in the same level of attenuation. In addition, these CI strains should be tested thoroughly for safety and efficacy before they can be considered for use in practice.

An interesting, possible prospect is investigating whether the developed CI strains can be used to protect laying hens as well as broilers, and whether these strains are able to induce a protective immune response when they are administered later in the chickens life, when the animals are old enough to mount an effective immune response. Both aspects could be of practical importance for the vaccine industry, as this would broaden the market.

Another important aspect of CI that remains to be elucidated, is the underlying mechanism. Currently, it remains poorly understood, but it shows similarities to the inhibition effect observed when a strain is grown in a stationary-phase broth culture of another strain [210]. The effect can thus be reproduced by inoculating stationary-phase cultures of *Salmonella* with small numbers of another *Salmonella* strain, which results in impaired growth of this second strain. This approach was followed by Nógrády et al. [235], who used a transposon bank to produce a large number of mutants and screen these for their ability to inhibit

growth of a second strain. They found genes contributing to anaerobic fumarate uptake and generation, the anaerobic L-arginine utilization pathway and flagellin synthesis to play a role in the *in vitro* inhibition effect. However, although mutants in these genes were deficient in growth inhibition *in vitro*, they were still able to inhibit growth of a challenge strain *in vivo*. Rychlik et al. [236] used a similar approach and found other genes to play a role in the *in vitro* inhibition effect. In this study, mutations of genes involved in respiration, amino acid biosynthesis, chemotaxis and nutrient uptake and its regulation were shown to result in strains which were no longer able to inhibit growth of a challenge strain. The effect of these mutations was however not investigated *in vivo*. Earlier studies [210, 237, 238] made similar observations for genes playing a role in the energy metabolism and genes involved in quorum sensing. A possible explanation for these observations is that, when the mutant strain is not able to utilize a certain carbon source or a specific nutrient, it can be used for growth by a second strain, resulting in a growth advantage for the second strain [237]. The defect in growth inhibition of mutants in genes involved in motility can be explained in a similar manner, as mutations in these genes results in an impaired motility and thus chemotaxis, limiting nutrient uptake and thus growth, allowing for a second strain to outgrow the mutant strain. Mutations in genes involved in respiration or proton translocating enzymes result in impaired growth as well, as these mutants are unable to use certain nutrients or have an impaired electro-chemical gradient across the plasma membrane. In addition, these studies suggest that quorum sensing does not play a role in CI either. As such, it is difficult to claim with certainty that any of these genes play an actual role in CI *in vivo* and consequently, further research to elucidate the underlying mechanism is necessary. Currently, proposed possible mechanisms for CI *in vivo* include competition for

nutrients, competition for attachment sites in the host GIT and possibly stimulation of the immune system [133].

## 4.6 Conclusion

Colonization-inhibition represents a powerful, unexploited tool that can be used to protect broilers early after hatching against *Salmonella* challenge. Not only is this the period in which chicks are most vulnerable for *Salmonella* infection, there are also no other effective control measures to fully protect young chicks that early in their life. In addition, it is possible to confer protection against multiple *Salmonella* serovars in a single treatment, provided the CI strains conferring protection against these serotypes are available. The developed *Salmonella* Enteritidis *hilAssrAfliG* deletion mutant strain is a safe and efficient strain and can consequently be used in broiler practice to help reduce *Salmonella* Enteritidis prevalence. The *Salmonella* Typhimurium *hilAssrAfliG* deletion mutant strain on the other hand, confers protection against *Salmonella* Typhmurium challenge, yet cannot be used in practice in its current form as it is not cleared by slaughter age. Finally, we found that CI strains should be administered as soon as possible in the broiler's life, which can be done by spraying a solution containing the CI strain on newly hatched chickens.

# Chapter 5: Summary & Samenvatting

# 5 Summary & Samenvatting

## 5.1 Summary

*Salmonella* contamination rates in poultry have declined drastically the past decade in the European Union thanks to the implication of member state control programs. Despite this, consumption of contaminated poultry meat remains an important cause of food-borne *Salmonella* infections in humans. Young chicks often become infected with *Salmonella* early in life, as they are highly susceptible to *Salmonella* infections due to the absence of natural intestinal microbiota and an underdeveloped immune system. In addition, there are no control measures available that effectively protect the chicks early after hatching. Therefore, a novel control method that protect broilers from day-of-hatch until slaughter age against infection with *Salmonella* might help contribute to further reduce of *Salmonella* prevalence in poultry. Colonization-inhibition (CI), a concept in which a live *Salmonella* strain is orally administered to day-old chickens and protects against subsequent challenge, can potentially be used as a novel control method to achieve this goal.

Before a *Salmonella* strain can be considered for use as a CI strain, it needs to fulfill several criteria. Firstly, the CI strain should be safe, meaning that this CI strain should no longer be virulent, cannot revert to virulence, should be cleared by slaughter age and does not interfere with testing procedures. Secondly, the CI strain should be able to offer protection against *Salmonella* early in the broiler life and this protection should last until slaughter age. Earlier research has shown that a *Salmonella* Enteritidis strain with a deletion in the *hilA* gene (coding for a key regulator for the *Salmonella* Pathogenicity Island 1) is a CI strain that

offers good protection against *Salmonella* Enteritidis challenge, but is still being shed and carried by slaughter age. Similarly, a *Salmonella* Enteritidis with a deletion in the *ssrA* gene (coding for a key regulator for the *Salmonella* Pathogenicity Island 2) offers protection against challenge, but is not cleared by slaughter age. Combination of these mutations might however result in a CI strain that fulfills both criteria of safety and efficacy. Finally, live vaccine strains lacking flagellin can easily be differentiated from wild-type strains and allow for serological differentiation between treated and infected animals. Introduction of such a mutation could thus confer several practical advantages to a putative CI strain.

The aim of this work was the development of CI strains that are safe for use in broiler practice and offer protection against *Salmonella* infection early in the chicken life. To do so, *hilA*, *ssrA* and *fliG* genes were deleted in a *Salmonella* Enteritidis and a *Salmonella* Typhimurium strain, after which safety and efficacy for both the resulting CI strains was investigated. In addition, we wanted to investigate whether simultaneous administration of these CI strains could offer protection against multiple *Salmonella* serovars.

In chapter one of this work, we investigated whether a *Salmonella* Enteritidis *hilAssrAfliG* deletion mutant strain is safe and offers recently hatched chicks protection against *Salmonella* Enteritidis until slaughter age. To investigate safety, we orally administered the *Salmonella* Enteritidis Δ*hilAssrAfliG* strain to a group of one-day-old broiler chicks and monitored faecal shedding and both spleen and caecal colonization by the strain until slaughter age. In addition, *hilA*, *ssrA* and *fliG* single deletion mutants and a *hilAssrA* deletion mutant were administered to other groups as well, allowing for a comparison of the different *Salmonella* Enteritidis deletion mutant strains. Shedding of the *Salmonella*

Enteritidis *hilAssrAfliG* deletion mutant was lower in comparison to the other deletion mutants, and ceased earlier; this strain was no longer shed after 21 days. Similarly, colonization of caecum and spleen was lower for the *Salmonella* Enteritidis *hilAssrAfliG* deletion mutant, and decreased rapidly after administration. It could no longer be detected after 28 days (well before slaughter age), indicating that the *Salmonella* Enteritidis *hilAssrAfliG* deletion mutant is a safe CI strain. To investigate whether the deletion mutant strain offers protection against *Salmonella* infection, we administered the *Salmonella* Enteritidis Δ*hilAssrAfliG* strain orally to one-day-old chicks and experimentally infected them with a wild-type *Salmonella* Enteritidis strain 24 h later in a seeder-bird model. Administration of the CI strain resulted in decreased faecal shedding of the wild-type *Salmonella* Enteritids strain and a significantly lower caecal colonization. This data indicates that a *Salmonella* Enteritidis Δ*hilAssrAfliG* strain is safe and can be used to protect chickens against *Salmonella* Entertidis infection.

In chapter two of this work, we investigated whether a *Salmonella* Typhimurium *hilAssrAfliG* deletion mutant strain is safe and offers recently hatched chicks protection against *Salmonella* Typhimurium until slaughter age. In addition, we investigated whether simultaneous administration of a *Salmonella* Enteritidis *hilAssrAfliG* deletion mutant strain and a *Salmonella* Typhimurium *hilAssrAfliG* deletion mutant strain offers protection against both *Salmonella* Enteritidis and *Salmonella* Typhimurium challenge. To investigate safety and efficacy, we administered the *Salmonella* Typhimurium *hilAssrAfliG* deletion mutant strain orally to one-day-old chickens, after which the chickens were experimentally infected with a *Salmonella* Typhimurium wild-type strain. Faecal shedding and spleen and caecal colonization of both CI and challenge strain were monitored until slaughter age. Shedding of

the wild-type strain decreased rapidly when chicks were treated with the CI strain, and it was no longer shed after 16 days. An even stronger protection was observed in caecum and spleen, as treatment with the CI strain prevented the challenge strain from colonizing these. However, the *Salmonella* Typhimurium *hilAssrAfliG* deletion mutant strain was still being shed and present in the caecum by slaughter age. To evaluate whether simultaneous administration of two CI strains offers protection against multiple *Salmonella* serotypes, we administered the *Salmonella* Enteritidis Δ*hilAssrAfliG* strain and the *Salmonella* Typhimurium Δ*hilAssrAfliG* strain simultaneous to one-day-old broiler chicks. One day later they were challenged with a *Salmonella* Enteritidis, Typhimurium or Paratypi B varietas Java strain. Treatment with both CI strains reduced caecal colonization of the *Salmonella* Enteritidis and Typhimurium challenge strains, but not of the *Salmonella* Paratypi B var. Java strain. This data indicates that the *Salmonella* Typhimurium *hilAssrAfliG* deletion mutant strain is a CI strain that offers pronounced protection against *Salmonella* Typhimurium infection. This strain is however not cleared by slaughter age, and can thus not be used in practice. Additionally, this data shows that simultaneous administration of the *Salmonella* Enteritidis Δ*hilAssrAfliG* strain and *Salmonella* Typhimurium Δ*hilAssrAfliG* strain can offer early protection against both *Salmonella* Enteritidis and Typhimurium infection.

In chapter three of this work, we investigated two practical application methods, drinking water and spray administration, that allow for early and uniform application of the *Salmonella* Enteritidis Δ*hilAssrAfliG* strain. To do so, we administered the strain to one-day-old chickens by coarse spray or in the drinking water for 24 h, after which the chicks were experimentally challenged with a wild-type *Salmonella* Enteritidis strain. Faecal shedding and both spleen and caecal colonization of both CI and challenge strain were monitored until

slaughter age. Administration of the strain using a coarse spray resulted in a strongly decreased feacal shedding of the challenge strain, while drinking water administration of the CI strain only reduced shedding of the challenge strain initially. Spray administration also reduced caecal colonization by the challenge strain significantly for the entire duration of the experiment, while this was not always the case for drinking water application. This data shows that CI strains should be administered early in the chicks life using a coarse spray, as this confers best protection against *Salmonella* challenge.

In conclusion, colonization-inhibition represents an unexploited, potentially powerful tool that can be used to protect broilers early after hatching against *Salmonella* challenge. In addition, it is possible to confer protection against multiple *Salmonella* serovars in a single treatment, provided the CI strains conferring protection against these serotypes are available. The developed *Salmonella* Enteritidis *hilAssrAfliG* deletion mutant strain is a safe and efficient strain and could consequently be used in broiler practice to help reduce *Salmonella* Enteritidis prevalence. The *Salmonella* Typhimurium *hilAssrAfliG* deletion mutant strain on the other hand, confers protection against *Salmonella* Typhmurium challenge, yet cannot be used in practice in its current form as it is not cleared by slaughter age. Finally, CI strains should be administered as soon as possible in the broiler's life, which can be done by spraying a solution containing the CI strain on newly hatched chickens.

## 5.2 Samenvatting

Het aantal *Salmonella* besmettingen van pluimvee is het laatste decennium drastisch gedaald in verschillende lidstaten van de Europese Unie door het invoeren van controleprogramma's. Desondanks blijft de consumptie van besmet pluimveevlees een belangrijke oorzaak van door voedsel overgedragen *Salmonella* besmettingen bij de mens. Vaak worden jonge dieren besmet met *Salmonella* op jonge leeftijd en zijn ze op slachtleeftijd nog steeds geïnfecteerd, wat tot contaminatie van het vlees kan leiden. Jonge kippen zijn namelijk uitermate gevoelig voor *Salmonella* infecties doordat hun darmflora en immuunsysteem onvoldoende ontwikkeld zijn. Bovendien zijn er geen efficiënte controlemaatregelen voorhanden die kippen op dat moment kunnen beschermen tegen *Salmonella*. Een nieuwe controlemaatregel die vleeskippen beschermt vanaf het moment van uitkippen tot op slachtleeftijd zou echter kunnen bijdragen tot een verdere reductie van het aantal *Salmonella* besmettingen in pluimvee. Kolonisatie-inhibitie, een concept waarbij een levende *Salmonella* stam wordt toegediend aan eendagskuikens en hen vervolgens beschermt tegen verdere *Salmonella* besmettingen, zou hiervoor gebruikt kunnen worden.

Alvorens een *Salmonella* stam kan gebruikt worden als CI stam dient deze aan verschillende voorwaarden te voldoen. Eerst en vooral dient deze stam veilig te zijn, wat inhoudt dat de stam niet langer virulent mag zijn, niet langer aanwezig mag zijn in de kip op slachtleeftijd, niet kan reverteren naar het oorspronkelijke virulente fenotype en niet interfereert met testprocedures voor *Salmonella*. Verder dient deze stam ook vroeg in het leven van de kip bescherming te bieden tegen *Salmonella* besmetting. Eerder onderzoek heeft aangetoond dat een *Salmonella* Enteritidis waarvan het *hilA* gen (een centrale regulator voor *Salmonella*

Pathogeniciteitseiland 1) gedeleteerd is goede bescherming biedt tegen *Salmonella* Enteritidis infectie, maar wordt nog steeds uitgescheiden en is nog steeds aanwezig op slachtleeftijd. Ook een *Salmonella* Enteritidis stam waarvan het *ssrA* gen (een centrale regulator voor *Salmonella* Pathogeniciteitseiland 2) gedeleteerd is biedt bescherming tegen *Salmonella* Enteritidis infectie, maar is ook nog steeds aanwezig op slachtleeftijd. Aanbrengen van beide mutaties in dezelfde stam zou echter kunnen resulteren in een CI stam die aan de criteria van veiligheid en bescherming voldoet. Tenslotte kunnen levende vaccins die geen flagellen hebben makkelijk onderscheiden worden van wild-type stammen en staan deze toe behandelde dieren en geïnfecteerde dieren serologisch van elkaar te onderscheiden. Aanbrengen van een mutatie die flagelline productie onmogelijk maakt zou potentiële CI stammen dus nog additionele praktische voordelen kunnen opleveren.

Het doel van dit werk was de ontwikkeling van veilige CI stammen die vleeskippen reeds kort na uitkippen beschermen tegen *Salmonella* infectie. Om dit te doen werden het *hilA*, *ssrA* en *fliG* gen gedeleteerd in een *Salmonella* Enteritidis en Typhimurium stam, waarna veiligheid van en bescherming geboden door de stammen werd onderzocht. Verder werd ook nagegaan of de gelijktijdige toediening van deze CI stammen bescherming biedt tegen verschillende *Salmonella* serotypes.

In hoofdstuk één van dit werk werd onderzocht of een *Salmonella* Enteritidis *hilAssrAfliG* deletie mutant veilig is en of deze kuikens vanaf uitkippen tot op slachtleeftijd kan beschermen tegen *Salmonella* infectie. Om de veiligheid van deze stam te onderzoeken werd deze toegediend aan één dag oude vleeskuikens, waarna caecum en milt kolonisatie en fecale uitscheiding van de stam werden opgevolgd tot op slachtleeftijd. Ook werden andere

groepen geïnoculeerd met een *hilA*, *ssrA*, *fliG*, *hilAssrA* deletiemutant of de oorspronkelijke wild-type stam, waardoor de veiligheid van deze deletiemutanten kon vergeleken worden. De *Salmonella* Enteritidis *hilAssrAfliG* deletie mutant werd steeds in mindere mate uitgescheiden dan de andere deletiemutanten en de wild-type stam, en hield ook vroeger op; na 21 dagen werd deze niet langer uitgescheiden. Verder was ook de kolonisatie van caecum en milt door de *Salmonella* Enteritidis *hilAssrAfliG* deletie mutant lager, en daalde dit snel na toediening. Deze stam kon niet langer teruggevonden worden na 28 dagen (ruim voor slachtleeftijd), wat aangeeft dat de *Salmonella* Enteritidis *hilAssrAfliG* deletie mutant een veilige CI stam is. Om na te gaan of deze stam ook bescherming biedt tegen *Salmonella* Enteritidis infectie werd de *Salmonella* Enteritidis Δ*hilAssrAfliG* stam oraal toegediend aan eendagskuikens, waarna deze 24 u later experimenteel geïnfecteerd werden met een wild-type *Salmonella* Enteritidis stam. Dit resulteerde in een verminderde fecale uitscheiding en in een verminderde caecale kolonisatie van de wild-type stam. Deze data geeft aan dat de *Salmonella* Enteritidis Δ*hilAssrAfliG* stam een veilige CI stam is en dat deze gebruikt kan worden om vleeskippen te beschermen tegen *Salmonella* Enteritidis infectie.

In hoofdstuk twee van dit werk werd onderzocht of een *Salmonella* Typhimurium *hilAssrAfliG* deletie mutant ook een veilige stam is en of deze stam kippen kan beschermen tegen *Salmonella* Typhimurium infectie vanaf uitkippen. Verder werd onderzocht of gelijktijdige toediening van een *Salmonella* Enteritidis Δ*hilAssrAfliG* stam en een *Salmonella* Typhimurium Δ*hilAssrAfliG* stam bescherming biedt tegen zowel *Salmonella* Enteritidis als Typhimurium infectie. Om de veiligheid en efficiëntie van de *Salmonella* Typhimurium Δ*hilAssrAfliG* stam na te gaan werd de stam oraal toegediend aan eendagskuikens, waarna de dieren al dan niet experimenteel geïnfecteerd werden met een wild-type *Salmonella*

Typhimurium stam. Uitscheiding van zowel de CI stam als de wild-type stam werden vanaf toediening tot op slachtleeftijd opgevolgd, alsook kolonisatie van caecum en milt. Behandeling met de CI stam reduceerde uitscheiding van de wild-type stam totdat deze niet langer werd uitgescheiden na 16 dagen. Een nog meer uitgesproken bescherming werd waargenomen in caecum en milt, waar de CI stam kolonisatie door de wild-type stam volledig verhinderde. De *Salmonella* Typhimurium *hilAssrAfliG* deletie mutant werd echter nog steeds uitgescheiden en was nog steeds aanwezig in het caecum op slachtleeftijd. Om na te gaan of gelijktijdige toediening van twee CI stammen bescherming biedt tegen meerdere *Salmonella* serotypes, werden de *Salmonella* Enteritidis Δ*hilAssrAfliG* stam en de *Salmonella* Typhimurium Δ*hilAssrAfliG* stam gelijktijdig oraal toegediend aan eendagskuikens. Eén dag later werden de dieren experimenteel geïnfecteerd met een wild-type *Salmonella* Enteritidis, Typhimurium of Paratyphi B varietas Java stam. Behandeling van de kippen met de CI stammen resulteerde in een gereduceerde caecale kolonisatie door de *Salmonella* Enteritidis en Typhimurium stammen, maar had geen effect op *Salmonella* Paratypi B var. Java kolonisatie. Deze data geeft aan dat de *Salmonella* Typhimurium *hilAssrAfliG* deletie mutant een CI stam is die goede bescherming biedt tegen *Salmonella* Typhimurium infectie, maar persisteert tot op slachtleeftijd en dus niet gebruikt kan worden in de praktijk. Verder bleek ook dat gelijktijdige toediening van een *Salmonella* Enteritidis Δ*hilAssrAfliG* stam en *Salmonella* Typhimurium Δ*hilAssrAfliG* stam bescherming kan bieden tegen zowel *Salmonella* Enteritidis als Typhimurium infectie.

In het derde hoofdstuk van dit werk werden twee praktisch relevante toedieningswijzen voor de *Salmonella* Enteritidis Δ*hilAssrAfliG* stam onderzocht en vergeleken, namelijk drinkwater en spray administratie. Hiervoor werd de *Salmonella* Enteritidis Δ*hilAssrAfliG*

stam toegediend aan eendagskuikens in het drinkwater gedurende 24 u of door de stam te sprayen op de kuikens, waarna ze experimenteel geïnfecteerd werden met een wild-type *Salmonella* Enteritidis stam. Uitscheiding van zowel de CI stam als de wild-type stam werden, net als kolonisatie van caecum en milt, opgevolgd vanaf toediening tot op slachtleeftijd. Toediening van de CI stam met behulp van een spray resulteerde in een sterke daling van fecale uitscheiding van de wild-type stam, terwijl uitscheiding van de wild-type stam enkel initieel daalde na toediening van de CI stam in het drinkwater. Administratie van de CI stam met behulp van een spray resulteerde ook in sterk gereduceerde caecale kolonisatie door de wild-type stam, terwijl dit niet steeds het geval was na drinkwater toediening. Deze data geeft aan dat CI stammen idealiter vroeg in het leven van de kippen toegediend worden met behulp van een spray, aangezien dit resulteert in betere bescherming tegen *Salmonella* infectie.

Kolonisatie-inhibitie is een momenteel onbenutte, doch efficiënte manier om kippen reeds vroeg in het leven te beschermen tegen *Salmonella* infectie. Ook kan kolonisatie-inhibitie bescherming bieden tegen verschillende *Salmonella* serotypes in één behandeling, op voorwaarde dat de CI stammen voorhanden zijn. Deze CI stammen dienen zo vroeg mogelijk in het leven aan vleeskippen toegediend te worden, wat best kan gebeuren met behulp van een spray. De ontwikkelde *Salmonella* Enteritidis *hilAssrAfliG* deletie mutant is een veilige CI stam die vroeg in het leven van vleeskippen bescherming kan bieden tegen *Salmonella* Enteritidis infectie en zou bijgevolg in de praktijk gebruikt kunnen worden om *Salmonella* prevalentie in pluimvee verder terug te dringen. De *Salmonella* Typhimurium *hilAssrAfliG* deletie mutant biedt uitgesproken bescherming tegen *Salmonella* Typhimurium infectie,

maar is nog steeds aanwezig op slachtleeftijd en kan als dusdanig niet gebruikt worden in de praktijk aangezien deze persisteert tot op slachtleeftijd.

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# Curriculum Vitae & Bibliography

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## Curriculum Vitae

Wolfgang De Cort werd op 13 oktober 1987 geboren te Sint-Niklaas. Na zijn middelbare studies Moderne Talen-Wetenschappen aan de Broederschool te Sint-Niklaas, startte hij in 2005 met de opleiding 'Bachelor in de Biochemie en Biotechnologie' aan de Universiteit Gent. Hierop aansluitend begon hij in 2008 aan de opleiding 'Master in de Biochemie en Biotechnologie, afstudeerrichting Microbiële Biotechnologie' en behaalde zijn diploma hiervoor in 2010. Na drie maanden gewerkt te hebben bij Alcon-Couvreur te Puurs, begon hij datzelfde jaar aan de opleiding 'Master na Master in de Milieusanering en Milieubeheer' aan Universiteit Gent en rondde deze opleiding later dat jaar met succes af.

In juli 2011 startte hij zijn doctoraatsonderzoek getiteld 'Bescherming van vleeskippen tegen verschillende *Salmonella* serotypes met behulp van kolonisatie-inhibitie' aan de vakgroep Pathologie, Bacteriologie en Pluimveeziekten met prof. dr. ir. Filip Van Immerseel en prof. dr. Richard Ducatelle als promotoren. Dit project werd gedeeltelijk gefinancierd door de Federale Overheidsdienst Volksgezondheid, Veiligheid van de Voedselketen en Leefmilieu (FOD VVVL).

Sinds 15 januari 2015 is hij tewerkgesteld op een nieuw project aan de vakgroep Pathologie, Bacteriologie en Pluimveeziekten getiteld 'Development of *Salmonella* live attenuated vaccine strains for various serotypes in layers and broilers'. Dit project heeft prof. dr. ir. Filip

Van Immerseel en prof. dr. Richard Ducatelle als promotoren en wordt gefinancierd door het Industrial Research Fund (IOF) van de Universiteit Gent.

Wolfgang De Cort is auteur van verschillende wetenschappelijke publicaties in internationale tijdschriften en was spreker op verschillende nationale en internationale congressen.

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### Scientific publications

**De Cort W.**, Geeraerts S., Balan V., Elroy M., Haesebrouck F., Ducatelle R. and Van Immerseel F. (2013) A *Salmonella* Enteritidis *hilAssrAfliG* deletion mutant is a safe live vaccine strain that confers protection against colonization by *Salmonella* Enteritidis in broilers. *Vaccine*. 31:5104-10.

**De Cort W.**, Mot D., Haesebrouck F., Ducatelle R., Van Immerseel F. (2014) A colonisation-inhibition culture consisting of *Salmonella* Enteritidis and Typhimurium Δ*hilAssrAfliG* strains protects against infection by strains of both serotypes in broilers. *Vaccine*. 32:4633-8.

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### Oral presentations at (inter)national conferences

**De Cort W.**, Haesebrouck F., Ducatelle R., Van Immerseel F. (2013) A *Salmonella* Enteritidis Δ*hilAssrAfliG* colonization inhibition strain - A safe live vaccine strain conferring good protection against colonization by *Salmonella* in broilers until slaughter age. World Veterinary Poultry Association (WVPA) Congress. August 19-23 2013. Nantes, France.

**De Cort W.**, Haesebrouck F., Ducatelle R., Van Immerseel F. (2014) Colonization-inhibition: an unexploited tool to protect broilers against *Salmonella*. XIVth European Poultry Conference. June 23-27 2014. Stavanger, Norway.

**De Cort W.**, Haesebrouck F., Ducatelle R., Van Immerseel F. (2014) Kolonisatie-inhibitie: een nieuwe manier om vleeskippen te beschermen tegen *Salmonella*. Studiemiddag World Poultry Science Association (WPSA) Belgium: Verslag en nieuws van het Europees pluimveecongres 2014. 14 oktober 2014. Geel, België.

# Dankwoord

## Dankwoord

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