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Flagellin and multidrug resistance mutants as future *Salmonella* vaccines for laying hens

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Abbreviation list

ABBREVIATION LIST

ATP	Adenosine TriPhosphate
BGA	Brilliant Green Agar
BPW	Buffered peptone water
cAMP	cyclic adenosine monophosphate
Cfu	Colony forming units
CI	Colonization Inhibition
CpG	Cytosine Guanine dinucleotide
CRP	cAMP Receptor Protein
EU	European Union
GMMA	Generalized Modules for Membrane Antigens
HBSS	Hanks Balanced Salt Solution
IFN	Interferon
IL	Interleukin
IVET	In Vivo Expression Technology
LB	Luria Broth
LPS	Lipopolysacharride
MAMP	Microorganism Associated Molecular Pattern
MDR	Multi Drug Resistance
MOI	Multiplicity Of Infection
MS	Member States

OEC	Oviduct Epithelial Cells
OMP	Outer membrane proteins
PAMP	Pathogen Associated Molecular Pattern
PMN	Polymorphonuclear Neutrophil
RND	Resistance Nodulation Division
SCV	Salmonella Containing Vacuole
SEM	Standard Error of the Mean
SPI	Salmonella Pathogenicity Island
TETRA	Tetrathionate
TLR	Toll Like Receptor
TMB	Tetramethylbenzidine
T3SS	Type 3 Secretion System
Th	T-helper

1 General introduction

General introduction

1.1 Salmonella: a diverse genus

This introduction does not include all aspects of *Salmonella* bacteriology, epidemiology and virulence. Only aspects that are of importance for this work will be mentioned. *Salmonellae* are prominent members of the family Enterobacteriaceae. They are gram-negative, non-sporogenic, facultative anaerobic, peritrichously flagellated (with a few exceptions) rods that produce gas from glucose and utilize citrate as their sole carbon source. *Salmonellae* generally produce hydrogen sulfide gas, decarboxylate lysine and ornithine, but are urease-negative and do not produce indole (Ruan, 2013). The genus *Salmonella* consists of only 2 species: *Salmonella enterica* and *Salmonella bongori*, based on DNA hybridization studies (Euzeby, 1999). The *Salmonella enterica* species is further subdivided in 6 subspecies (*enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae* and *indica*). Subspecies *enterica* contains the majority of human pathogenic *Salmonella*, whereas the other subspecies are mainly associated with cold-blooded vertebrates. Subspecies *enterica* is divided in approximately 2500 serotypes based on flagellar (H), capsular (Vi) and somatic (O) antigens. Serotypes can be further divided based on their susceptibility to antimicrobials and phages. Serotypes can also be divided in biovars. *Salmonella enterica* serotype Gallinarum is divided into biovars Gallinarum and Pullorum. These biovars cause distinct diseases, with biovar Gallinarum eliciting fowl-typhoid and Pullorum being a dysentery agent (Shivaprasad, 2000).

These 2500 different serovars can also be divided into typhoidal and non-typhoidal *Salmonella.* Despite their genetic similarity, these two groups elicit very different diseases and distinct immune responses in humans. Typhoidal salmonellosis is caused by *Salmonella enterica* serotype Typhi. It is restricted to humans, causing 13.5 million annual episodes of typhoid fever, especially in low-and middle-income countries (Ceyssens et al, 2015). It is not necessarily food borne. Other serotypes belonging to this group include serotypes Sendai and Paratyphi A B or C.

Non-typhoidal salmonellosis, results in gastroenteritis and is caused by ingestion of a variety of serotypes. These serotypes differ greatly in their natural reservoirs, their ability to provoke infections, and their resistance to antimicrobials (Parry and Threlfall, 2008). Non-typhoidal *Salmonella* infections in humans have an incubation period of 12-72 hours, and illness duration is typically 4-7days. Fecal excretion usually persists for days or weeks after recovery from illness. Life-threatening invasive infections may occur in vulnerable patients. Antibiotic treatment does not reduce symptom duration, can prolong shedding, and is usually not indicated except in case of complicating extra-intestinal infections (Guerrant et al., 2001). Human salmonellosis caused by *Salmonella* Enteritidis is linked to contaminated eggs and egg products.

1.2 Population dynamics of *Salmonella enterica* serotypes: shift, trends and prevalence

1.2.1 Salmonellosis in the human population in Belgium

The Belgian National Reference Centre for *Salmonella* received 16 544 human isolates of *Salmonella enterica* between January 2009 and December 2013. A schematic overview is presented in figure 1. A total of 377 different serotypes were identified, but the landscape is dominated by *Salmonella enterica* serovars Typhimurium (55%) and Enteritidis (19%) in a ratio inverse to European Union averages (Ceyssens et al., 2015). An explanation for this discrepancy can be found in the national vaccination program in layer flocks at the beginning of the millennium, causing a drastic reduction in *Salmonella* Enteritidis (Collard et al., 2008). Other non-typhoid *Salmonella* serotypes are far less commonly encountered and account for a maximum of 2.1% of all isolates. (Johnson et al., 2011).

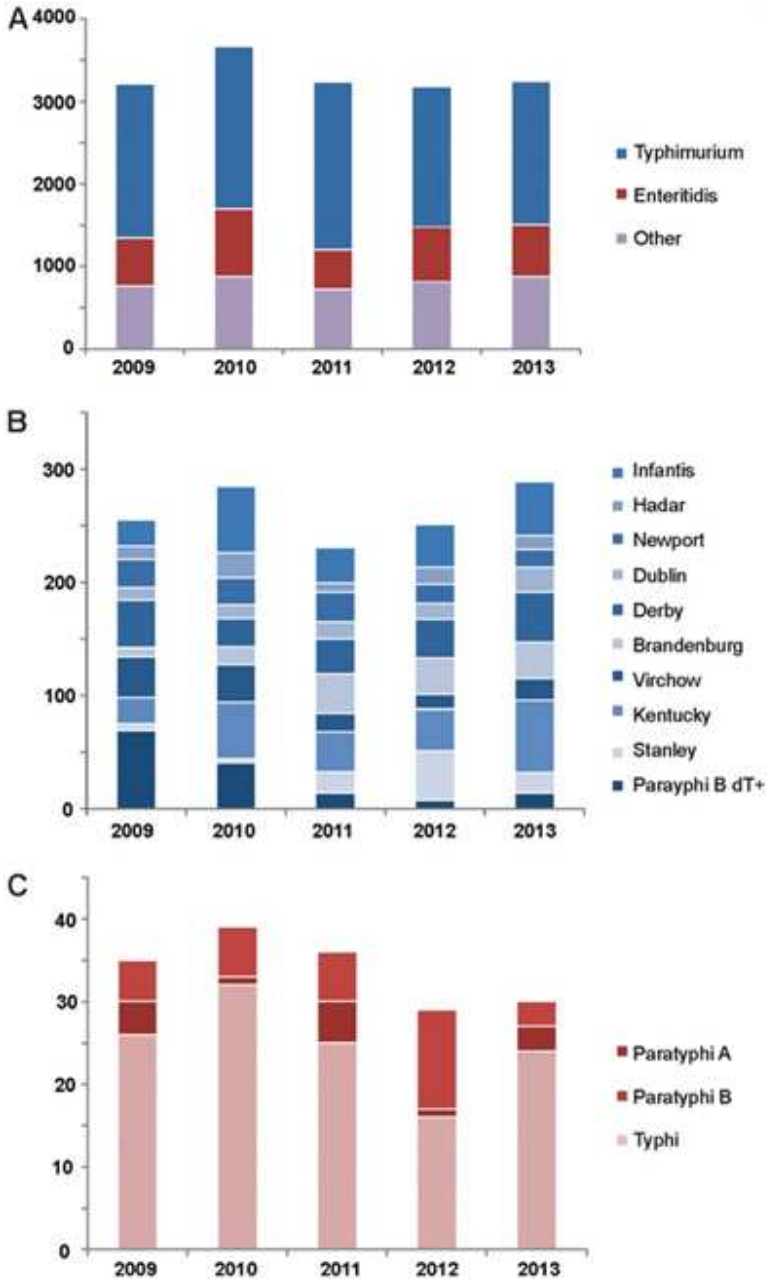

Figure 1: Epidemiology of human salmonellosis in Belgium from 2009 to 2013. The number of annually submitted strains remained fairly constant, varying between 3,182 and 3,668 isolates. In this vast collection, 377 different serotypes were identified. Results are shown grouped by the total number of isolates (with the contribution of the two major *Salmonella* serotypes indicated) (A), prevalence of 10 important non-typhoid, non-Enteritidis and non-Typhimurium *Salmonella* serotypes (B), and three typhoid *Salmonella* serotypes (C) (Ceyssens et al., 2015).

While the serotype landscape remained largely stable for the past 5 years, apart from specific outbreaks, serotype-dependent trends of antibiotic resistance are emerging. A particular threat for public health are circulating clonal lineages of cephalosporine- and fluoroquinolone-resistant *Salmonella* Infantis and *Salmonella* Kentucky strains, respectively, and intermediate fluoroquinolone- resistant *Salmonella* Paratyphi A and B isolates (Ceyssens et al., 2015).

1.2.2 Salmonellosis in humans and poultry in the EU

From 2003

on, the reporting of investigated food-borne outbreaks has been mandatory for European Union (EU) member states (MS). Since 2005, campylobacteriosis has been the most commonly reported zoonosis with an increase in confirmed human cases in the EU since 2008. Salmonellosis remains the second most common zoonosis in humans in the EU with 88 715 confirmed cases and 1 049 food-borne outbreaks reported in 2014 (EFSA, 2015). As in previous years, the two most commonly reported *Salmonella* serovars in 2014 were *Salmonella* Enteritidis and *Salmonella* Typhimurium, representing 44.4% and 17.4% respectively, of all reported serovars in confirmed human cases (table 1). An increase in the absolute number of *Salmonella* Typhimurium (typically attributed to the pig and cattle reservoirs) cases is also observed. This is partly related to the emergence of monophasic variants (Messens et al., 2013). Since 2014 this serotype is the third most important serotype and often carries multi drug resistance. Whether currently used vaccines offer protection against this serotype was not yet been investigated at the start of these PhD studies.

Since 2008, a mandatory *Salmonella* control program is to be implemented in laying hen flocks in the EU, with specific targets set for the different member states depending on the level of contamination of their laying hen flocks. Most MS met their *Salmonella* reduction targets for poultry (flockprevalence <2% for layers, <1% for broilers and breeders) but isolates of *Salmonella* Infantis increased at EU level (EFSA, 2015). Indeed, the most commonly reported serovar was *Salmonella* Infantis, accounting for 38.3% of all 5 377 reported isolates, followed by *Salmonella* Mbandaka (12.1%) and *Salmonella* Enteritidis (11.9%). Although *Salmonella* Infantis

is the most common detected serovar in Gallus gallus species, it only accounts for 2.5% of human salmonellosis cases in the EU (table 1). Nevertheless, the steady increase in *Salmonella* Infantis reports over the past few years is a matter of concern (EFSA, 2015).

The distribution of serovars in the poultry production listed in the EU summary report on zoonoses, zoonotic agents and food-borne outbreaks in 2014 mainly concerns Gallus gallus, including breeding hens, layers and broilers. Between 2013 and 2014, the total number of *Salmonella* isolates from laying hens went down from 758 to 598, which is a reduction of 21.1%. Although the absolute number of *Salmonella* Enteritidis isolates reduced, the proportion of *Salmonella* isolates from laying hens being typed as *Salmonella* Enteritidis has actually increased from 37.2% to 43%, and this serovar is recognized as being the only one of major significance in terms of contamination of eggs, because of its special ability to invade, survive and multiply within intact eggs.

The proportion of *Salmonella* isolates from G. gallus being typed as *Salmonella* Typhimurium was 3.9%, while it was 10.4% from laying hens. *Salmonella* Typhimurium therefore seems to be overrepresented in laying hen flocks compared to broiler and breeder flocks.

Table 1: Distribution of reported confirmed cases of human salmonellosis in the EU. Member State (MS): 25 MS and two non-MS; Austria, Belgium, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden and United Kingdom (EFSA, 2015).

Serovar	2014			2013			2012		
	Cases	MS	%	Cases	MS	%	Cases	MS	%
Enteritidis	32,878	27	44.4	29,09	27	39.5	32,917	27	41.0
Typhimurium	12,867	27	17.4	14,852	27	20.2	17,975	27	22,4
Monophasic Typhimurium 1,4, [5], 12:i:-	5,770	13	7.8	6,313	14	8.6	5,836	12	7.3
Infantis	1,841	26	2.5	2,226	25	3.0	1,929	26	2.4
Stanly	757	23	1.0	714	21	1.0	969	20	1.2
Derby	753	23	1.0	813	21	1.1	730	21	0.9
Newport	752	20	1.0	818	21	1.1	754	21	0.9
Kentucky	605	21	0.8	651	23	0.9	626	23	0.8
Virchow	509	22	0.7	571	22	0.8	532	20	0.7
Bovismorbificans	441	22	0.6	412	20	0.6	410	20	0.5
Java	388	15	0.5	581	24	0.8	445	18	0.6
Agona	378	23	0.5	401	18	0.5	452	18	0.6
Saintpaul	374	19	0.5	448	17	0.6	354	18	0.4
Muenchen	368	17	0.5	434	14	0.6	242	20	0.3
Napoli	333	14	0.4	290	17	0.4	365	16	0.5
Brandenburg	294	20	0.4	111	13	0.2	302	17	0.4
Chester	294	18	0.4	267	19	0.4	106	13	0.1
Hadar	286	16	0.4	238	10	0.3	300	20	0.4
Braenderup	276	17	0.4	245	19	0.3	454	17	0.6
Oranienburg	261	17	0.4	274	15	0.4	311	16	0.4
Other	13,599	-	18.4	13,883	-	18.9	14,286	-	17.8
Total	74,024	27	100.0	73,632	27	100.0	80,295	27	100.0

Eggs and egg products continue to be the most frequently identified food vehicle, associated with 44% of the reported outbreaks, mainly caused by *Salmonella* Enteritidis (table 2).

Table 2: Percentage of human salmonellosis cases in EU attributable to the four main animal reservoirs included (Messens et al., 2013).

	Percentage of human cases(%) Mean
Pigs	28.2
Broilers	2.4
Laying hens	65.0
Turkeys	4.5

1.2.3 Global salmonellosis

A recent study estimated that approximately 93.8 million human cases of gastroenteritis and 155 000 deaths occur due to non-typhoidal *Salmonella* infection around the world each year (Hoelzer et al., 2011). In Switzerland, salmonellosis and campylobacteriosis case curves crossed in 1995; in Austria it was in 2006. The reason for this striking difference might be that Switzerland addressed the epidemic of *Salmonella* Enteritidis in eggs at a very early stage (Schmutz et al., 2016). In China, a laboratory-based surveillance of non-typhoidal *Salmonella* infections was carried out for the first time in 2008. *Salmonella* Enteritidis and *Salmonella* Typhimurium were the most common serotypes, similar to most other countries (Ran et al., 2011).

Over the last several decades, there have been significant global shifts in the predominant *Salmonella* serovars associated with both poultry and human infections. Some of the most commonly detected serovars in chickens over the last 25 years are also among the top five serovars associated with human infections (*Salmonella* Enteritidis and *Salmonella* Heidelberg; Foley et al., 2011). *Salmonella* Kentucky has recently become the most commonly detected serovar in chickens, while *Salmonella* Typhimurium and Enteritidis remain the most common cause of human infections.

1.3 Molecular pathogenesis highlighting major virulence factors

Salmonella pathogenesis has been studied mostly as it relates to human infections, while there is more limited information about the mechanisms of colonization and pathogenesis in food animals such as chickens. Section 1.3.1 and 1.3.2 describe the general pathogenesis. The remainder of the current section focuses on *Salmonella* responses to the poultry host.

1.3.1 SPIs, fimbriae

Once the *Salmonella* bacterium orally infects its host, the species encounters extremes of pH, oxygen tension, bile salts, and competing microorganisms in the gastro-intestinal environment. This hostile environment serves as a signal for *Salmonella* to initiate transcription of genes specifically adapted for host interactions. Attachment of bacteria to the host cell surface is believed to be a first and essential step in the pathogenesis and occurs mainly through fimbriae. Fimbriae are a family of polymeric proteinaceous surface organelles expressed by many bacteria. *Salmonella* Enteritidis has 10 putative fimbrial operons (Folkesson et al., 1999). These fimbrial operons can be divided according to their assembly pathways: the chaperone-usher pathway, the extracellular nucleation pathway and a special system of type IV pili, which is similar to the type II secretion system.

Fimbriae assembled by the chaperone-usher pathway are directed to the periplasm through the general secretion pathway via an N-terminal secretion sequence that is cleaved off during transport. The function of these fimbrial adhesins is primarily achieved through binding to a specific receptor on the host cell. In general, the nature of these receptors may be a distinct membrane protein, sugar residues or lipid structures. However, all fimbrial adhesins characterized so far in *Salmonella* exhibit lectin-like functions. StdA binds to alfa (1-2) fucosylated receptors, PefA binds to the Lewis X blood group antigen and FimH, which is encoded by the fim operon, is highly specific for mannose residues.

A second group of fimbriae are named the thin aggregative fimbriae, whose structures are assembled through the nucleation-precipitation pathway. These thin aggregative fimbriae are fimbrial adhesins with a diameter of 3-4 nm and lead to auto-aggregation of *Salmonella*, biofilm

formation and adhesion to various surfaces and are expressed and assembled in response to nutrient limitation, low osmolarity and low temperature. They interact with different extracellular matrix proteins such as fibronectin or laminin and might allow the colonization of wounds.

A last class of fimbrial adhesins are the type IV pili, but these are only detected in the strictly human-adapted serovar Typhi (Wagner et al, 2011).

After attachment, the Type 3 Secretion System (T3SS), a multiprotein complex, is expressed and facilitates epithelial uptake and invasion. This apparatus acts as a molecular syringe to transport toxins and other effector proteins into intestinal epithelial cells and is associated with *Salmonella* Pathogenicity island-1 (SPI-1), which harbors virulence genes involved in *Salmonella* adhesion, invasion and toxicity. Upon activation, membrane ruffling is induced and the *Salmonella* bacterium is engulfed by the host cell membrane in a membrane-bound compartment termed the *Salmonella* containing vacuole (SCV). Once internalized into host cells, *Salmonella* cells express a second T3SS encoded on SPI-2 that is responsible for secreting effector proteins that modulate trafficking of the SCV to avoid fusion with the lysosomes (Raspoet R., 2014). This is a strategy to avoid immunologic recognition of *Salmonella* microbial associated molecular pattern (MAMPs) by the important Toll-like receptor4 (TLR4) which recognizes lipopolysaccharide (LPS) and TLR5, which recognizes flagellin.

1.3.2 MAMPs

The *Salmonella* bacterium contains different MAMPs. The major MAMPs are LPS, flagellin and unmethylated CpG motifs in the DNA. TLR4 is activated by LPS, TLR5 by flagellin and although TLR9 (which recognizes Cytosine Guanine dinucleotide (CpG) motifs in mammals) is not present in the chicken genome this recognition capacity is fulfilled by chicken TLR21 (Temperley et al., 2008). All of these TLR-MAMP interactions are important for the induction of responses in a range of cell types including epithelial, macrophage and polymorphonuclear neutrophil (PMN) cells.

The majority of *Salmonella* serovars possess up to 10 randomly positioned flagella on their cell surface, which confer motility to these bacteria. The ability of certain serovars to display flagellin phase variation is another mechanism of the organisms to minimize the host immune response by creating phenotypic heterogeneity of the flagellar antigens (Foley et al, 2013). Even more, genetically modified aflagellate *Salmonella* Typhimurium was able to cross the gut more efficiently, supporting the idea that TLR5-flagellin interactions are an important event in starting a pro-inflammatory reaction and restricting flagellate serovars (Enteritidis and Typhimurium) largely to the intestine. This may also partly underpin the ability of non-flagellate biovars (Gallinarum and Pullorum) to rapidly escape the gut and colonize the deep tissues (Broz et al., 2012). The avian-adapted egg-contaminating biovars *Salmonella* Pullorum and *Salmonella* Gallinarum lack flagella and associated motility. Flagellation has been shown to contribute to virulence in birds (Horiyama et al., 2010). The exact role of flagella in *Salmonella* pathogenesis and their possible role in adhesion and invasion of oviduct cells remain unclear.

1.3.3 Role of MDR pumps in reproductive tract colonization in laying hens

In the final stage of the pathogenesis in laying hens, *Salmonella* Enteritidis reaches the reproductive tract (figure 2), most likely by taking advantage of the macrophages. Once inside the reproductive tract, *Salmonella* Enteritidis invades and resides within primary chicken oviduct epithelial cells (Li et al., 2009). The region of colonization in the reproductive tract determines the site of incorporation into the forming egg. Infection of the ovary would lead to incorporation of *Salmonella* Enteritidis into the yolk, while persistence in the magnum, isthmus or uterus gives rise to contamination of the egg white, inner shell membranes or egg shell respectively. *Salmonella* Enteritidis has been isolated from both the yolk and the albumen, but according to most authors, the albumen is most frequently contaminated (De Buck et al., 2004a; Humphrey et al., 1991). Yolk contamination could occur due to ovary colonization. Degeneration of follicles in the ovary however, has often been observed after experimental *Salmonella* infections, most likely caused by extensive growth in the nutrient-rich yolk at chicken body temperature (Kinde et al., 2000). Secondary immigration from egg white to egg yolk during storage of eggs seems more plausible (Humphrey et al., 1991). *Salmonella* Enteridis is more

often associated with the tubular gland cells of the isthmus than with other parts of the oviduct (De Buck et al., 2004b). Once inside the egg white, *Salmonella* Enteritidis uses multi drug efflux pumps to neutralize the antibacterial proteins present in egg white. Until now, 9 of these pumps have been identified in *Salmonella*. Two of these pumps belong to the major facilitator (EmrAB and MdfA), 1 to the multidrug and toxic compound extrusion (MdtK), 1 to the ATP-binding cassette efflux (ABC) and 5 to the RND (AcrAB, AcrD, AcrEF, MdetABC, MdsABC) transporter family (Nishino et al., 2007). Two pumps (MdfA and MdtK) span the cytoplasmic membrane, while the other 7 transporters are multicomponent systems spanning both the inner and outer membrane. Except for MdsAB, which is capable of using MdsC, all multicomponent system pumps require TolC as outer membrane channel for their function (Horiyama et al., 2010). Besides a role in bacterial pathogenicity by exporting host-derived antimicrobial agents and thus allowing the bacteria to colonize and survive in hostile host niches, MDR-pumps also confer antibiotic resistance. This will be discussed below (1.6.1).

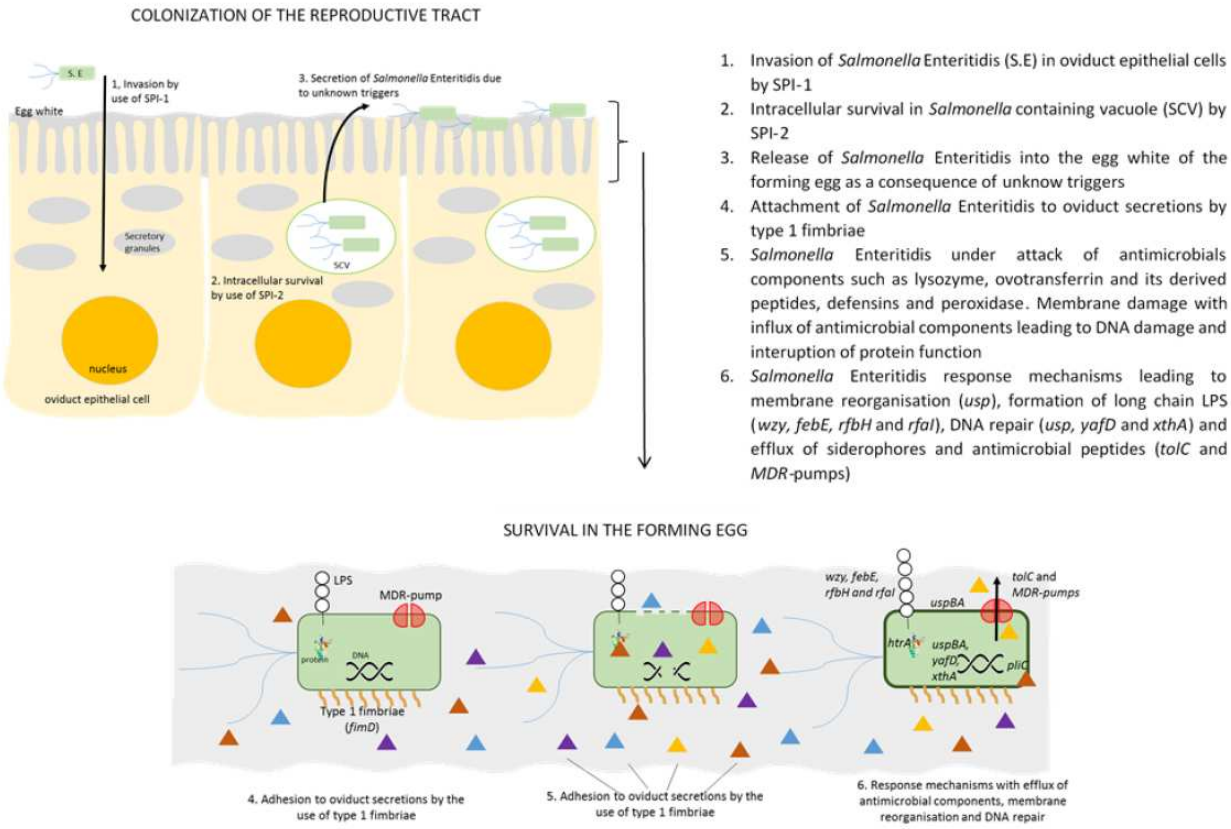

Figure 2: Summary of *Salmonella* Enteritidis oviduct colonization and egg contamination (Raspoet et al., 2014).

1.4 Salmonella pathogenesis: highlighting the differences in most important *Salmonella* serovars in laying hens

Bacteria can either infect a broad range of hosts, or become specialized, infecting one or few hosts, the latter usually being associated with more severe disease presentation. The severity of *Salmonella* infections indeed depends strongly on the infecting serovar (Chappell et al., 2009). The broad host range *Salmonella* serovar Enteritidis is able to infect plants and different species of warm and cold blooded animals, while *Salmonella* Gallinarum is restricted to birds, as *Salmonella* Dublin is found mostly in cattle. Consequently, *Salmonella* Enteritidis and *Salmonella* Gallinarum follow a very different course. *Salmonella* Gallinarum causes Fowl Typhoid, a severe systemic infection affecting birds of all ages, typified by hepatosplenomegaly, anemia and in the

later stages hemorrhage of the intestinal tract. Poultry infected with *Salmonella* Pullorum and *Salmonella* Gallinarum, experience drastic weight loss and sharply decreased egg production. A mortality rate of 60% is observed after experimental infection of 3 week old outbred chickens (Wigley et al., 2005). When replication of *Salmonella* Gallinarum is not controlled, this usually results in death of the animal. *Salmonella* Enteritidis infection is most often not associated with mortality but leads to persistence in the gut and reproductive tract and consequently egg infection. There are typically no clinical signs in birds infected with *Salmonella* Enteritidis. Therefore farmers often are not aware of the public health threat posed by *Salmonella* Enteritidis infected laying hens and their produce. Nevertheless, *Salmonella* Enteritidis and *Salmonella* Gallinarum are closely related genetically, presenting 99.7% homology between orthologous genes. Reasons for their different pathological behavior are still poorly understood. Within *Salmonella* subspecies however, there are patterns of genome evolution that accompany host adaptation. Some differences at the genomic and proteomic levels that have been identified will be described below.

As pathogens acquire virulence determinants they become increasingly adapted to a specific host. Evolution of pathogenicity of *Salmonella* is strongly associated with the acquisition of mobile genetic elements called SPIs. Many of these SPIs were acquired very early in the evolution of *Salmonella* and so their complement is found to be conserved across this species. These SPIs encode secretion systems allowing the bacteria to enter and survive in cells, and although they are still present in host restricted serovars, they might function differently. Several studies have shown that *Salmonella* Gallinarum is less invasive than *Salmonella* Enteritidis when tested in cells of avian or human origin. Apparently, the *Salmonella* T3SS-1 is slower in *Salmonella* Gallinarum compared to *Salmonella* Enteritidis (Allen-Vercoe and Woodward, 1999). Genome comparison of four *Salmonella* Gallinarum and two *Salmonella* Enteritidis strains revealed that all *Salmonella* Gallinarum genomes display the same point mutations in each of the main T3SS-1 effector genes (SipA, SopA, SopD, SopE and SopE2; Rossignol et al., 2014).

Host restricted pathogens also often exhibit extensive genome decay, through insertion sequence element proliferation, genomic rearrangement and pseudogene formation. As a consequence, loss of metabolic capacity is seen with host adapted serovars, mostly function loss through pseudogene formation (Lillehoj et al., 2000). Pseudogene formation largely occurred after serovar diversification. *Salmonella* Gallinarum has a large number of pseudogenes in its genome compared to the broad-host-range serovar *Salmonella* Enteritidis. Moreover, *Salmonella* Gallinarum has lost many metabolic pathways such as 1,2-propanediol degradation and ornithine decarboxylation, leading to restriction of usable carbon and energy sources. These limited metabolic capabilities could explain *Salmonella* Gallinarum' s reduced ability to colonize the gut (Atterbury et al., 2009).

Different host range serovars also vary in their interaction with the immune system of the host. Invasion of *Salmonella* biovars Pullorum or Gallinarum in the gut does not cause substantial intestinal inflammation, unlike *Salmonella* Typhimurium or *Salmonella* Enteritidis. The former two are not recognized by TLR5 due to lack of flagellin, which plays a key role in the initiation of inflammatory responses. A key aspect of systemic disease is survival and multiplication within macrophages, although *Salmonella* Dublin organisms in calves at least translocate from the gut to the spleen as extra-cellular bacteria (Barrow et al., 2012). It is known that biovar Pullorum persists within macrophages and the immune response to the organism is different from the response after infection with *Salmonella* Typhimurium. Sometimes clearance from the tissues is not complete and *Salmonella* Pullorum is able to persist in the tissues until sexual maturity of the bird. Wigley and colleagues found that the pathogen persists in splenic macrophages in young convalescent birds until onset of lay when a transient immunosuppression associated with a surge in sex hormones in the female enables the bacteria to escape and infect the oviduct resulting in vertical transmission. This group also found that the immune response induced by *Salmonella* Pullorum is associated with higher levels of interleukin-4 (IL-4) and reduced interferon gamma (IFNgamma) indicating a T-helper 2 (Th2)-type response in contrast to the more common Th1-type response associated with serovars such as *Salmonella* Typhimurium (Wigley et al., 2001).

The broad host range serotypes Enteritidis and Typhimurium appear to have similar virulence mechanisms and pathogenicity. Both pathogens have the highly conserved pathogenicity island-encoded type III secretion mechanisms and virulence effector proteins, and both harbor a large virulence plasmid, are motile and produce a galactose-rhamnose-mannose repeat unit of the LPS O-chain backbone decorated with a dideoxyhexose that determines serotype (Galan and Curtiss, 1991). Nevertheless, *Salmonella* Enteritidis is the predominant serotype contaminating eggs, while Typhimurium is far less commonly found in eggs. At the onset of the present PhD studies it was still unclear how *Salmonella* Enteritidis is predominantly the cause of egg-associated salmonellosis.

1.5 *Salmonella* Enteritidis: the egg autocrat

Salmonella Enteritidis is indeed the only human pathogen that contaminates eggs routinely, even though the on-farm environment of the chicken is a rich source of a range of different *Salmonella* serotypes and other pathogens. Different serotypes have been evaluated for their egg colonization capacities and results show that *Salmonella* Enteritidis is superior in reproductive organ colonization and egg white survival compared to other serotypes (De Vylder et al., 2013).

Egg contamination associated with *Salmonella* Enteritidis is believed to occur before deposition of the shell, by internal (vertical) transmission to the contents of the egg (yolk or albumen) via the reproductive tract (figure 3). The bacteria can reside inside the cells of the oviduct and escape the host defense mechanisms, but once inside the egg, bacteria face a hostile environment (Raspoet et al., 2011). Egg white proteins, such as lysozyme and ovotransferrin are important for anti-bacterial defense. Lysozyme is a muramidase capable of rupturing the peptidoglycan layer. Ovotransferrin causes an iron-deficient environment through chelation of iron and interacts with the bacterial cytoplasmic membrane, inducing damage to biological functions (Gantois et al., 2009). Additional minor egg proteins and peptides have recently been found to play known or potential roles in protection against bacterial contamination, mainly showing proteinase-inhibiting activity (Baron et al., 2016).

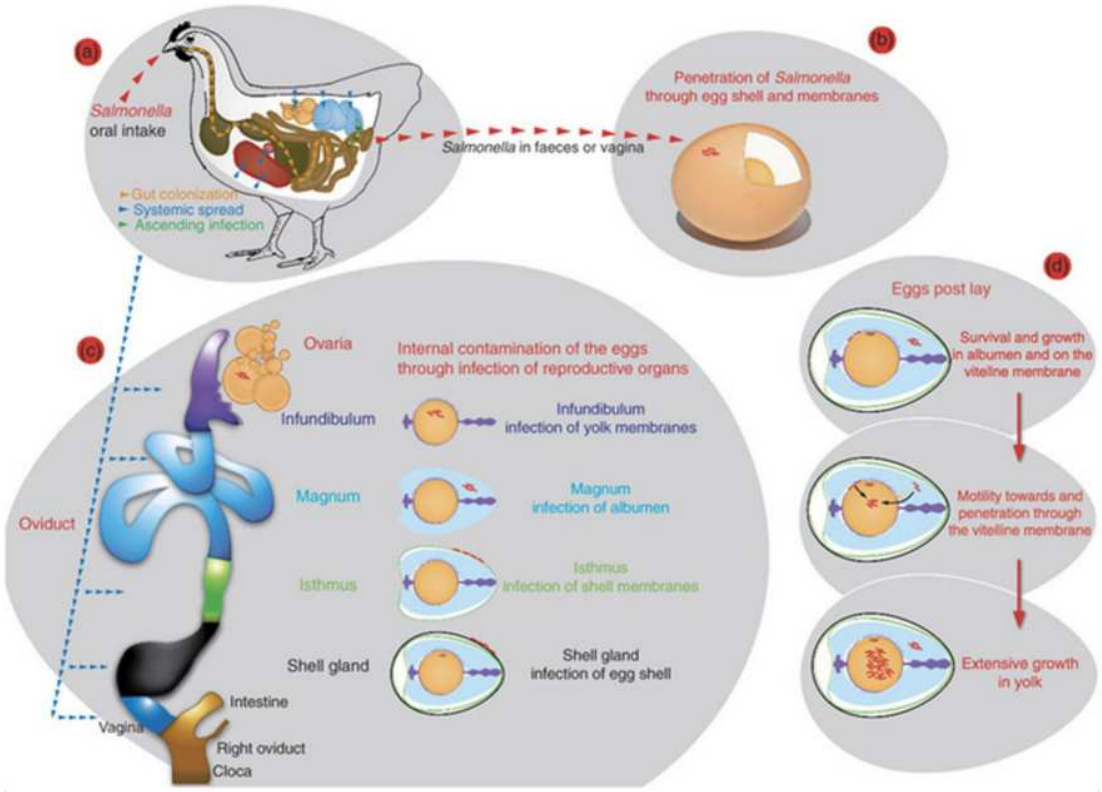

Figure 3: Overview of egg contamination by Salmonella (Gantois et al., 2009): a) Salmonella contamination of the reproductive organs of a hen via systemic spread after gut colonization or via an ascending infection; b) horizontal transmission route; c) vertical transmission route; d) survival and growth of Salmonella in the egg contents.

Numerous attempts have been made to identify genes encoding proteins important for egg white survival of *Salmonella* Enteritidis. Based on current literature, the main approaches used are directed mutagenesis (Cogan et al., 2001; Kang et al., 2006; Lu et al., 2003), insertional mutagenesis (Chappell et al., 2009; Clavijo et al., 2006), IVET (Gantois et al., 2008), and a microarray-based transposon library screening (Raspoet et al., 2014). These studies based on mutagenesis differ in terms of methods of mutant construction, screening approaches, strains, and incubation conditions (inoculum size, temperature). Taken together, a great diversity of genes involved in the survival of *Salmonella* Enteritidis in egg white have been identified (table 3). The genes presented are mainly implicated in cell wall structure or function, cell wall proteins or metabolism.

Table 3: Mutants of *Salmonella enterica* serovar Enteritidis SE2472[a] (Clavijo, R.I., 2006)

Category	Mutant	Gene	Function and/or feature
Cell wall structure or function	ES1	SEN3892	Homologous to mechanosensitive ion channel
	ES2	*prgH*	Component of type III secretion apparatus
	ES10	*glnH*	Glutamine-binding periplasmic protein precursor
	ES11	*prgJ*	Invasion protein of type III secretion apparatus
	ES15	*proY*	Proline-specific permease
	ES21	*modF*	Putative molybdenum transporter
	ES30	*bcfC*	Fimbrial usher protein
	ES46	*spaP*	Membrane protein of type III secretion system
	ES53	*waaJ*	LPS synthesis
	ES54	*yijC*	Transcription factor regulating fat production
Putative cell wall proteins	ES17	SEN1188	Putative inner membrane protein
	ES31	*yigQ*	Putative periplasmic protein or exported protein
	ES33	SEN1861	Putative inner membrane lipoprotein
	ES37	STM3980	Putative outer membrane protein
	ES41	SEN0784	Putative inner membrane protein
	ES50	SEN1204	Putative membrane protein
Metabolism	ES3	*ordL*	Homologous to DadA involved in phenylalanine metabolism
	ES5	*tdk*	Thymidine kinase
	ES7	*yejD*	Ribosomal small subunit pseudouridine synthase
	ES12	*ydiB*	Putative shikimate 5-dehydrogenase involved in aromatic amino acid synthesis
	ES20	*ybdL*	Putative aminotransferase involved in phenylalanine metabolism
	ES22	*tyrR*	Regulator of aromatic amino acid biosynthesis and transport
	ES25	*cadA*	Lysine decarboxylase
	ES52	*lysC*	Lysine sensitive aspartokinase III
Unknown function	ES6	SEN2128	Putative cytoplasmic protein
	ES19	SEN2997	Putative ATP-dependent RNA helicase-like protein
	ES27	*ygdI*	Putative lipoprotein
	ES28	SEN2263	Transcriptional regulator, function unknown
	ES35	*ybbN*	Putative thioredoxin protein
	ES51	*rssC*	Putative cytoplasmic protein
SE specific	ES16	SEN4287	Possible restriction endonuclease gene
	ES47	Prot6E gene	Fimbrial biosynthesis

[a] A summary of the characteristics of mutants isolated from screening a Tn mutant library for mutants with decreased survival in egg albumen compared to the wild-type *Salmonella enterica* serovar Enteritidis is presented. The gene that was disrupted by the Tn insertion in each mutant is listed. If the Tn insertion was present in a gene that is uncharacterized and unnamed, the annotation of *Salmonella enterica* serovar Enteritidis or *Salmonella enterica* serovar Typhimurium genome is used. Salient features of the ORFs disrupted by the Tn insertion in each mutant are summarized.

1.6 Combating *Salmonella*

1.6.1 Antimicrobials

Antibiotics were used from the 1960s to reduce mortality in young birds, caused by a variety of pathogens including *Salmonella* (Bleuel et al., 2005). Resistant bacteria were selected over time due to extensive use. In the 1990s, the prevalence of multidrug-resistant *Salmonella* serotypes increased dramatically in many countries, with documented outbreaks associated with drug resistant *Salmonella* in poultry meet, beef and pork. This emerging resistance to antibiotics in *Salmonella* has also been found in strains isolated from humans and is thus a potentially serious public health problem (Yamasaki et al., 2011). The emergence of resistant isolates is now a major concern (Dias de Oliveira et al., 2005; Schmutz et al., 2016). The resistance is mediated by transmissible plasmids. Resistance to drugs is often associated with multidrug efflux pumps that decrease drug accumulation in the bacterium. In gram-negative bacteria, transporters belonging to the Resistance Nodulation Division (RND) family are particularly effective in generating resistance through forming a tripartite complex with periplasmic proteins and the outer membrane protein channel TolC (figure 4). The RND transporters have broad substrate specificity and require TolC for their function (Nikaido H., 2011). In *Salmonella enterica*, the function of all RND transporter systems requires TolC, except for MdsABC, which requires either MdsC or TolC for drug resistance. Furthermore these drugs also cause disruption of the gut flora, enhancing *Salmonella* colonization and increasing susceptibility in birds. While antibiotic usage to eliminate *Salmonella* in poultry is now strictly forbidden in the EU, in other parts of the world this is still common practice. According to Article 2 of Regulation (EC) No 1177/2006 (Commission Regulation (EC) No 1177/2006), antimicrobials shall not be used as a specific method to control *Salmonella* in poultry. There is a significant correlation between the use of the aminoglycoside apramycin and the isolation of resistant *Salmonella*, especially *Salmonella enterica* serotype Typhimurium. Amnioglycoside resistance in these bacteria is due to the acquisition of a gene encoding an acetylating enzyme. Another pool of resistence genes are bèta-lactamase genes which are encoded on mobile genetic elements, such as plasmids, transposons and integrons, which often also carry additional resistance genes (Smet et al, 2009).

The consequences of selection of resistance can range from prolonged illness and side effects, due to the use of alternative, and possibly more toxic drugs, to death, following complete treatment failure. To reduce the risk of selecting resistant bacteria, the use of antibiotics must be restricted. Furthermore, in order to ensure that EU targets for reducing *Salmonella* are met, all Member States national control programs should include biosecurity measures designed to prevent *Salmonella* infection on poultry farms. The introduction of such measures also has a positive effect in terms of preventing other diseases. Specific EU guidelines have been published by the Commission services for farms where broilers and laying hens are kept.

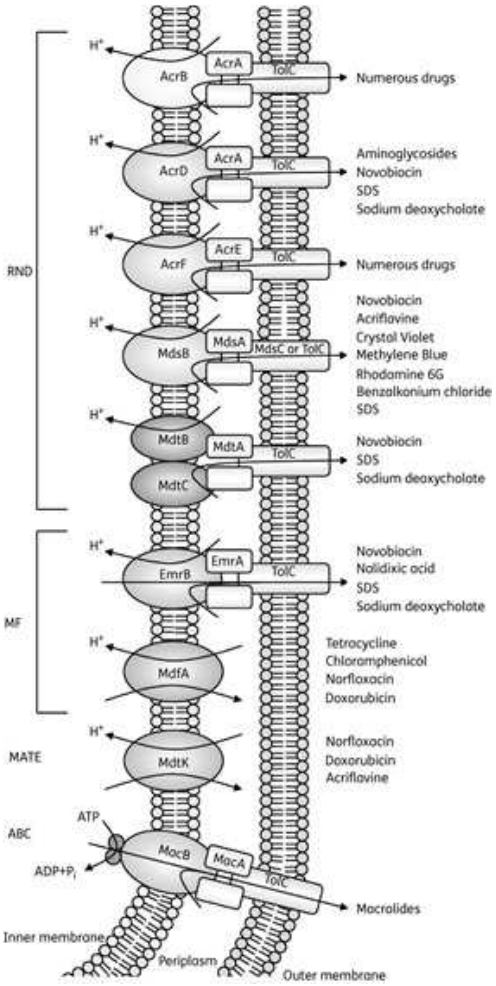

Figure 4: The RND transporters AcrB, AcrD, and MdtABC capture antimicrobials in the periplasm and then export them to the growth medium through the outer membrane channel TolC (Horiyama et al., 2010).

1.6.2 Non-antibiotic feed additives

Various substances have been investigated for their inhibitory effects on *Salmonella* infection and faecal shedding. Butyrate can reduce *Salmonella* colonization in chickens *in vivo* via up-regulation of host defense peptides and by the suppression of SPI-1 (Gantois et al., 2006; Sunkara et al., 2011). Acidified feed inhibits *Salmonella* shedding (Willamil et al., 2011). The cereal type in feed influences *Salmonella* colonization in broilers (Teirlynck et al., 2009). Other soluble plant non-starch polysaccharides have been shown to block pathogen-epithelium interactions. Adding polysaccharide hydrolyzing enzymes into the diets may modify the microfloral and physicochemical balance in the gastrointestinal tract (Parsons et al., 2014). Another strategy is to create passive immunity of birds through feeding them aspecific antibodies produced from eggs of hyperimmunized hens. Other strategies simply recommend the use of genetically resistant chicken lines. Feed additives such as prebiotics, probiotics, and synbiotics that modify the gut microflora are also being investigated, and the success of these approaches differs with the additive used.

1.7 Currently used vaccines: never change a winning team?

1.7.1 Historical overview

In the 1960s and 1970s killed vaccines against *Salmonella* Gallinarum were used in order to limit *Salmonella* Gallinarum associated mortality. In the 1980s *Salmonella* Enteritidis arised and became the most important bacteria causing zoonotic disease. This serotype mostly does not cause any clinical signs in chickens. Humans get contaminated by the consumption of eggs and egg products. Reducing mortality was the main benefit of the killed vaccines but due to the limited effect on faecal shedding and lack of effectively stimulating cytotoxic T-cells, the industry quickly turned to live vaccines, prepared by bacterial culture under conditions of iron starvation or in the presence of a mutagenic product (Barrow et al., 2007). Live vaccines have been shown to generate higher levels of protection in birds and instead of needing to inject the vaccine, they can be administered in the drinking water (Barrow et al., 1990; Methner et al., 2011). These vaccines are often produced on the basis of metabolic drift mutations. They are

often antibiotic resistant and with undefined mutations. A successful decrease of *Salmonella* Gallinarum was seen through extensive use of the *Salmonella* Gallinarum 9R vaccine developed by Williams Smith in 1956, with J. F. Tucker. A decrease of *Salmonella* Enteritidis infections has been seen after vaccination with live *Salmonella* Enteritidis vaccines (Nassar et al., 1994).

Due to the successful decrease of salmonellosis by live attenuated vaccines, the use of these vaccines in commercial poultry increased worldwide and is regarded as one of the most important prophylactic measures to protect chickens against *Salmonella* infections and to protect the public from egg-borne *Salmonella* infections (Vandeplas et al., 2010). Currently used live vaccines contain strains harboring (undefined) point mutations. Although in previous years, vaccination was proven to be safe, current data suggest that these types of vaccines can regain virulence. Safety is undeniably a major concern of live vaccines, including the possible risk of reversion to virulence (Van Immerseel et al., 2013; White et al., 1997). A solution could be to delete whole genes. Since the scientific understanding of the organism has exploded in the past 25 years, an increasing number of defined deletion vaccines have been developed and investigated. These vaccines were mainly evaluated for their ability to reduce shedding. Few studies evaluated the protection against egg contamination.

1.7.2 Possible attenuations in *Salmonella*

Selecting genes that can be deleted from the *Salmonella* bacterium is not an easy task. The basic criteria needed to be fulfilled for vaccines should be kept in mind. An ideal *Salmonella* vaccine should offer protection against mucosal and systemic infection, preferably during the whole life span of the chicken, while being avirulent to both man and animals. Finally, reduction of intestinal colonization to reduce or completely prevent shedding and egg contamination, congruence with other control measures, and low cost of application are of importance (Van Immerseel et al., 2005). Protection against most or even all serovars of *Salmonella* capable of causing foodborne illness in humans could top off the list. Currently, no vaccine or vaccination program is capable of providing this type of protection. Luckily, in recent years, knowledge on

the function of *Salmonella* genes and the host response to *Salmonella* infections, combined with molecular biological techniques has led to the development of more sophisticated vaccines.

Many live *Salmonella* vaccine strains have been experimentally tested with different results (table 4). Deleting genes important for metabolism, virulence, or survival in the host organism is the usual strategy. Indeed, a number of vaccines contain strains with gene deletions that are important for metabolism, like aroA, aroC and aroD (in *Salmonella* Enteritidis). The reduced virulence of aro mutants has been explained by their inability to produce aromatic metabolites, mainly aromatic amino acids such as phenylalanine, tyrosine, and tryptophan. This extreme attenuation most likely led to some cases where the aro mutants were not sufficiently immunogenic and did not efficiently protect animals from subsequent infection (Hormaeche et al., 1991; Nnalue, 1990). Despite this, inactivation of aro genes is one of the most frequently used methods for *Salmonella* attenuation. Other vaccine strains compromised for metabolic functions contain crp derived mutations in *Salmonella* Typhimurium. The crp gene encodes the cAMP receptor protein (crp), which regulates transcription of a magnitude of operons involved in transport of sugars and catabolic functions (Schroeder and Dobrogosz, 1986).

Table 4: A small selection of experimental and commercial live attenuated *Salmonella* vaccines (Desin et al., 2014)

Type of vaccine	Route of delivery	Frequency of immunization	Challenge	Effect
Salmonella Enteritidis ΔaroA	Oral	Once, 1 day of age	Oral, S. Enteritidis 10^8 CFU	Reduction of colonization
Salmonella Typhimuium Δcyalcrp	Oral	Twice (1, 14 days)	Oral, S. Enteritidis 10^6 CFU	Reduction of spleen colonization only
Ts S. Enteritidis mutant	Oral	Twice (1, 14 days)	Oral, S. Enteritidis 10^9 CFU	Reduction of colonization
Nobilis® SG 9R	sc.	Twice (6, 14-16 weeks)	Field conditions	Protection: 2.5% flocks positive relative to control (11.5%)
TAD Salmonella vac®E	Oral	Three doses (1day, 6 weeks, 16 weeks)	iv., S. Enteritidis 10^7 CFU	Protection: 12/28 liver, 6/28 oviduct, 9/35 egg samples positive relative to control (25/30, 15/29 and 15/35, respectively)
Megan® Vac 1	Oral	Three doses (1 day, 2 weeks, 5 weeks)	Field conditions	Protection: 38\% cecal and 14% reproductive tract samples positive versus 68 and 52% control, respectively
S. Enteritidis ΔphoP/fliC	Oral	Twice (1, 21 days)	Oral, S. Enteritidis 10^8 CFU	Reduction of colonization
S. Enteritidis Δlon/cpxR	Oral	Twice (1 day of age)	Oral, S. Enteritidis 10^9 CFU	Reduction of colonization
Salmonella Gallinarum ΔcobS/cbiA	Oral	Twice (5, 25 days)	Oral, S. Enteritidis 10^8 CFU	Reduction of colonization
S. Enteritidis ΔSPI-1, ΔSPI-2	Oral	Twice (1, 21 days)	Oral/ iv., S. Enteritidis 10^7 CFU	Reduction of colonization

Another range of deletions include virulence factors. *Salmonella* Enteritidis secretion systems are known to be important virulence factors in chickens but since structural components are protective antigens in other bacterial species, their deletion may not be beneficial (Methner et al., 2011). Other vaccines containing strains impaired for virulence lack the phoP gene, among other attenuations. The PhoP/PhoQ system is directly involved in the regulation of the SPI-2 pathogenicity island and highlights PhoP/PhoQ's central role in *Salmonella* virulence. The phoP/fliC double gene deleted strain allows differentiation of vaccinated from infected animals, through the absence of fliC, which is a major component of flagellin. Flagellin is one of the MAMPs, recognized by TLR5, leading to the production of anti-flagella antibodies (Gewirtz et al., 2001). Deletion of flagella in *Salmonella* Typhimurium however, led to a less efficient recognition by the host immune system and a temporary increase in the virulence in the early stages of chicken infection (Karasova et al., 2009; Kodama and Matsui, 2004). Deletion of this important MAMP thus raises concerns about increased virulence and shedding of flagella defective mutants (Iqbal et al., 2005; Methner and Barrow, 1997). Additional independent attenuations are thus needed. A final range of deletions result in a decreased survival of *Salmonella* in the environment. Lon for example is an evolutionarily conserved stress protein induced by multiple stressors and helps to remove damaged and abnormal proteins during

stress (Si et al., 2015). The cpdB gene enables *Salmonella* Enteritidis to grow on 2", 3"-cAMP as a sole source of carbon and energy (Si et al., 2015). A major drawback of most current used live vaccines in the field is that immunized animals, producing antibodies against the vaccine strain are no longer distinguishable from field-exposed animals by serological tests (Adriaensen et al., 2007; Matulova et al., 2013). Taken together, there still appears to be a need for new deletion mutants that better fulfill the requirements as set forward at the start of this paragraph.

1.7.3 Currently used vaccines: modes of action

Since live vaccines are most widely used, this section focuses on the mode of action of live vaccines. Protective mechanisms observed by live vaccines can be divided into mechanisms effective during the "immunity gap" and protection based on a humoral and, more importantly, cell-mediated immunity.

Protective mechanisms effective during the "immunity gap", the time between administration of the vaccine and development of the adaptive immune response, relies on the principle of colonization inhibition (CI, Methner et al., 2011). Oral administration of a live attenuated *Salmonella* strain to day-old chicks confers protection against a *Salmonella* infection within hours after administration. This protective mechanism cannot be based on an adaptive immune response (see below). The actual mode of action remains unclear but it is assumed that bacteriological exclusion phenomena play a role.

During the immunity gap, chickens are most vulnerable for infections, and contamination during this period results often in persistent infection. CI is highly effective especially between strains of the same serotype (Cox et al., 1990; Vandeplas et al., 2010). The molecular basis of colonization inhibition is still relatively poorly understood. Oral administration of live vaccines to newly-hatched chickens results in massive multiplication in the gut for a few days with a resulting competitive exclusion effect against related bacteria. This is thus thought to be largely a bacteriological exclusion, with heterophil infiltration into the gut mucosa also inhibiting invasion of *Salmonella* strains and other bacteria to internal organs.

Long term protection against salmonellosis requires the host' s immunity of both cellular and humoral arms (Babu et al., 2003). It was shown that an increase of T-cell subsets is seen 7 days post-inoculation, peaking at day 10 after inoculation with a live vaccine. This increased cell mediated immunity is only associated with live vaccines and could explain why these live vaccines are more effective than killed vaccines for the control of *Salmonella* infections. Cell mediated immune responses are generally more important in controlling organisms which replicate intracellularly (Imre et al., 2015). It has also been observed that CD8+ T-cells play an important role in the immunological defense after primary infection in young chicks and that clearance of *Salmonella* Typhimurium infections in chickens correlates with high cell-mediated responses (Barrow, 2007). In addition, intraperitoneal administration of recombinant IFNgamma decreases *Salmonella* colonization, underlying the importance of cell-mediated immune mechanisms in the systemic clearance of *Salmonella*. Finally heterophil-depleted chickens are much more susceptible to *Salmonella* Enteritidis, further illustrating the importance of cell mediated immunity in *Salmonella* infections in poultry (Methner et al., 2011).

The peak of CD8+ T-cells is then followed by an increase in B-cell numbers at day 14. An adaptive immune response thus takes at least 10 days to develop. Serum titers of IgM and IgG are directly related to the size of the inoculum. These antibodies remain in the serum for at least 35 weeks pi (Cox and Pavic, 2010). Protection against infection is generated by preventing translocation from the gastrointestinal tract. Secretory IgA functions by inhibiting the adherence of coated micro-organisms to mucosal cells. This kind of protection is of primary importance to avoid a bacterial infection (Desmidt et al., 1998). 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.

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2 Scientific aims

Scientific aims

Salmonellosis is still the second most commonly reported zoonotic disease, following campylobacteriosis. The first attempts to control *Salmonella* enterica serovar Enteritidis were initiated in the 1980s in the EU, and control measures were developed, including stringent biosecurity programs, the use of feed additives and vaccination programs using inactivated and live vaccines. Thanks to the implementation of control programs, an epidemiologic turnaround for *Salmonella* Enteritidis infections has been achieved. Initially, the EU target was to cover only *Salmonella* Enteritidis and *Salmonella* Typhimurium. In addition, for breeding flocks of Gallus gallus, *Salmonella* Hadar, *Salmonella* Infantis and *Salmonella* Virchow were considered, as these serovars were, together with the former, the five most frequently reported *Salmonella* serovars in human salmonellosis in the EU. Most of the legislation related to *Salmonella* control in the EU dates back to the beginning of the 21st century. The control programs that were set up and implemented in response to this legislation, including vaccination programs in layers, have drastically changed the epidemiological situation. On the one hand, *Salmonella* Enteritidis infections in layers have become almost rare events. On the other hand, multiple other serotypes have emerged in humans. Therefore, the general aim of this thesis was to evaluate the efficacy of existing vaccines and the exploration of novel vaccine approaches that may be better adapted to the evolving epidemiological situation. The *Salmonella* monophasic strains with antigenic formula 1,4,[5],12:i:- are important epidemiological developments. These strains are variants of *Salmonella* Typhimurium and EU legislation thus also states that these need to be controlled. These monophasic *Salmonella* Typhimurium strains have been shown to have similar virulence and antimicrobial resistance characteristics to other strains of *Salmonella* Typhimurium and thus are considered to pose comparable public health risks to that of other epidemic *Salmonella* Typhimurium strains. Current vaccines have been developed and tested against Enteritidis and Typhimurium infections, but their efficacy against emerging monophasic variants has not yet been investigated. A first aim was thus to study the efficiency of a widely used commercial live Typhimurium vaccine against infection with this new arising monophasic

variant. This was done by evaluating shedding and organ colonization in three independent trials with different infection doses, after vaccination at day 1.

Currently monophasic *Salmonella* Typhimurium 1,4,[5],12:i:- variants are emerging worldwide. These variants are lacking the *fljB*-encoded second phase antigen. It has been suggested that the lack of flagella changes virulence characteristics of *Salmonella* but the exact role of flagella in the pathogenesis of *Salmonella* infections in chickens is not yet completely clear. The aflagellate *Salmonella* Gallinarum is causing severe systemic disease with reproductive tract pathology. Little was known yet about the role of flagellin in oviduct colonization by non-host specific serotypes such as *Salmonella* Enteritidis. Therefore the second aim of this work was to evaluate the role of flagellin in oviduct colonization by analyzing the expression of flagellar genes in oviduct cells and studying the response of oviduct cells to flagellin. This information could be important for future vaccine development.

Egg white survival is a key feature of *Salmonella* Enteritidis that gives strains of this serotype a unique opportunity to be transmitted to the egg-consuming host. Various genes have been identified that play a role in egg white survival. Mutating these genes thus enable a vaccine to be safe for humans and allow the creation of vaccines that will not enter the food chain through eggs. Multidrug resistance pumps (MDR) are bacterial systems that export host antimicrobial proteins and antibiotics as protection mechanism. MDR pump mutants have been shown to be attenuated and cannot survive in egg white, making these strains potentially valuable safe live vaccines. A third aim of the PhD thesis was to evaluate whether MDR pump mutants also protect chickens against egg contamination, after oral vaccination.

3 Experimental studies

3.1 Oral administration of the *Salmonella* Typhimurium vaccine strain Nal2/Rif9/Rtt to laying hens at day of hatch reduces shedding and caecal colonization of *Salmonella* 4,12:i:-, the monophasic variant of *Salmonella* Typhimurium.

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Oral administration of the *Salmonella* Typhimurium vaccine strain Nal2/Rif9/Rtt to laying hens at day of hatch reduces shedding and caecal colonization of *Salmonella* 1,4,[5],12:i:-, the monophasic variant of *Salmonella* Typhimurium.

Abstract

A new monophasic variant of *Salmonella* Typhimurium, serotype 1,4,[5],12:i:-, is rapidly emerging. This serotype is now considered to be among the 10 most common serovars isolated from humans in many countries in Europe and in the United States. The public health risk posed by these emerging monophasic *Salmonella* Typhimurium strains is considered comparable to that of classical *Salmonella*Typhimurium strains. The serotype 1,4,[5],12:i:- is frequently isolated from pigs but also poultry are carrying strains from this serotype. In the current study, we evaluated the efficacy of the *Salmonella* Typhimurium strain Nal2/Rif9/Rtt, a strain included in the commercially available live vaccines AviPro *Salmonella* Duo and AviPro *Salmonella* VacT, against infection with the emerging monophasic variant in poultry. Three independent trials were conducted. In all trials, laying type chicks were orally vaccinated with the *Salmonella* Typhimurium strain Nal2/Rif9/Rtt at d hatch, while the birds were challenged the next d with a different infection dose in each trial (low, high, and intermediate). For the intermediate-dose study, a seeder bird model was used in which one out of 3 animals were infected while all individual birds were infected in the other trials. Data obtained from each independent trial show that oral administration of the *Salmonella* Typhimurium strain Nal2/Rif9/Rtt at d hatch reduced shedding, caecal, and internal organ colonization of *Salmonella* Typhimurium 1,4,[5],12:i:- , administered at d 2 life. This indicates that *Salmonella* Typhimurium strain Nal2/Rif9/Rtt can help to control *Salmonella* 1,4,[5],12:i:- infections in poultry.

Introduction

For more than 20 years health agencies and the animal production industry are combating *Salmonella* infections. In the European Union (EU), the implementation of *Salmonella* control programs in poultry (and pigs) has led to a strong decrease in the number of human salmonellosis cases (EFSA, 2010a). Although *Salmonella* Enteritidis and Typhimurium still continue to be the most commonly reported *Salmonella* serovars in human cases, atypical pathogenic *Salmonella* strains have emerged. Current studies in numerous countries worldwide confirm the rapid emergence and dissemination of a monophasic variant of *Salmonella* Typhimurium, i.e. serotype 1,4,[5],12:i:- (Bone et al., 2010; Hopkins et al., 2012; Mossong et al., 2007; Peters et al., 2010). This variant has been detected in Spain and Portugal since 1997 (Usera et al., 2002) and is now the third most commonly isolated serotype causing human and animal salmonellosis in the EU (EFSA, 2010b; EFSA, 2014). Of a total of 92,916 cases of human salmonellosis that were reported by the European Union Member States in 2012, the monophasic strain *Salmonella* Typhimurium 1,4,[5],12:i:- was responsible for 7.2% of the cases (EFSA, 2010b; Anonymous, 2014).

While most *Salmonella* serovars are biphasic and express two distinct flagellar antigens encoded by fliC (phase-1 flagellin) and fljB (phase-2 flagellin), monophasic strains fail to express either the phase-1 or phase-2 flagellar antigen. Cases of human infection caused by the emerging monophasic variants have been linked to a number of sources, predominantly pigs (EFSA, 2010a; Mandilara et al., 2013). Strains from this serotype have also been found in chicken meat, broilers and recently in laying hens (Le Hello et al., 2012). This shows that the monophasic variant 1,4,[5],12:i:- represents a significant and potential emerging threat to humans, not only through porcine meat, but also through chicken product consumption. Consequently it has been included in actions implementing the legislation of the EU to detect and control *Salmonella* serovars of public health significance in laying hens (Anonymous, 2011; Parsons et al., 2014).

While control programs have been efficient in reducing the prevalence of *Salmonella* Enteritidis in laying hen flocks and as a consequence contamination of eggs and egg products, data on effects of control measures for *Salmonella* 1,4,[5],12:i:- in layers are scarce. Control of *Salmonella* in the primary production of chickens should mainly be based

on biosecurity measures and the administration of feed additives. In laying hens vaccination is an important tool to protect against colonization. While vaccination of layers against *Salmonella* is mainly used to control egg contamination, vaccines also aim to reduce gut colonization and shedding. Booster immunizations of live vaccine strains are used in the field to decrease *Salmonella* colonization in adult birds, but administration of live vaccine strains at day-of-hatch can also protect chickens against early colonization with *Salmonella*, a process called colonization-inhibition (De Cort et al., 2013). To our knowledge not a single vaccine study has been performed until now, with the objective of reducing the colonization of the emerging monophasic variant in chickens. While the efficacy of the commercial live vaccines *Salmonella* TAD® VacE and VacT (later renamed to AviPro® *Salmonella* VacE and VacT) to protect laying hens from oviduct colonization and egg contamination by *Salmonella* Enteritidis has been proven (Gantois et al., 2006), no data have been published yet on potential effects of this vaccine on caecal, spleen and liver colonization by the monophasic serotype 1,4,[5],12:i:-. Therefore, in the present study two short-term (two weeks) trials, either using a high or a low infection dose, and 1 longer term study (6 weeks) were carried out to evaluate the protective effect against gut and internal organ colonization after vaccination with *Salmonella* Typhimurium strain Nal2/Rif9/Rtt, a strain contained in the commercially available live vaccines AviPro® *Salmonella* Duo and AviPro® *Salmonella* VacT, at day of hatch.

Materials and Methods

Experimental Birds

One-day-old Lohmann Brown laying type chicks were obtained from a local commercial hatchery (De Biest, Kruishoutem, Belgium). Experimental groups were housed in separate rooms in containers (3 m^2) on wood shavings. Commercial feed and drinking water was provided ad libitum. The animals received 12 h of light per day. The birds were confirmed to be *Salmonella*-free by bacteriological analysis of cloacal swabs. All of the animal experiments in this study followed the institutional guidelines for the care and use of laboratory animals and were approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University, Belgium. Euthanasia was performed humanely with an overdose of sodium pentobarbital (Sigma-Aldrich, St. Louis, MO).

Bacterial Strains and Growth Conditions

Salmonella Typhimurium strain Nal2/Rif9/Rtt, contained in the commercially available live vaccines AviPro® *Salmonella* Duo and AviPro® *Salmonella* VacT, is a metabolic drift mutant of *Salmonella* Typhimurium produced by chemical mutagenesis (Linde, 1981) and is resistant to nalidixic acid and rifampicin. The vaccine strain was suspended in sterile Hank's Balanced Salt Solution (HBSS, Invitrogen, Paisley, England) according to the manufacturer's protocol to obtain the appropriate dilution. The monophasic variant of *Salmonella* Typhimurium 1,4,[5],12:i:- (strain number 06-01900) was used as a challenge strain and is resistant to carbenicillin. The strain was originally isolated from a hospitalized human patient with diarrhea. It exhibits the characteristics of the new epidemic type (seroformula 1,4,[5],12:i:-). Before use in the trials, the strain was statically incubated overnight at 37°C in Luria Bertani (LB) medium (Sigma, St. Louis, MO, USA). After overnight incubation, ten-fold dilutions were plated on brilliant green agar (BGA, Oxoid, Hampshire, UK) and incubated overnight to determine the titer. The culture of the challenge strain was put at 4°C overnight, and the bacterial suspension was diluted in phosphate buffered saline (PBS, Sigma, St. Louis, MO, USA) to the desired colony forming units (cfu) per ml.

Experimental Design

In total 3 different independent experimental studies were set up in order to evaluate the colonization-inhibiting potential of the vaccine strain against the *Salmonella* Typhimurium 1,4,[5],12:i:- strain.

Trial 1. Trial one was conducted to evaluate the ability of the *Salmonella* Typhimurium strain Nal2/Rif9/Rtt to protect against a low dose challenge of *Salmonella* Typhimurium 1,4,[5],12:i:-. One-day-old chicks were orally immunized through crop instillation of 0.5 ml containing 10^8 cfu *Salmonella* Typhimurium strain Nal2/Rif9/Rtt (n=30). The control group (n=30) was kept as non-immunized control and was given 0.5 ml sterile PBS. The next day, the groups were infected with the monophasic variant of serotype Typhimurium, serotype 1,4,[5],12:i:-, through crop instillation of 0.5 ml containing 10^3 cfu (low dose). Cloacal swabs were taken one week after the infection and analyzed as described below. At the same time, 10 animals per group were euthanized. Samples of the spleen and caeca were aseptically removed and analyzed as described below (bacterial recovery from organs). The remaining

animals were euthanized 14 days post infection (pi). Enumeration of *Salmonella* in the spleen and caeca was performed as described below (bacterial recovery from organs).

Trial 2. In the second trial chicks were orally immunized on day of hatch as described above for trial 1 (n=30), or kept as non-immunized controls (n=30). The next day, the groups were infected with the monophasic variant of serotype Typhimurium 1,4,[5],12:i:-, through crop instillation of 0.5 ml containing 10^8 cfu (high dose). Cloacal swabs were taken at day 3, 6 and 11 pi. Samples of the spleen and caeca were aseptically removed on day 7 (n=10) and 14 (n=20) pi.

Trial 3. In trial 3, one-day-old chicks were orally immunized on day of hatch through crop instillation of 0.5 ml containing 10^8 cfu *Salmonella* Typhimurium strain Nal2/Rif9/Rtt (n=75) or kept as non-immunized controls (n=75). Twenty-four hours later, 15 randomly selected chicks in each group were tagged and infected with 10^5 cfu (intermediate dose, seeder birds) and housed together with the non-infected chicks. Cloacal swabs were taken at day 3, 9, 16, 23 and 30. Samples of the spleen, caeca and liver were taken at day 7, 21 and 42. At each sampling 1/3 of the chicks were euthanized (of which 5 were seeder birds at each time point). At the end of the trial, litter samples were collected.

Bacteriological Analysis of Cloacal Swabs

Cloacal swabs were taken at different time points and bacteriologically examined to evaluate the shedding of the *Salmonella* strains. In order to quantify shedding of the challenge strain (*Salmonella* Typhimurium 1,4,[5],12:i:-), the swabs were directly inoculated on Brilliant Green Agar (BGA) plates supplemented with 100 µg/ml carbenicillin. Additionally in the third trial, the swabs were directly inoculated on BGA supplemented with 100 µg/ml rifampicin to quantify shedding of the *Salmonella* Typhimurium strain Nal2/Rif9/Rtt. Swabs negative after direct inoculation were pre-enriched in buffered peptone water (BPW, Oxoid, Basingstoke, Hampshire, UK) and incubated overnight at 37°C. One ml of this BPW suspension was further enriched by adding nine ml tetrathionate-brilliant green broth (TETRA, Oxoid, Basingstoke, Hampshire, UK). After overnight incubation at 37°C a loopful of this suspension was plated on BGA supplemented with the appropriate antibiotic. Litter samples were plated out on BGA supplemented with 100 µg/ml rifampicin to detect the *Salmonella* Typhimurium strain Nal2/Rif9/Rtt in the third trial.

Bacteriological Analysis of Organs

Samples of caecum and spleen were manually homogenized in BPW (10 % weight/volume suspensions) and 10-fold dilutions were made in HBSS (Invitrogen, Paisley, England). Six droplets of 20 µl of each dilution were plated on BGA supplemented with 100 µg/ml carbenicillin (for quantification of the *Salmonella* 1,4,[5],12:i:- strain) or 100 µg/ml rifampicin (for quantification of the *Salmonella* Typhimurium vaccine strain Nal2/Rif9/Rtt in the third trial). 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. Negative samples were enriched as described above.

Statistical Analysis

GraphPad Prism 5 software was used for statistical analysis. Data of cfu *Salmonella*/gram tissue were log-transformed and analyzed by a student's t–test to determine differences between the groups. Differences with p-values below 0.05 were considered to be statistically significant. After enrichment samples were classified as either positive or negative. A Fisher's exact test was used to determine significant differences ($p<0.05$). Cloacal swabs were analyzed in the same way.

Results

Analysis of Cloacal Swabs: Evaluating Shedding of *Salmonella* 1,4,[5],12:i:- and the Vaccine Strain (*Salmonella* Typhimurium strain Nal2/Rif9/Rtt)

During the first trial (low challenge dose), shedding of the challenge strain was only observed in the challenge-control group (Table 1). In the second trial (high challenge dose), one chick died in the vaccinated group during administration of the vaccine. There was a significant difference in shedding of the challenge strain at day 3 ($p<0.0001$), 6 ($p<0.0001$) and 11 ($p=0.0019$) between the vaccinated and the control group. In the third trial (seeder bird model, intermediate dose) there was a significant difference in shedding of the challenge strain between vaccinated and control animals at day 9 ($p=0.0005$), 23 ($p=0.0181$) and 30 ($p=0.0181$). Shedding of the vaccine strain *Salmonella* Typhimurium Nal2/Rif9/Rtt could not be detected anymore in trial 3 at day 23, while only 1 animal out of 50 was positive at day

16. At the end of the third trial litter samples were collected and analyzed. The vaccine strain *Salmonella* Typhimurium Nal2/Rif9/Rtt could not be isolated.

Table 1. The number of cloacal swabs positive for *Salmonella* 1,4,[5],12:i:- and *Salmonella* Typhimurium strain Nal2/Rif9/Rtt at direct plating and after enrichment Trial 1: vaccination at day 1 (10^8 cfu) and infection the next day (10^3 cfu); Trial 2: vaccination at day 1 (10^8 cfu) and infection the next day (10^8 cfu); Trial 3: vaccination at day 1 (10^8 cfu), infection at day 2 (seeder birds were infected with 10^5 cfu of the challenge strain)

	Strain	days pi	3	6	7	9	11	16	23	30
trial 1		control			0/30[a] (3)[b]					
		VacT			0/30 (0)					
trial 2	*Salmonella* Typhimurium 1,4,[5],12:i:-	control	30/30 (30)	29/30 (30)			17/20 (18)			
		VacT	17/29*** (29)	15/29*** (21)**			6/20** (17)			
trial 3		control				28/50 (50)		24/50 (50)	10/25 (15)	10/25 (17)
		VacT				19/50 (39)**		19/50 (36)	2/25* (18)	2/25* (12)
	Salmonella Typhimurium strain Nal2/Rif9/Rtt	control	0/75 (0)			0/50 (0)		0/50 (0)	0/25 (0)	0/25 (0)
		VacT	17/75 (57)			15/50 (45)		0/50 (1)	0/25 (0)	0/25 (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 for the monophasic variant between the control and vaccinated group ($p<0.0001$)

**Significant difference in positive samples for the monophasic variant between the control and vaccinated group ($p<0.005$)

*Significant difference in positive samples for the monophasic variant between the control and vaccinated group ($p<0.05$)

Analysis of Gut and Internal Organ Samples: Evaluation of the Colonization-Inhibiting Potential of *the Salmonella* Typhimurium Strain Nal2/Rif9/Rtt.

In the first trial, bacterial enumeration of the organs showed that vaccination significantly decreased colonization of the caeca on day 7 (at direct plating and after enrichment, $p=0.0351$ and $p=0.0039$, respectively) and day 14 (at direct plating and after enrichment, $p=0.0471$ and $p=0.0033$, respectively; table 2).

Table 2. The number of caecal or spleen samples positive at direct plating and after enrichment for *Salmonella* Typhimurium 1,4,[5],12:i:- during trial one and two Trial 1: vaccination at day 1 (10^8 cfu) and infection the next day (10^3 cfu) Trial 2: vaccination at day 1 (10^8 cfu) and infection the next day (10^8 cfu)

	Organs	Groups	trial 1	trial 2
Day 7 post inoculation	Caecum	Control	5/10[a] (7)[b]	10/10 (10)
		Vaccinated	0*/10 (0**)	10/10 (10)
	Spleen	Control	0/10 (1)	4/10 (10)
		Vaccinated	0/10 (0)	0/10 (0**)
Day 14 post inoculation	Caecum	Control	5/20 (10)	20/20 (20)
		Vaccinated	0*/20 (1**)	15**/19 (15)
	Spleen	Control	0/20 (1)	3/20 (14)
		Vaccinated	0/20 (0)	0/19 (1***)

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

[b]Number of positive samples after enrichment

***Significant difference in positive samples for the monophasic variant between the control and vaccinated group ($p<0.0001$)

**Significant difference in positive samples for the monophasic variant between the control and vaccinated group ($p<0.005$)

*Significant difference in positive samples for the monophasic variant between the control and vaccinated group ($p<0.05$)

In the high challenge dose study (second trial), a reduction in spleen colonization was seen in vaccinated animals at day 7 ($p=0.0001$; after enrichment; table 2). On day 14 a significantly lower caecum ($p=0.0006$; at direct plating) and spleen ($p<0.0001$; after enrichment) colonization was seen in the vaccinated group. Colonization of the challenge strain in the caeca was lower in the vaccinated group on day 7 ($p<0.0003$) and day 21 ($p=0.0105$) in the third trial (figure 1).

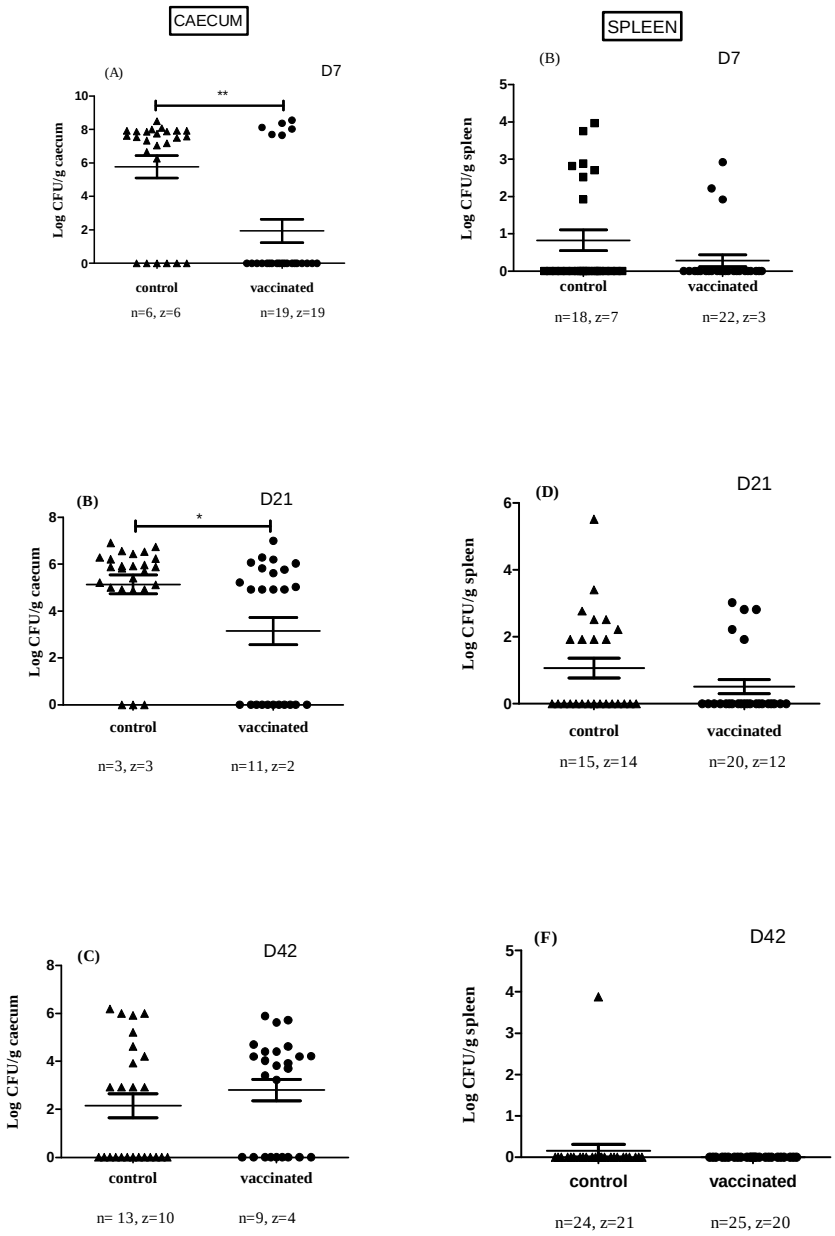

Figure 1. Caecal (A,C,E) and spleen (B,D,F) colonization of the *Salmonella* Typhimurium 4,12:i:- challenge strain in trial 3. Animals (n=25) were orally challenged 24 hours after vaccination (*Salmonella* Typhimurium strain Nal2/Rif9/Rtt) or not (control). Subfigures A and B represent colonization on day seven, C and D on day 21, and E and F on day 42. Represented values are ^{10}log of cfu/g sample. The middle horizontal line represents the mean, the error bars represent the standard error of the mean (SEM). 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. Asterisks indicate a difference between the groups. (* equals p<0.05 and ** equals p<0.005)

DISCUSSION

In the current study, it was shown that oral administration of the *Salmonella* Typhimurium strain Nal2/Rif9/Rtt, included in the commercially available live vaccines AviPro® *Salmonella* Duo and AviPro® *Salmonella* VacT, at day of hatch, reduces colonization with a strain of the monophasic variant of *Salmonella* Typhimurium, 1,4,[5],12:i:- after challenge at day 2. It is of paramount importance that day-old chicks are protected as early as possible because infection with field strains often occurs within the first week of life. At this age, the autochthonous intestinal microbiota is not fully mature and the animal's immune system is not yet fully developed (Bar-Shira and Friedman, 2006). Early protection of chickens after oral administration of a live vaccine strain at day 1 against a challenge strain administered already at day 2 can be conferred by a phenomenon called colonization-inhibition (Bohez et al., 2008; Bohez et al., 2007; De Cort et al., 2013; Methner et al., 1999). The exact mechanism is unknown but the exclusion phenomenon can be modelled *in vitro* in test tubes, indicating a microbiological exclusion effect (Barrow et al., 1987). This colonization-inhibition phenomenon has until now only been recognized between strains of the same serotype (Barrow et al., 1987). The *Salmonella* 1,4,[5],12:i:- serotype is Typhimurium-like and can thus, as shown in this study, also be controlled in the early immune deprived stage by using live *Salmonella* Typhimurium vaccines.

In addition, live vaccines may stimulate innate immunity, which may help to protect against invasion and systemic spread of *Salmonella* to internal organs (Methner et al., 1997). Indeed, in different studies the expression of CXC chemokines and subsequent infiltration of the intestinal mucosa by immune cells, of which heterophilic granulocytes are the first, were observed after administration of live *Salmonella* Typhimurium strains (Withanage et al., 2004; Withanage et al., 2005). Although our study did not investigate long-term protection conferred by the live vaccine, typically the observed protective effect would require cell-mediated immune responses (Chappell et al., 2009).

In practice, the live vaccines containing the *Salmonella* Typhimurium strain Nal2/Rif9/Rtt are recommended to be administered at day 1, week 7 and week 16. Vaccination with the commercially available AviPro® *Salmonella* VacT and AviPro® *Salmonella* Duo is recommended for vaccination of layer flocks, parent flocks and grandparent flocks against

Salmonella Typhimurium. Data provided in the current study show that early vaccination already protects the animals against challenge with a *Salmonella* 1,4,[5],12:i:- at day 2 post-challenge. This also implies that the live vaccine can in theory protect broilers when delivered at day 1. Although the colonization-inhibition phenomenon can thus help in protecting young chickens against infection, also other methods need to be implemented on-farm to control *Salmonella*. These include biosecurity measures and potentially the use of feed additives that limit *Salmonella* colonization.

In summary, oral administration of the *Salmonella* Typhimurium strain Nal2/Rif9/Rtt, a strain present in the commercially available live vaccines AviPro® *Salmonella* Duo and AviPro® *Salmonella* VacT, at day of hatch, is able to limit shedding and caecal colonization of a *Salmonella* 1,4,[5],12:i:- strain that is administered at day 2 of life. This is of value for layers and breeders as well as for broilers and can be part of a control program for the new emerging serotype 1,4,[5],12:i:-.

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3.2 *Salmonella* Enteritidis flagellar mutants have a colonization benefit in the chicken oviduct.

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Salmonella Enteritidis flagellar mutants have a colonization benefit in the chicken oviduct.

Abstract

Egg borne *Salmonella* Enteritidis is still a major cause of human food poisoning. Eggs can become internally contaminated following colonization of the hen's oviduct. In this paper we aimed to analyze the role of flagella of *Salmonella* Enteritidis in colonization of the hen's oviduct. Using a transposon library screen we showed that mutants lacking functional flagella are significantly more efficient in colonizing the hen's oviduct *in vivo*. A micro-array analysis proved that transcription of a number of flagellar genes is down-regulated inside chicken oviduct cells. Flagella contain flagellin, a pathogen associated molecular pattern known to bind to Toll-like receptor 5, activating a pro-inflammatory cascade. *In vitro* tests using primary oviduct cells showed that flagellin is not involved in invasion. Using a ligated loop model, a diminished inflammatory reaction was seen in the oviduct resulting from injection of an aflagellated mutant compared to the wild-type. It is hypothesized that *Salmonella* Enteritidis downregulates flagellar gene expression in the oviduct and consequently prevents a flagellin-induced inflammatory response, thereby increasing its oviduct colonization efficiency.

Introduction

Salmonella (S.) enterica is a major cause of food poisoning worldwide. Most outbreaks are due to subspecies *enterica* serovar Enteritidis contamination of eggs (De Reu et al., 2006). Contaminated eggs however, usually don't present any signs of microbial alteration (EFSA, 2007.). Furthermore, laying flocks infected with *S.* Enteritidis usually show no symptoms, nor a decline in egg production (Kaiser et al., 2000). How *S.* Enteritidis is capable of causing such insidious infections in laying hens is a puzzling question that remained largely unanswered since at least two decades. More recently, it was shown that strains from the serotype Enteritidis are superior to other serotypes in colonizing the oviduct of chickens without causing overt clinical signs (Gantois et al., 2008b; Okamura et al., 2001; Raspoet et al., 2011). The isthmus and magnum of the oviduct are the predominant colonization sites (De Buck et al., 2004a).

Very little is known about the mechanisms allowing *S.* Enteritidis to persistently colonize the hen's oviduct. Temporary regression of certain highly expressed beta defensins of the chicken oviduct cells by the *S.* Pathogenicity Island-2 encoded type III secretion system avoids antimicrobial killing (Ebers et al., 2009). For sure, one of the hallmarks of oviduct colonization by *S.* Enteritidis is the relative lack of inflammation and cellular damage, which may play a role in the persistence of oviduct colonization and consequently stable egg production (Kaiser et al., 2000). Nevertheless, *S.* Enteritidis does carry microbial associated molecular patterns (MAMPs), such as LPS and flagellin, which bind to Toll-like receptors (TLRs) on epithelial cells. Binding of MAMPs to TLRs should normally initiate the innate immune response, leading to inflammation and tissue damage. Flagellin is the main structural protein of the bacterial flagellum and binds to TLR5. Flagellin/TLR5 signaling triggers several mechanisms that activate the pro-inflammatory cascade in various epithelial cells (Eaves-Pyles et al., 2001; Hayashi et al., 2001; Steiner, 2007). The importance of the TLR-5 activation pathway in clearance of bacterial pathogens is well documented (Vijay-Kumar et al., 2007).

The TLR5 receptor has been identified in the theca and granulosa of the ovary as well as in the glandular epithelial cells of the oviduct in laying hens (Woods et al., 2009). Considering the presence of TLR5, it is even more remarkable that *S.* Enteritidis is able to avoid inflammation while colonizing the hen's oviduct. Therefore, in the present study we

investigated the role of flagella in oviduct colonization by *S.* Enteritidis. More specifically, a transposon library screen was performed in order to evaluate the behavior of flagellar gene mutants in oviduct colonization. Using a microarray, flagellar gene transcription was evaluated in oviduct cells. We investigated the role of flagella in adhesion to and invasion in oviduct gland cells. Finally, we studied the effect of flagellin on oviduct cells *in vivo* by comparing inflammatory cell infiltration after injection of an aflagellated mutant (Δ*fliG*) in oviduct ligated loops compared to the *S.* Enteritidis wild-type. Based on these data, it was concluded that *S.* Enteritidis downregulates flagellar gene expression in the chicken oviduct, hereby avoiding inflammation, which may be essential for persistent colonization.

Materials and Methods

Salmonella Enteritidis strain and ΔfliG mutant construction

Salmonella (S.) Enteritidis phage type 4 strain 147 was used for the experiments. This strain is streptomycin resistant and was originally isolated from egg white. *S.* Enteritidis phage type 4 strain 147 is known to colonize the gut and internal organs to a high level (Bohez et al., 2008; Methner et al., 1995). *S.* Gallinarum strain was originally isolated from egg white. Δ*fliG* is an aflagellate mutant of *S.* Enteritidis 147 phage type 4 lacking the *fliG* gene. This gene encodes one of the switch proteins of *Salmonella* bacteria located towards the cytoplasmic face of the M ring of the flagellar basal body (Francis et al., 1992). This mutant was constructed according to the one step inactivation method previously described (Datsenko and Wanner, 2000). The targeted gene was deleted from start to stop codon, as confirmed by sequencing.

Evaluation of the behavior of S. Enteritidis flagellar gene mutants in isolated oviduct cells (in vitro) and in the hen's oviduct (in vivo), using a transposon library

Primary chicken oviduct epithelial cells (OEC) were harvested from seven Lohman Brown pullets (obtained from a local hatchery) according to the isolation method developed by Jung-Testas (Jung-Testas et al., 1986). One day after the final estradiol-benzoate administration the 13 to 15 week old chickens were euthanized with an overdose of pentobarbital. Non-adhering oviduct cells were removed and seeded in tissue culture 24-well plates at 1 x 10^6 cells/ml. The 24-well plates had been coated for 24 hours with Bovine Collagen Solution (Purecol®, Advanced Biomatrix, San Diego, USA, 1ml/well). Two days post-

isolation, the wells were evaluated for confluent growth and used for *in vitro* experiments. The experimental protocol was approved by the ethical committee of the Faculty of Veterinary Medicine, Ghent University (no 2013_34).

Details on library construction can be consulted elsewhere (Badarinarayana et al., 2001; Chan et al., 2005; Lawley et al., 2006; Raspoet et al., 2014). For the identification of *S.* Enteritidis genes involved in intracellular oviduct cell persistence, oviduct cells were isolated and cultured. The *S.* Enteritidis transposon library (initial library) was grown for 7 h at 37°C in LB medium (Sigma-Aldrich, ST. Louis, USA) with agitation in the presence of streptomycin (200 µg/ml) and kanamycin (30 µg/ml). The bacterial suspension was added to the oviduct tubular gland cells at a concentration of 10^7 cfu/ml (multiplicity of infection (MOI) 10:1). The plates were centrifuged for 10 min at 524 *g*. The cells were incubated for 1 h at 37°C and rinsed three times with Hanks Balanced Salt Solution (HBSS), and then cell culture medium containing gentamicin (100 µg/ml; Gibco/Invitrogen) was added. After 1 h, the gentamicin concentration was lowered to 30 µg/ml, and the cells were incubated for another 14 h. The plates were rinsed three times with HBSS, and the cells were lysed using 1% Triton X-100 (Sigma-Aldrich, ST. Louis, USA). The plates were placed on an MTS 2/4 digital microtiter plate shaker (IKA, Staufen, Germany) for 10 min at maximum speed. Afterward, HBSS was added, and the bacteria were collected. Harvested intracellular bacteria (output library) were grown in LB medium with streptomycin and kanamycin for 7 h and then used for a second round of invasion. In all, three subsequent enrichment passages were performed, and the experiment was repeated in five independent replicates.

For the identification of genes involved in oviduct colonization *in vivo,* three 21-week-old commercial laying hens (Lohman Brown) were pre-medicated intramuscularly with buprenorphine hydrochloride at 0.05 mg/kg (Temgesic; Schering-Plough, Kenilworth, NJ) and atropine at 0.05 mg/kg. Anesthesia was induced by the administration of isoflurane (Schering-Plough). After intubation with a 3.0-mm uncuffed tracheal tube (Hudson RCI, Temecula, CA), a continuous oxygen flow of 1.5 to 2.0 liters/min was administered carrying 1.5 to 3% isoflurane. The oviduct segments were carefully exposed. The oviduct was inoculated with 1 ml of the bacterial suspension at the isthmus-magnum transition zone (Vicryl^{TM} Plus, Johnson & Johnson, Diegem, Belgium). A 7-h-old culture of the *S.* Enteritidis transposon library was centrifuged and diluted in HBSS until 10^7 colony forming units

(cfu)/ml were obtained. After inoculation, the oviduct was reintroduced into the abdomen, and the abdominal wall was sutured. After recovery from anesthesia, the birds were placed in separate cages on wood shavings. The animals had unrestricted access to drinking water and feed. The hens were euthanized 2 days after infection by an overdose of sodium pentobarbital (Sigma-Aldrich, St. Louis, USA). The oviducts were aseptically removed and opened longitudinally. Oviducts were rinsed three times in HBSS supplemented with 100 µg/ml gentamicin to kill extracellular bacteria. Tubular gland cells were isolated according to the isolation method developed by Jung-Testas (Jung-Testas et al., 1986) but with an additional 50 µg of gentamicin/ml in all enzyme solutions and without penicillin and streptomycin until the cells were lysed with 1% Triton X-100 for 10 min, after which the bacteria were harvested. Microarray hybridization was performed as (Raspoet et al., 2014).

Enteritidis *gene transcription analysis in oviduct cells*

The *S.* Enteritidis 147 strain was grown overnight in Luria Bertani (LB) broth (Sigma-Aldrich, ST. Louis, USA), supplemented with streptomycin (100µg/ml, Sigma-Aldrich, ST. Louis, USA). After overnight incubation, bacterial cultures were centrifuged at 4000 g for 10 minutes and re-suspended in cell culture medium without foetal calf serum (FCS). Ten-fold dilutions were plated on LB supplemented with streptomycin (100µg/ml) and incubated overnight to determine the number of cfu. The culture was kept at 4°C overnight. The bacterial suspensions were diluted in cell culture medium to the desired cfu/ml. Primary chicken magnum cells were seeded at 1 x 10^6 cells/ml and were allowed to adhere for 48h (37°C, 5% CO_2). Subsequently the cells were washed twice with HBSS. Infection was carried out using an MOI of 10:1. The cells were incubated for 4 hours with the bacteria after centrifugation (5 min, 1200 g). A gentamicin protection assay was performed and after 4 hours the cells were lysed and intracellular bacteria were recovered (Metcalfe et al., 2010). A detailed description of the microarray procedure is described in the study of Raspoet *et al.* (Raspoet et al., 2014). For the comparative control, the strain was grown in LB medium until mid-exponential phase was reached (OD_{600}: 0.6). Significantly different transcribed *Salmonella* genes in magnum cells 4h post-infection relative to the comparative control were identified ($p<0.05$). Signal values of the output library were normalized against those of the initial library and used to identify mutants for which the gene value had at least a 2-fold increase (fold difference < 0.5) after the selection procedure compared to the initial library grown in LB. Significance of

the centered data, at p≤0.001 for *'in vitro'* tests and p≤0.05 for *'in vivo'* tests, was determined using a parametric-based statistical test adjusting the individual p-value with the Benjamini and Hochberg false discovery rate multiple test correction (Noda et al., 2010). As the microarray is mainly annotated for *S.* Typhimurium, gene sequences were used in a BLAST search to look for their *S.* Enteritidis (SEN) homologues.

Adhesion to and invasion of S. Enteritidis wild type and ΔfliG in isolated oviduct cells

The *S.* Enteritidis 147 strain and the *ΔfliG* mutant were grown overnight in LB broth (Sigma, ST. Louis, USA), supplemented with streptomycin (100µg/ml, Sigma, ST. Louis, USA). *S.* Gallinarum was also grown overnight in LB broth (Sigma, ST. Louis, USA) without the addition of antibiotics. After overnight incubation, bacterial cultures were centrifuged at 4000 g for 10 minutes and re-suspended in cell culture medium without FCS. Ten-fold dilutions were plated on LB supplemented with streptomycin (100µg/ml) or without antibiotics for the *S.* Gallinarum strain and incubated overnight to determine the number of cfu. The cultures of the strains were kept at 4°C overnight. The bacterial suspensions were diluted in cell culture medium to the desired cfu per ml. Two 24-well plates of primary chicken OEC were seeded at 1 x 10^6 cells/ml and were allowed to adhere for 48h (37°C, 5% CO2). Subsequently the cells were washed twice with HBSS and incubated for 2 hours with the bacteria. Infection was carried out at an MOI of 10:1. Following the 2h incubation, the inoculum was removed from each well and oviduct cells were washed 3x with HBSS. Bacterial invasion and adhesion was determined as described by Metcalfe *et al*. (Metcalfe et al., 2010). Cfu were counted after incubation for 24h at 37°C. Intracellular and associated bacteria were quantified by calculating the number of cfu in the homogenate. Adherent bacteria were calculated by subtracting the intracellular bacteria from the associated bacteria. In total 3 biological repeats were performed.

2.5 Determination of inflammation in a ligated loop model

Commercial Lohmann Brown laying hens (obtained from a local hatchery) of 21 weeks old were brought under anesthesia as described in section 2.2. Three loops/chicken (experimental, in-between and control loop) were ligated in the magnum using surgical suture (Vicryl™ Plus, Johnson & Johnson, Diegem, Belgium). The ligated loops were 1.5 to 2.0 cm long. Sufficient blood supply was ensured to all separate loops. In total 6 hens were

used for this experiment. On 3 separate days, each day 2 hens were used and ligated loops were constructed in which either 1 ml of the *S.* Enteritidis 147 wild type (10^7 cfu/ml) or the *Δfli*G (10^7 cfu/ml) mutant were injected in the experimental loop using a 27 gauge needle. Each time the included control loop contained pure HBSS. The bacterial cultures were prepared overnight as described in section 2.1. After injection of the loops, 2 ml of HBSS containing 400 µg/mL of gentamicin (Thermo Fisher Scientific, Erembodegem, België) was sprayed over the serosal side of the loops and the loops were reintroduced into the abdomen. After 6 hours the hens were euthanized. Samples of the ligated loops were put in formalin. A haematoxylin-eosin staining was performed on the oviduct ligated loop samples. This allowed visualization of recruited immune cells. A scoring system based on histopathological descriptions for experimental infection of the chicken described by Withange *et al*. (Withanage et al., 2005) was used to evaluate the inflammatory state of the oviduct tissue (table 1). After looking at all the samples, scoring was performed blinded for 10 random fields (20X enlargement) by a board certified pathologist. The experimental protocol was approved by the ethical committee of the Faculty of Veterinary Medicine, Ghent University (EC2015/25).

Table 1. Scoring system used for evaluation of inflammation in the oviduct wall.

score	histopathology (haematoxylin-eosin)
0	Normal
1	Small increase in dispersed heterophils
2	Increased numbers of heterophils throughout the tissue, small foci of heterophils.
3	Small increase in heterophil numbers in longitudinal folds or underlying epithelium
4	Increased numbers of heterophils associated with epithelium and lamina propria
5	Extensive influx of heterophils, necrotic damage

Statistical analysis

All data were analyzed with GraphPad Prism 5 software. For the invasion and adhesion tests, a non-parametric Kruskal-Wallis test was performed, followed by a Dunns multiple comparison test to determine significant differences. The same statistical tests were used to determine significant differences between the groups for the scoring of the oviduct loops. For all tests, differences with p-values below 0.05 were considered to be statistically significant.

Results

Evaluation of the behavior of S. Enteritidis gene mutants in isolated oviduct cells (in vitro) and in the hen's oviduct (in vivo), using a transposon library

The technique using the transposon library identifies mutants harboring transposon insertions in genes that are either important for persistence or multiplication in oviduct cells, or mutants that have an advantage in oviduct colonization. Mutants harboring insertions in genes, leading to decreases in persistence in oviduct cells were described in a paper by (Raspoet *et al*., 2014). Here we report mutations leading to a significantly increased intracellular presence in oviduct cells *in vitro* and an increased colonization level in the hen's oviduct *in vivo*. The list of genes that are truncated and lead to increased intracellular presence in isolated oviduct cells (*in vitro*) as well as a significantly increased intracellular presence in the hen's oviduct (*in vivo*), is shown in table 2. The genes flgE, flgL, fliF encode

structural proteins; flhB, flgN, fliI, flgM and fliK are related to the assembly and function of flagellin.

Table 2. Genes involved in intracellular persistence in isolated oviduct cells (*in vitro*) as well as a significantly increased intracellular presence in the hen's oviduct (*in vivo*).

gene symbol	locus tag	gene description
flgE	SEN1871	flagellar hook protein FlgE
flgL	SEN1864	flagellar hook-associated protein FlgL
flgM	SEN1876	anti-sigma-28 factor FlgM
flgN	SEN1877	flagella synthesis protein FlgN
flhB	SEN1089	flagellar biosynthesis protein FlhB
fliF	SEN1040	flagellar MS-ring protein
fliI	SEN1037	flagellum-specific ATP synthase
fliK	SEN1035	flagellar hook-length control protein

Mutations in the genes listed in Table 2 resulted in a significantly increased intracellular persistence of *Salmonella* in oviduct cells in an *in vitro* assay ($p<0.001$) and after inoculation in the hen's oviduct *in vivo* ($p<0.05$).

S. Enteritidis *flagellar gene transcription analysis in oviduct cells*

Salmonella flagellar genes of which the transcription significantly differed intracellularly in oviduct cells relative to the LB control are listed in table 3. In total 27 flagellar genes were downregulated inside oviduct cells. Gene characteristics range from assembly of the flagellar body (flgA, flgD, flgL, flgN, fliH, fliO, fliP), structural proteins (flgB,flgE to flgJ, fliE to fliG, fliJ, fliK to fliN, fliS to fliY) to regulation of its activity (fliA, fliG, fliM, fliZ).

Table 3. List of flagella-related genes of which the transcription significantly differed intracellulary in oviduct cells relative to the LB control.

Gene symbol	Fold decrease vs LB	Gene symbol	Fold decrease vs LB
flgA	5.38	*fliG*	6.78
flgB	10.72	*fliH*	4.02
flgD	33.92	*fliJ*	6.76
flgE	15.22	*fliK*	3.93
flgF	9.79	*fliL*	6.25
flgG	5.72	*fliM*	23.28
flgH	7.22	*fliN*	3.58
flgI	7.67	*fliO*	2.56
flgJ	9.52	*fliP*	3.71
flgL	8.12	*fliS*	14.84
flgN	3.29	*fliT*	5.00
fliA	2.88	*fliY*	2.20
fliE	3.09	*fliZ*	7.43
fliF	4.37		

The expression of flagella-related genes was significantly (more than two-fold, $p<0.05$) down regulated in hens magnum cells 4h post infection compared with log phase LB.

Quantification of intracellular and adherent bacteria

The fraction of bacteria that were able to adhere and invade in chicken OEC was determined. There was no significant difference in the percentage of adherent bacteria to the oviduct cells between the strains (figure 1). The *S.* Enteritidis 147 parent strain was significantly more invasive in oviduct cells compared to the aflagellated *S.* Gallinarum strain ($p<0.05$). This was not the case for the aflagellated *S.* Enteritidis 147 *Δfli*G mutant strain.

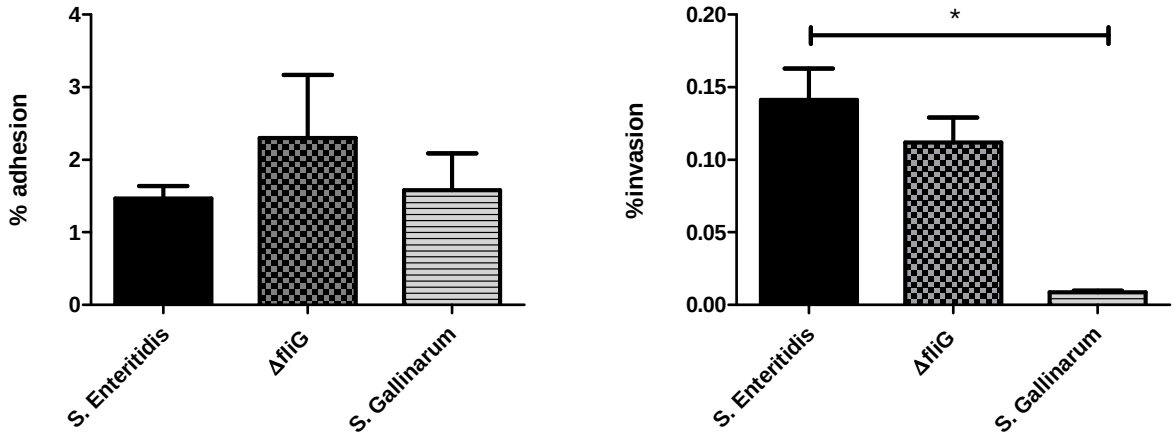

Figure 1. Percentage of adhesion and invasion in oviduct epithelial cells. Values shown are means and SEM from three independent experiments for *S.* Enteritidis 147, *S.* Gallinarum, *ΔfliG* (deletion strain in *S.* Enteritidis 147). Asterisks indicate significance (p<0.05).

Scoring of inflammation in the ligated oviduct loop

Results obtained from scoring the inflammatory state of the oviduct wall are presented in figure 2. *S.* Enteritidis attracted significantly more heterophils than its aflagellated mutant *ΔfliG*.

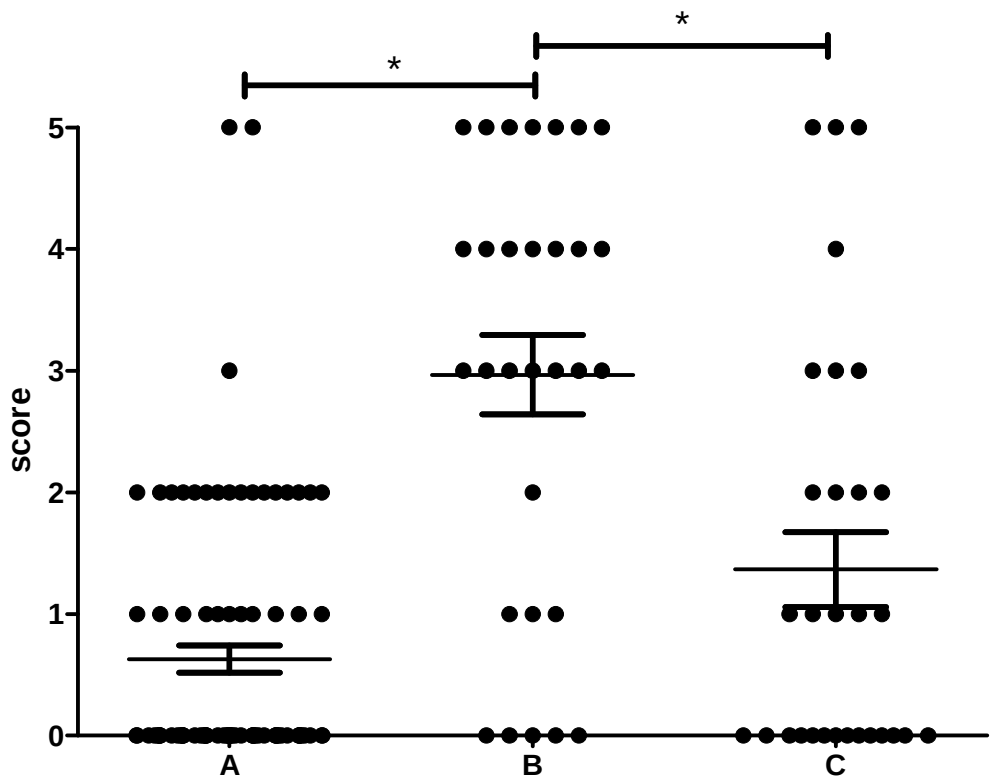

Figure 2. Heterophilic granulocyte infiltration scored in ligated oviduct loops. Scoring was performed 6 hours after injection of HBSS (A), *S.* Enteritidis (B) and *ΔfliG* mutant of *S.* Enteritidis (C). In total, 3 loops were analyzed per strain. Haematoxylin-eosin staining was performed on the oviduct samples. Scoring was done blinded and based upon 10 random fields (20X enlargement) of each oviduct sample. Significant differences are indicated by an asterisk.

DISCUSSION

Multiple hypotheses have been put forward in order to explain why the *Salmonella (S.)* serotype Enteritidis has been successful in contaminating eggs. The best known hypotheses are based on the observations that the serotype Enteritidis is capable of colonizing the chicken oviduct without causing pathological changes and its superior survival in egg white as compared to other serotypes (Coward et al., 2013; De Vylder et al., 2013; Raspoet et al., 2014; Raspoet et al., 2011). The strategy used by *S.* Enteritidis to persistently colonize the chicken oviduct without causing inflammation however, remained hitherto largely

unexplained (De Buck et al., 2004b; Gantois et al., 2008a). In the present study, we investigated the role of flagella in chicken oviduct colonization. Although the presence of flagella has been reported to be essential for the full invasive potential of *Salmonella* strains in various tissue cultures (Jones et al., 1992; Schmitt et al., 2001), no studies regarding flagellin were done yet with primary chicken oviduct epithelial cells (OEC). *S.* Enteritidis grown in peritoneal cavities of chickens do not express flagella (Chart et al., 1993). Here we report that absence of flagella in *S.* Enteritidis does not significantly affect invasiveness in chicken OEC. However, we also found that expression of flagella by *S.* Enteritidis is downregulated following colonization of the chicken oviduct and in chicken OEC. Moreover, using a transposon library screen, we showed that flagellar mutants have a colonization advantage in the chicken oviduct. Downregulation of flagella expression thus appears to be important for successful oviduct colonization by *S.* Enteritidis. In addition, an aflagellate mutant *ΔfliG* of *S.* Enteritidis attracted less heterophilic granulocytes in a ligated oviduct loop model, thus escaping the host's primary inflammation reaction.

This is in accordance with the behavior of aflagellate mutants in other *Salmonella* serotypes. Indeed, lack of flagella in *S.* Dublin correlates with a reduced early inflammation in the ceca of mice (Yim et al., 2014). Similarly, lack of flagella in *S.* Typhimurium is associated with reduced heterophil influx in experimentally infected chickens (Pan et al., 2012). Conversely, when flagella are expressed in a mutant of the naturally aflagellated *S.* serovar Gallinarum biovar Gallinarum, the flagellated mutant induces a higher expression of inflammatory cytokines in chicken kidney cells compared to the parent strain. Also, mortality rates are lower in birds challenged with a flagellated Gallinarum mutant compared to the wild-type Gallinarum strain (de Freitas Neto et al., 2013).

Recruitment of heterophilic granulocytes is an essential primary response to infectious insult in the chicken, as heterophilic granulocytes exhibit a range of activities including adhesion, chemotaxis, phagocytosis and microbicidal activity through degranulation and oxidative burst (Genovese et al., 2013). Swaggerty *et al.* selected broilers for higher levels of pro-inflammatory mediators. This resulted in progeny with increased *in vitro* heterophil function and an increased resistance against *S.* Enteritidis challenge infection (Swaggerty et al., 2006; Swaggerty et al., 2014). In the case of *S.* Enteritidis colonization of the oviduct in the laying hen however, it appears that downregulation of the flagella expression hampers efficient

clearance of the bacteria by the heterophilic granulocytes. Taken together, the present studies indicate that *S.* Enteritidis is capable of avoiding an effective inflammatory response when colonizing the chicken oviduct and when invading chicken OEC through downregulation of flagellar gene expression. Further studies are needed to identify the signaling and sensing mechanisms involved in the downregulation of flagella expression by *S.* Enteritidis in the environment of the chicken oviduct.

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3.3 Prevention of egg contamination by *Salmonella* Enteritidis after oral vaccination of laying hens with *Salmonella* Enteritidis Δ*tolC* and Δ*acrABacrEFmdtABC* mutants

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Abstract

Vaccination of laying hens has been successfully used to reduce egg contamination by *Salmonella* Enteritidis, decreasing human salmonellosis cases worldwide. Currently used vaccines for layers are either inactivated vaccines or live attenuated strains produced by mutagenesis. Targeted gene deletion mutants hold promise for future vaccines, because specific bacterial functions can be removed that may improve safety and allow differentiation from field strains. In this study, the efficacy of *Salmonella* Enteritidis *ΔtolC* and *ΔacrABacrEFmdtABC* strains in laying hens as live vaccines was evaluated. The mutants are deficient in either the membrane channel TolC (*ΔtolC)* or the multi-drug efflux systems acrAB, acrEF and mdtABC (*ΔacrABacrEFmdtABC).* These strains have a decreased ability for gut and tissue colonization and are unable to survive in egg white, the latter preventing transmission of the vaccine strains to humans. Two groups of 30 laying hens were orally inoculated at day one, 6 weeks and 16 weeks of age with 10^8 cfu of either vaccine strain, while a third group was left unvaccinated. At 24 weeks of age, the birds were intravenously challenged with $5x10^7$ cfu *Salmonella* Enteritidis PT4 S1400/94. The vaccine strains were not shed or detected in the gut, internal organs or eggs, 2 weeks after the third vaccination. The strains significantly protected against gut and internal organ colonization, and completely prevented egg contamination by *Salmonella* Enteritidis under the conditions of this study. This indicates that *Salmonella* Enteritidis *ΔtolC* and *ΔacrABacrEFmdtABC* strains might be valuable strains for vaccination of layers against *Salmonella* Enteritidis.

Introduction

Salmonella Enteritidis first emerged in the 1980s as a significant threat to public health worldwide. Eggs were identified as the main food vehicle causing human illness (Braden, 2006; Greig and Ravel, 2009). A sustained commitment of the authorities, implementation of *Salmonella* control programs and serious investment in *Salmonella* research led to international progress in decreasing the incidence of both egg contamination (Esaki et al., 2013) and human infections (O'Brien, 2013). Vaccination in particular contributed to the decline in the number of recorded human cases of *Salmonella* Enteritidis (Cogan and Humphrey, 2003). Both inactivated and live vaccines have been shown to reduce *Salmonella* colonization in layers and contamination of eggs (Atterbury et al., 2009; de Freitas Neto et al., 2008; Gantois et al., 2009a). Several live vaccines were developed and proven to be efficient against *Salmonella* colonization (Gantois et al., 2006; Kilroy et al., 2015; Matsuda et al., 2011). Live vaccines may stimulate both cell-mediated and humoral immunity, can induce rapid protection by colonization-inhibition and are easy to administer, i.e. through the drinking water (Atterbury et al., 2009; Van Immerseel et al., 2005). A major concern of live vaccines however is safety, including the possible risk of reversion to virulence (Van Immerseel et al., 2013). Whole gene deletion mutants are generally considered to be less capable of reversion to a virulent phenotype as compared to strains harboring point mutations or undefined genetic alterations. For *Salmonella* Enteritidis, a lot of knowledge has been generated on the function of many of the chromosomal genes, and targeted deletions of specific genes related to virulence or persistence in a host have been used to construct live vaccine strains (De Cort et al., 2013; De Cort et al., 2014; Hassan and Curtiss, 1997; Nassar et al., 1994; Parker et al., 2001). In the case of *Salmonella* vaccines for laying hens, the issue of vaccine safety has an additional dimension, as safety should not only include the target species, but also the risk of transmission to humans through consumption of the eggs. Deleting genes important for virulence in mammals, but also deleting genes that are involved in egg white survival can be a key issue because this will prevent transmission of the vaccine strains to the egg consumers.

Egg white survival is a key characteristic of *Salmonella* Enteritidis transmission to humans. Because of the high pH, iron restricting conditions and the presence of a variety of antimicrobial molecules, egg white is an antimicrobial matrix (Pang et al., 2013).

Lipopolysaccharide (LPS) structure (Gantois et al., 2009b), lysozyme inhibitors (Callewaert et al., 2008) and protein and DNA damage repair mechanisms (Clavijo et al., 2006; Lu et al., 2003) are important in egg white survival of *Salmonella*. Deleting genes encoding these functions could thus generate strains with a deficient egg white survival. Recently obtained data suggested that the multi-drug resistance (MDR) pump systems and the TolC outer membrane channel, through which MDR pumps export antibacterial molecules out of the bacterial cell, are also involved in egg white survival (Raspoet, 2014). Siderophore export through TolC counteracting iron-deprivation in egg white, or MDR pump-mediated export of antimicrobial molecules out of the bacterial cell may be involved in this (Clavijo et al., 2006; Li et al., 2015).

In the current study, we aimed to evaluate the efficiency of the *Salmonella* Enteritidis *ΔtolC* and *ΔacrABacrEFmdtABC* strains, the latter devoid in 3 MDR efflux pumps, as live vaccines for protection against *Salmonella* Enteritidis egg contamination and tissue colonization in laying hens.

Materials and Methods

Vaccine and challenge strains

The vaccine strains *ΔtolC* and *ΔacrABacrEFmdtABC* are defined mutants of *Salmonella* Enteritidis 147 phage type 4. The wild type strain 147 was originally isolated from egg white and is resistant to streptomycin. The strain is known to colonize the gut and internal organs to a high level (Bohez et al., 2008; Methner et al., 1995). All mutations were constructed according to the one step inactivation method previously described by Datsenko and Wanner (Datsenko and Wanner, 2000). Briefly, for the *ΔtolC* mutant, a kanamycin resistance cassette, flanked by FRT-sites, was amplified from the pKD4 plasmid with specific primers, homologous with the flanking region of the target gene. The resulting PCR product was used for recombination on the *Salmonella* Enteritidis 147 strain chromosome using the pKD20 helper plasmid encoding the λ Red system, promoting recombination between the native gene and PCR adjusted antibiotic resistance cassette. Recombinant clones were selected on kanamycin containing plates. Replacement of the target gene by the resistance cassette was confirmed by PCR. The deletion was P22-transduced into a new *Salmonella* Enteritidis 147 strain. The antibiotic resistance cassette was eliminated using the pCP20 helper plasmid,

encoding the FLP-recombinase, mediating recombination between the FRT-sites flanking the kanamycin resistance cassette. For the *ΔacrABacrEFmdtABC* strain, the procedure was carried out in 3 steps, successively deleting the *acrAB*, *acrEF* and *mdtABC* genes. P22 transduction was done in the stepwise generated mutants. All targeted genes were completely deleted from start to stop codon, as confirmed by sequencing analysis. *Salmonella* Enteritidis S1400/94 was used as a challenge strain. The characteristics of this strain have been described previously (Allen-Vercoe and Woodward, 1999).

The challenge and vaccine strains were incubated overnight with gentle agitation (60 rpm) at 37°C in Luria Bertani (LB) medium (Sigma, ST. Louis, MO, USA). To determine bacterial titers, ten-fold dilutions were plated on brilliant green agar (BGA, Oxford, Basingstoke, Hampshire, UK) for the challenge strain. The vaccine strains were plated on LB supplemented with 1% lactose, 1% phenol red and 100 µg/ml streptomycin to determine the titer, because these strains do not grow on traditional *Salmonella* culture media. The vaccine and challenge strains were diluted in HBSS (Hanks Balanced Salt Solution, Invitrogen, Paisley, England) to 10^8 cfu/ml.

Experimental birds

Ninety (90) day-old Lohmann Brown laying hens (De Biest, Kruishoutem, Belgium) were randomly divided into 3 groups and housed in separate units. Commercial feed and drinking water was provided ad libitum. The animal experiment in this study followed the institutional guidelines for the care and use of laboratory animals and was approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University, Belgium (EC2013/135). Euthanasia was performed with an overdose of sodium pentobarbital in the wing vein.

Experimental setup

Two different groups (n=30) of animals were orally immunized at day of hatch, at 6 weeks of age and at 16 weeks of age through crop instillation of 0.5 ml containing 10^8 cfu *Salmonella* Enteritidis 147 *ΔtolC* (group 1) or *Salmonella* Enteritidis 147 *ΔacrABacrEFmdtABC* (group 2). A third group of birds (n=30) was kept as non-immunized but *Salmonella* challenged positive controls (group 3). At the age of 18 weeks, serum samples were taken for quantification of

anti-*Salmonella* Enteritidis antibodies in an LPS-ELISA (Desmidt et al., 1996). At the same time, cloacal swabs were taken in each group and bacteriologically analyzed for the presence of the vaccine strains. At 21 weeks of age, all the hens were in lay. Eggs were collected daily during 3 weeks for bacteriological detection of the vaccine strain in the egg content. At 24 weeks of age, all the animals were intravenously inoculated in the wing vein with 0.5 ml containing 5 x 10^7 cfu of the *Salmonella* Enteritidis challenge strain S1400/94. This protocol was already used previously to produce high levels of internal egg contamination (De Buck et al., 2004; Gantois et al., 2006). The eggs were collected daily during 3 weeks after inoculation and analyzed for the presence of the challenge strain. Three weeks after challenge inoculation, all the animals were euthanized by an overdose of pentobarbital in the wing vein. Samples of the spleen, oviduct, ovary, uterus and caecum were aseptically removed for bacteriological quantification of challenge and vaccine strain bacteria.

ELISA to quantify anti-LPS antibodies

For analysis of anti-*Salmonella* LPS antibodies in serum samples, a previously described indirect ELISA protocol was used (Desmidt et al., 1996). Three 96 well-plates (Sigma, St. Louis, MO, USA) were coated with 100 µl of an LPS solution (10 µg/ml) in 0.05 M carbonate-bicarbonate (pH 9.6; coating buffer) and incubated for 24 hours at 4°C. The LPS was purified from *Salmonella* Enteritidis PT4 strain. The plates were rinsed four times with phosphate buffered saline (PBS, Sigma, St. Louis, MO, USA) supplemented with 0.1% Tween-20 (Sigma, St. Louis, MO, USA; washing buffer) between each step. In the first step, 100 µl PBS (Sigma, St. Louis, MO, USA) supplemented with 1% bovine serum albumin (BSA, Sigma, St. Louis, MO, USA; blocking buffer) was added to the wells for one hour at 37°C. The blocking buffer was then removed. Secondly, serum samples of animals from the different groups were diluted in blocking buffer (1:200) and added to the plates (100 µl). As an internal negative control, serum from a *Salmonella* free chick was used. Serum from a chick that had been infected experimentally with *Salmonella* Enteritidis PT4, strain 76Sa88, was used as an internal positive control. The plates were incubated on a shaking platform for 2 hours at 37°C. Thirdly, peroxidase-labelled rabbit anti-chick IgG (100 µl, Sigma, St. Louis, MO, USA) was diluted (1:2000) in blocking buffer and added to the wells for 1 hour and 30 min while shaking at 37°C. Finally 50 µl of TMB substrate (Fisher Scientific, Erembodegem, Belgium) was added to the wells. The reaction was blocked with 50µl of sulfuric acid (0.5M). The

absorbance was measured in an ELISA reader at 450nm. Every sample was analyzed in duplicate. Data were shown as S/P ratios, thus (OD(sample)-OD(negative control))/(OD(positive control)-OD(negative control)). Negative values were considered as zero.

Bacteriological examination of the challenged birds

Cloacal swabs taken at week 18 were incubated overnight at 37°C in buffered peptone water (BPW, Oxoid, Basingstoke, Hampshire, UK). Afterwards a loopful was plated on LB plates supplemented with 1% lactose, 1% phenol red and 100 µg/ml streptomycin (Sigma, St.Lous, MO, USA) for the detection of the vaccine strains *Salmonella* Enteritidis 147 *Δtol*C and *ΔacrABacrEFmdtABC*.

Samples of caecum, spleen, ovary, oviduct and uterus were pre- enriched and homogenized in BPW (10% weight/volume suspensions) and 10-fold dilutions were made in HBSS (Invitrogen, Paisley, England). Six droplets of 20 µl of each dilution were plated on BGA (for quantification of the challenge strain) or on LB supplemented with 1% lactose, 1% phenol red and 100 µg/ml streptomycin (for quantification of the vaccines). 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. Samples that tested negative after direct plating for the challenge strain were enriched in tetrathionate brilliant green broth (Oxoid, Basingstoke, UK) by overnight incubation at 37°C. After incubation, a loopful of the tetrathionate brilliant green broth was plated on BGA.

Egg production and bacteriological examination of eggs

Eggs were collected daily for 6 weeks from week 21 onwards and the egg production was determined. Each day, eggs of six hens per group were pooled in one batch, yielding an egg per batch number that varied between one and six. Upon collection, lugol solution and 95% ethanol were used to decontaminate the surface of the eggshell. After decontamination of the eggshell, the eggs were broken aseptically and the total content of the eggs was pooled and homogenized per batch. A volume of 40 ml of BPW was added for each egg to the pooled egg content and incubated for 48h at 37°C. To detect the vaccine strains, a loopful of the BPW broth was plated on LB plates supplemented with 1% lactose, 1% phenol red and

100µg/ml streptomycin. To detect the challenge strain, a loopful of the BPW broth was plated on BGA. Additionally, further enrichment was done overnight at 37°C in tetrathionate brilliant green broth and after incubation, a loopful of broth culture was streaked onto BGA.

Statistical analysis

SPSS 22.0 software was used for statistical analysis. Cloacal swabs, batches of eggs and data of cfu *Salmonella*/gram tissue of the caecum, spleen, ovary, oviduct and uterus after enrichment were categorized as either positive or negative. A binary regression model was used to determine differences between the groups. For all tests, differences with p-values below 0.05 were considered to be statistically significant.

Results

Detection of anti-Salmonella LPS antibodies in serum

Data derived from the LPS-ELISA are shown in figure 1. The data are represented as S/P ratios, thus (OD(sample)-OD(negative control))/(OD(positive control)-OD(negative control)).

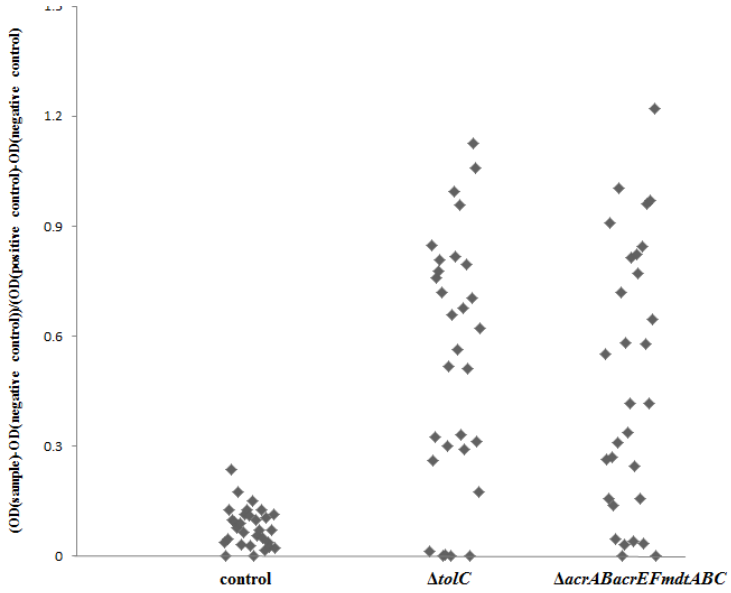

Figure 1. (OD(sample)-OD(negative control))/(OD(positive control)-OD(negative control) measured in the ELISA detecting anti-*Salmonella* LPS antibodies. Serum of 18-week old laying hens, vaccinated at day 1, 6 weeks of age and 16 weeks of age with *Salmonella* Enteritidis 147 *ΔtolC* and *Salmonella* Enteritidis 147 *ΔacrABacrEFmdtABC* was analysed.

Analysis of cloacal swabs and eggs for the presence of vaccine strains

Not a single *Salmonella* vaccine isolate was obtained from cloacal swabs or egg content samples.

Clinical signs and egg production after challenge

Over the whole experiment, there was no reduction in feed and water intake in either of the groups. The egg production rate after infection in the unvaccinated control group dropped to 59% in the first week post-infection (pi) and raised to 75% and 86% in the second and third week pi. The egg production rate did not decrease significantly after challenge in the vaccinated groups compared to before challenge. The egg production percentage in the group vaccinated with the *ΔtolC* strain was 60%, 100% and 90%, in the first, second and third week after challenge. In the group vaccinated with the *ΔacrABacrEFmdtABC* strain, the egg production percentage was 56%, 70% and 68% respectively. Some eggs were thin-shelled and malformed during the first week after infection. At the end of the experiment, 11 hens died in the group of animals vaccinated with the *Salmonella* Enteritidis 147 *ΔacrABacrEFmdtABC* strain because of cannibalism.

Isolation of the challenge strain from egg contents

Not a single *Salmonella* positive egg batch was detected from animals vaccinated with the *Salmonella* Enteritidis 147 *ΔtolC* and *Salmonella* Enteritidis 147 *ΔacrABacrEFmdtABC* strains (table 1). During the first week, three egg batches out of 26 were *Salmonella* positive in the non-vaccinated control group at direct plating. In the third week pi, no positive egg batches were found.

Table 1. Percentage of egg content batches positive for the challenge strain *Salmonella* Enteritidis S1400/94 after enrichment.

Group	Week 1	Week 2
Non-vaccinated	70[a](74)[a]	0(17)[a]
ΔtolC	0[b](0)[b]	0(0)[b]
ΔacrABacrEFmdtABC	0[b](0)[b]	0(0)[b]

Animals were vaccinated at day one, 6 weeks and 16 weeks of age with 10^8 cfu of either *Salmonella* Enteritidis 147 *ΔtolC* or *Salmonella* Enteritidis 147 *ΔacrABacrEFmdtABC* strains or kept as non-immunized controls. Results are shown for egg content samples, plated on BGA after BPW (48h, 37°C) incubation. Percentage of batches positive after enrichment in tetrathionate brilliant green broth (37°C, overnight) are shown between brackets. Different superscripts within a column indicate significant differences between the groups ($p<0.05$)

Isolation of the challenge strain from the organs at 3 weeks post-infection

No samples were positive at direct plating. Table 2 presents the percentage of *Salmonella*-positive organ samples after enrichment, in vaccinated and non-vaccinated groups, at 3 weeks post challenge. Vaccination with the *Salmonella* Enteritidis 147 *ΔtolC* strain significantly decreased the number of *Salmonella* positive samples in the spleen, caecum and ovary as compared to the control group. Vaccination with the *ΔacrABacrEFmdtABC* strain significantly reduced the number of *Salmonella* positive samples in the caecum, ovary and oviduct.

Table 2. Percentage of *Salmonella*-positive samples after enrichment.

	control	*ΔtolC*	*ΔacrABacrEFmdtABC*
uterus	13.3	10	15.9
spleen	80	50[*]	63.2
caecum	30	6.6[*]	0[*]
ovary	70	36.6[*]	31.6[*]
oviduct	46.6	30	5.3[*]

Samples of uterus, spleen, caecum, ovary and oviduct were taken, 3 weeks post-infection with *Salmonella* Enteritidis S1400/94. Animals were vaccinated at day 1, week 6 and week 16 with either *Salmonella* Enteritidis 147 *ΔtolC* or *Salmonella* Enteritidis 147 *ΔacrABacrEFmdtABC*. Statistically significant differences ($p<0.05$) in percentage of positive organ samples between vaccinated groups and the non-vaccinated control group are indicated with an asterisk.

DISCUSSION

Current commercial live vaccines contain strains harboring undefined mutations in one or more genes on the chromosome or defined point mutations. Strains harboring (undefined or defined) point mutations might, however, revert to a virulent phenotype and are thus considered to be unsafe (Audisio and Terzolo, 2002; Van Immerseel et al., 2013). Future live vaccines should therefore contain fully defined strains carrying (multiple) gene deletions for purposes of safety. Deletion of entire genes additionally permits differentiation from wild type strains, allowing quality control. Numerous experimental vaccines were already tested in various animal hosts, including chickens, but data on the protection of these live vaccines against egg contamination are scarce (Gantois et al., 2006; Hassan and Curtiss, 1997; Nassar et al., 1994).

Successful attenuation of the wild type strain requires prior knowledge of the pathogen's virulence factors. A vaccine strain used for the prevention of (vertical) egg contamination of *Salmonella* Enteritidis ideally colonizes and induces local immunity in the reproductive tract. From a public health point of view, it may not persist here and preferably does not survive in egg white. A logical approach is to eliminate genes playing a role in egg white survival. In the current study defined mutants in MDR transporters and the TolC outer membrane channel were used as vaccine strains. The TolC promoter is activated after contact with egg white at 42°C, but not under standard *'in vitro'* culture conditions (Raspoet, 2014). The TolC outer membrane channel is used by MDR transporters (eg *acrAB, acrEF, mdtABC*) to export host antibacterial compounds and bacterial molecules such as siderophores, and is involved in survival in harmful environments, including egg white (Pan et al., 2010). The *ΔtolC* and Δ*acrABacrEFmdtABC* vaccine strains can no longer survive in egg white, thereby eliminating the risk of human exposure through eggs (Raspoet, 2014). To our knowledge, these genes

were never associated with protective immunity in chickens, allowing wild type-like antigen presentation.

The actual immune mechanism explaining the protection against *Salmonella* Enteritidis colonization observed in the current trial is not completely clear. Immunization with *Salmonella* vaccines can induce variable humoral and cell-mediated responses that do not always correlate with acquired resistance to re-infection (Mastroeni et al., 2001). A role for humoral responses in the clearance of *Salmonella* infections has been shown for using inactivated vaccines, which are less able to induce cellular responses but are still partially protective (Feberwee et al., 2001). Cell-mediated immunity was not investigated during this trial but for *Salmonella* in poultry, susceptibility to the infection is correlated with a fall in $CD4^+$ and $CD8^+$ T-lymphocytes and $\gamma\delta$ T-lymphocytes in the oviduct, and with T-lymphocyte hyporesponsiveness (Johnston et al., 2012). Live vaccines have been shown to increase numbers of $CD4^+$ and $CD8^+$ T-lymphocytes to a certain level in the gut wall (Berndt and Methner, 2001). Future studies should further investigate the role of the humoral and cellular immune responses during vaccine-induced protection. Possibly a combination of cell-mediated immunity and a strong humoral response are yielding additional protective effects.

To conclude, data from this trial indicate that *Salmonella* Enteritidis *ΔtolC* and *ΔacrABacrEFmdtABC* strains are safe vaccines that can induce protection against internal organ colonization after intravenous inoculation of a *Salmonella* Enteritidis challenge strain. The vaccine strains were able to completely prevent egg contamination with *Salmonella* Enteritidis in the current *in vivo* trial.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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AUTHOR'S CONTRIBUTIONS

SK participated in the design of the study, performed the experiments, analyzed the data and drafted the manuscript. RR, FVI, RD and FH coordinated the study, participated in the design of the study, helped to interpret the results and edited the manuscript. All authors read and approved the final manuscript.

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4 General discussion

General discussion

Vaccination against *Salmonella* has been a successful control method in poultry for years. Literature on vaccination against *Salmonella* colonization is very broad. In this work we aimed to highlight and discuss challenges in vaccine development. Although the prevalence of *Salmonella* serotypes, has declined in both poultry and humans due to vaccination programs, there are some challenges in vaccine development that will gain importance in the future. First, new serotypes are constantly emerging and current vaccines were not developed to control these serotypes. Secondly, there is a constant pressure to guard and improve vaccine safety. The general purpose of the present study was to lay the scientific foundation for novel vaccination strategies to protect laying hens and their eggs from *Salmonella* contamination under continuously evolving current and future epidemiological conditions. For live vaccines, it is a common belief that defined deletion mutants are safer than undefined mutants, since the chance of reversion to virulence is minimized (Van Immerseel et al., 2013). In the current work it was aimed to specifically gain scientific insights into the role of flagella in the pathogenesis of *Salmonella* infections in laying hens, with special emphasis on those aspects that have an impact on vaccination. Secondly, as non-phasic or monophasic strains are emerging it was aimed to evaluate the effect of a currently used live Typhimurium vaccine against infection with the monophasic variant and to evaluate the efficacy of a newly developed *Salmonella* Enteritidis defined mutant in multi-drug resistance pumps against egg contamination by *Salmonella* Enteritidis.

4.1 Understanding the dynamics of *Salmonella* serotypes in poultry

Understanding the historical factors that contributed to population shifts of *Salmonella* serotypes provides insights for developing strategies to control current *Salmonella* problems. The predominant serovars in the first half of the last century, *Salmonella* Gallinarum biovars Gallinarum and Pullorum, were successfully eradicated from commercial poultry in most countries in the EU through *Salmonella* control programs, but still are a problem in many other

countries worldwide. In the last couple of decades of the 20th century, *Salmonella* Enteritidis became the predominant serovar in poultry and eggs worldwide, not only colonizing birds but also causing salmonellosis in humans. *Salmonella* Enteritidis consequently has been targeted by a number of control programs over the past few decades with great success. The decrease of *Salmonella* Enteritidis unfortunately coincides with the emergence of different strains belonging to various serotypes. Often multi-drug resistance is seen in the new emerging strains (Mandilara et al., 2013).

Evaluating the protection of currently used vaccines against these emerging strains is important in order to find out whether novel strategies for vaccination need to be developed. *Salmonella* Typhimurium strain Nal2/Rif9/Rtt, present in the commercially available live vaccines AviPro *Salmonella* Duo and AviPro *Salmonella* VacT was proven to be efficacious after challenge with a low, intermediate and high dose of the emerging monophasic variant of *Salmonella* Typhimurium serotype 4,12: 1,4,[5],12:i:-. The monophasic variants are Typhimurium strains that have mutations in genes involved in flagella production, while almost all other antigens are well conserved. Protection of a vaccine strain against different *Salmonella* serotypes or variants in laying hens however is not self-evident. Oral vaccination of a *Salmonella* Typhimurium cya/crp double mutant for instance, significantly reduced levels of the *Salmonella* Typhimurium wild-type strain but was ineffective against *Salmonella* Enteritidis challenge (Hassan and Curtiss, 1994). Furthermore, if the immune responses are specifically directed at surface antigens, other serotypes can conquer the available niche. It is known that colonization-inhibition is a serotype-specific phenomenon. This type of protection as well as immune-related protection (antibodies or cell-mediated immunity) requires cell surface molecules that are conserved amongst strains against which one wants to protect. Once a given serotype is cleared following vaccination, other serotypes expressing different surface antigens may conquer the available niche. Indeed, *Salmonella* Heidelberg shares some common surface antigens with *Salmonella* Enteritidis that *Salmonella* Kentucky does not, which may help explain why *Salmonella* Kentucky has increased in recent years in the US (Foley et al., 2011).

4.2 Current limitations, pitfalls and shortcomings of vaccination

Various prophylactic measures have been employed to control *Salmonella* infections in poultry production in general and in laying hens in particular. The aim of all control programs should be to provide and implement an integrated strategy including a series of complementary prophylactic measures. Such prophylactic measures are to produce *Salmonella*-free day-old chicks, *Salmonella*-free poultry feeds and *Salmonella*-free poultry houses. Within such a program, rigorous and planned vaccination is exceptionally important and should start as early as possible. Day-old chicks are most susceptible to *Salmonella* infection because the autochthonous intestinal microbiota has not yet developed sufficiently. Commercial poultry are often infected with *Salmonella* within the first week of life when the infectious dose required is several orders of magnitude less than that of an adult chicken (Cox et al., 1990).

Several commercial vaccines based on live attenuated *Salmonella* strains are available and approved for vaccination of poultry. In Belgium, live oral vaccines are currently on the market for active immunization of laying hens to reduce mortality, colonization, shedding and faecal excretion of *Salmonella* Typhimurium and/or Enteritidis. Acquired immunity develops within 15 days of the first vaccination. Vaccination is effective in reducing the overall number of positive birds and the level of colonization. These vaccine strains contain several point mutations in genes encoding essential enzymes and metabolic control centers of the bacterium, resulting in prolonged generation times and corresponding reductions in virulence (Linde et al., 1997). Although only very sporadically reported for specific live vaccines, the possible risk of reversion to virulence of current undefined vaccines combined with the fact that eggs and egg-products continue to be the most important source of *Salmonella* infection, has created a market pull towards developing defined deletion vaccines able to prevent egg colonization. Ideally such a vaccine should contain a strain that is able to offer optimal immune protection but cannot survive in egg white. The latter directly avoids transmission of the vaccine strains to the egg consumers. Previous studies from our group showed that colonization of and survival in egg white is critically important for contamination of table eggs by *Salmonella* Enteritidis (De Buck et al., 2004; De Vylder et al., 2013). Genes important for survival in egg white therefore need to be identified. A number of genes were found to be important for colonization and egg white

survival. This provides vital new data for the design of strategies to control *Salmonella* in laying hens and reduce transmission to humans (Chaudhuri et al., 2013). In the current PhD, *Salmonella* Enteritidis Δ*tolC* and Δ*acrABacrEFmdtABC* vaccine strains were created and evaluated for their protection against (vertical) egg contamination in a 6 months *in vivo Salmonella* Enteritidis challenge model. The mutants are deficient in either the membrane channel TolC (*ΔtolC)* or the multi-drug efflux systems acrAB, acrEF and mdtABC (*ΔacrABacrEFmdtABC).* Unlike most other defined deletion strains used for vaccination of laying hens, these deleted genes are not involved in metabolism or virulence. Furthermore, all surface antigens are intact and their multiplication under physiological circumstances is not compromised. These strains have a decreased ability for gut and tissue colonization, and are unable to survive in egg white, the latter preventing transmission of the vaccine strains to humans. After vaccination, egg contamination was completely prevented in a 6 months *in vivo* challenge model and the vaccine strains were not able to survive in egg white (Kilroy et al., 2016). Moreover, organ colonization was also significantly reduced but could not be completely prevented. Protection against infection of other *Salmonella* serovars needs to be evaluated, even if these serotypes are rarely associated with laying hens, eggs and egg products.

4.3 The role of flagellin in vaccination and infection.

Completely eliminating salmonellosis is likely to be an utopia, since this pathogen is present in the environment and an internal gut colonizer. Minimizing the global salmonellosis burden requires different approaches depending on the *Salmonella* serotype and the respective host. Vaccines containing different defined deletion strains are needed. This has major implications for vaccine design (Morgan et al., 2004). Additionally, vaccines containing different serotypes with the same gene deletion do not necessarily result in the same type of attenuation or protection, since the genetic background of the serotype has a major impact (Foley et al., 2013). Moreover, it remains uncertain whether a double or triple mutant would combine the characteristics of the corresponding single mutations and if the additional deletion might affect the capability to induce an effective adaptive immune response. Indeed, immunization with the

double attenuated ΔphoPfliC mutant compared to the single phoP mutant did not reduce *Salmonella* contamination (Methner et al., 2011).

Another limitation of currently registered live or inactivated vaccines for laying hens, is the lack of serological differentiation between vaccinated and infected animals. Deleting flagellin would allow such serological differentiation and could be a straightforward solution. However, earlier studies show that attenuated and less invasive flagellin mutants induce a lower influx of granulocytes in the gut mucosa and, as a result, a less effective invasion-inhibition effect especially against heterologous *Salmonella* serovars (Methner et al., 2010). Furthermore, non-motile *Salmonella* serovars causing systemic disease in poultry are emerging. The absence of flagella would enable these variants to invade without the stimulation of a pro-inflammatory response from the host (Iqbal et al., 2005). Implications for vaccine development need to be elucidated, but mutating flagellin could help to escape the host's immune response. Indeed, we showed that flagellar gene transcription is downregulated inside the chicken oviduct and aflagellated mutants have a colonization advantage in laying hens. While interaction of flagellin with the gut mucosa has been studied in detail at the start of this thesis, little information was available about the interaction of flagellin with the oviduct tissues. Primary cultivation of chicken oviduct epithelial cells showed that flagellin is not important for invasion in or adhesion to oviduct cells. In an attempt to understand the innate immune responses against flagellin, aflagellated mutants were introduced in the oviduct of laying hens and compared to the wild-type. Injection of an aflagellated mutant indeed attracted significantly less heterophils compared to the wild-type strain, endorsing the hypothesis that flagellin downregulation is a possible immune escape mechanism employed by *Salmonella* Enteritidis to avoid host immune reaction.

4.4 A new era in *Salmonella* vaccination of laying hens?

The industry could turn to a new generation of vaccines that contain defined deletions which are less likely to revert to virulence and thus safer than current licensed vaccines for laying hens. Although the *Salmonella* Enteritidis Δ*tolC* and Δ*acrABacrEFmdtABC* strains completely

prevented (vertical) egg contamination, carcasses and eggs can still be (horizontally) contaminated because these vaccines reduce, but do not eliminate shedding or prohibit colonization of the gastrointestinal tract. Researchers could look for additional adjuvants that increase protection but do not cause side effects. As vaccines have become more advanced, there is a need for more advanced adjuvants to potentiate those vaccines has developed.

Designing the ultimate vaccine preparation for use against *Salmonella* colonization in laying hens and egg transmission is not an easy task. As of today, no vaccine provides complete protection or cross-protection against all serogroups (Gast, 2007). Current vaccines are not protecting against other serovars with different O and H antigens (Noda et al., 2010). Ideally, the vaccine should offer protection against infection by more than one serotype such as *Salmonella enterica* serovar Infantis, which also has been isolated from laying hens frequently and is becoming more and more important. Proof-of-principle studies have demonstrated efficacy, in animal models, of live-attenuated and subunit vaccines that target the O-antigens, flagellin proteins, and other outer membrane proteins of *Salmonella* Typhimurium and *Salmonella* Enteritidis. The relatively poor immunogenicity of purified O-antigens can be significantly enhanced through chemical linkage to carrier proteins. The subunit glycoconjugation approach specifically links LPS-derived O polysaccharide to carrier proteins and has been successful in increasing immunogenicity of purified O-antigens. Another delivery strategy for non-typhoidal *Salmonella* vaccines are the Generalized Modules for Membrane Antigens (GMMA; Tennant et al., 2016). This technology presents surface polysaccharides and outer membrane proteins in their native conformation and is self-adjuvanting, as it delivers multiple MAMP molecules.

To comply with increasing public demand for cross protective vaccines against multiple *Salmonella* serovars, also the development of component vaccines, with highly conserved antigens, has great value, although they need to be injected instead of added to the drinking water, causing practical limitations and leading to higher labor costs. Outer membrane proteins (OMPs) are considered effective antigens to stimulate immune responses because they are exposed on the bacterial surface and easily recognized by the host immune system. OmpA for instance is considered essential for the conservation of cell structure by physical linkage

between the outer membrane and peptidoglycan. It is reported to function in host-pathogen interactions, including the adhesion and invasion of epithelial cells. OmpA is also known as an immune target and is involved in evasion and biofilm formation (Smith et al., 2007). OmpA is well conserved among *Salmonella* serovars and shows a strong humoral response, however the bacterial shedding after challenge was not reduced by vaccination with OmpA. A potential reason is that the anti-OmpA antibody did not reach or recognize the OmpA on the outer membrane of live *Salmonella* due to the presence of other properties, such as LPS, pili, flagella and other porin proteins, which could have masked the OmpA. These antigenic component vaccines can be delivered through liposomes. Oral immunization with liposome-associated rSefA, which encodes the main subunit of the SEF14 fimbrial protein elicits both systemic and mucosal antibody responses and results in reduced bacterial colonization in the intestinal tract and reduced excretion of *Salmonella* Enteritidis in the feces. Significantly less fecal excretion of bacteria was observed in immunized chickens for 4 weeks after challenge in contrast with the unimmunized controls (Pang et al., 2013). Another genetic engineering technology that allows immune reactions against outer membrane proteins is ghost vaccination. Controlled expression of the PhiX174 lysis gene E in gram-negative organisms induces trans-membrane tunnel formation, expulsion of the cytoplasmic contents and ultimately leads to the generation of non-living envelopes called bacterial ghosts. As the non-enzymatic activity of protein E does not cause any physical or chemical denaturation of the bacterial surface proteins during the lysis process, the resulting bacterial ghosts have the same antigenic determinants as their replicating counterparts (Witte et al., 1992). The efficacy of *Salmonella* Enteritidis ghost vaccination was evaluated in laying hens by characterizing the nature of the adaptive immune response. Chickens from the immunized group demonstrated significant increases in *Salmonella* Enteritidis-specific plasma IgG, intestinal secretory IgA and lymphocyte proliferative response, and different populations of cytokines. Furthermore, the immunized group exhibited decreased challenge strain recovery of the internal organs compared to the non-immunized group (Jawale and Lee, 2014).

To overcome the need for intramuscular injection, the development of self-destructing *Salmonella* vaccines are in full development. These self-destructing vaccines form a biological

containment system using recombinant *Salmonella* strains that are attenuated yet capable of synthesizing protective antigens. The system is composed of two parts. The first component is the attenuated strain, which features a number of mutations in genes required for synthesis of the peptidoglycan layer of the bacterial cell wall, mutations that enhance bacterial cell lysis and antigen delivery, mutations ensuring that the bacteria do not survive *in vivo* or after excretion and mutations allowing maximum antigen production. The second component is a plasmid encoding genes that can lead to bacterial cell lysis through concerted activities. The regulated delayed attenuation and programmed self-destructing features designed into these *Salmonella* strains enable them to efficiently colonize host tissues and allow release of the bacterial cell contents after lysis. These vaccines are able to stimulate mucosal, systemic, and cellular protective immunities (Kong et al., 2012).

4.5 A holistic management approach to control

The future for *Salmonella* control in laying hens will consist of the maintenance of high standards of management to prevent introduction and spread of infection and the continuous exploration of new approaches. Biosecurity should play a crucial role. Novel ideas such as using lytic bacteriophages have been assessed experimentally, producing some reductions in levels of colonization and remarkable effects on carcass decontamination (Atterbury et al., 2007). Control of *Salmonella* as a zoonosis in general is definitely not limited to vaccination and protection of the laying hens, nor to vaccination of other poultry and other farm animal species. Continuing research on vaccine development for prevention of *Salmonella* infections in human populations will benefit communities where these infections are endemic, in both the developing and industrialized world. Experience gained in vaccine development for laying hens may contribute to the development of novel strategies for protecting the human population, which may also include vaccination of the human population with more advanced vaccines than the currently still commonly used inactivated vaccines for typhoid fever. It should be emphasized however that successful vaccination is closely correlated with optimal husbandry conditions and the maintenance of high sanitary standards. The overall *Salmonella* burden of a laying hen population can only be reduced by long-term, comprehensive vaccination of flocks,

which will ultimately minimize contamination of foods of animal origin with *Salmonella*. Future research should further focus on finding good adjuvantia, not only to enhance protection of these live defined attenuated vaccines but also to protect against a broader range of serotypes.

4.6 References

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Summary

Summary

Salmonella enterica subspecies *enterica* serovar Enteritidis is a pandemic pathogen, present in countries with industrial poultry production since the 1990s. Ingestion of this foodborne pathogen by humans results in gastroenteritis and is linked to contaminated eggs and egg products. Salmonellosis caused by *Salmonella* Enteritidis in chickens however does not lead to clinical symptoms but causes enormous economic losses. Consequently, there is continuing interest in finding ways of preventing flock infection of laying hens with *Salmonella*. Control of *Salmonella* infections in poultry farms begins with good farming practices and appropriate management. In laying hens vaccination is an important tool to protect against *Salmonella* colonization. Vaccination against *Salmonella* Enteritidis is vastly undertaken in many countries around the world. Studies have reported that cases of human salmonellosis due to food poisoning decreased significantly after the implementation of a widespread vaccination program in commercial layers. Although great efforts have been made, recently, atypical pathogenic *Salmonella* strains emerged. At the start of this doctoral thesis, studies in numerous countries worldwide confirm the rapid emergence and dissemination of a monophasic variant of *Salmonella* Typhimurium, i.e. 1,4,[5],12:i:-. Cases of human infection caused by the emerging monophasic variant have been linked to a number of sources, predominantly pigs. Strains from this serotype have also been found in chicken meat, broilers and recently in laying hens. This shows that the monophasic variant 1,4,[5],12:i:- represents a significant and potential emerging threat to humans, not only through porcine meat, but also through chicken product consumption. Consequently it has been included in actions implementing the legislation of the EU to detect and control *Salmonella* serovars of public health significance in laying hens, broilers, breeders and turkeys.

While the efficacy of the commercial live vaccines AviPro® *Salmonella* VacE and VacT to protect laying hens from oviduct colonization and egg contamination by *Salmonella* Enteritidis has been proven, no data have been published yet on potential effects of this vaccine on caecal, spleen and liver colonization by the emerging monophasic serotype 1,4,[5],12:i:-. Therefore, in the first

study of this thesis (**chapter 3.1**), two short-term (two weeks) trials, either using a high or a low infection dose, and 1 longer term study (6 weeks) were carried out to evaluate the protective effect against gut and internal organ colonization after vaccination with *Salmonella* Typhimurium strain Nal2/Rif9/Rtt, a strain contained in the commercially available live vaccines AviPro® *Salmonella* Duo and AviPro® *Salmonella* VacT, at day of hatch. Oral administration of the vaccine strain at day of hatch, reduced colonization with a strain of the monophasic variant of *Salmonella* Typhimurium, 1,4,[5],12:i:-, after challenge at day 2. The *Salmonella* 1,4,[5],12:i:- serotype is Typhimurium-like and can thus, as shown in this study, also be controlled in the early immune deprived stage by using live *Salmonella* Typhimurium vaccines. This is of value for layers as well as for broilers and can be part of a control program for the new emerging serotype 1,4,[5],12:i:-.

These monophasic *Salmonella* Typhimurium 1,4,5,12:i:- variants are lacking the *fljB*-encoded second phase antigen. It has been suggested that the lack of flagella changes virulence characteristics of *Salmonella* but the exact role of flagella in the pathogenesis of *Salmonella* infections in chickens was not yet completely clear. Little was known yet about the role of flagellin in oviduct colonization. The glandular epithelial cells of the oviduct in laying hens express Toll-like receptors (TLRs). These interact with MAMPs, like LPS and flagellin. Binding of MAMPs to TLRs should normally initiate the innate immune response, leading to inflammation and tissue damage. *Salmonella* Enteritidis however is able to colonize the oviduct without causing an inflammatory reaction. Indeed we found that expression of flagella by *Salmonella* Enteritidis is downregulated following colonization of the chicken oviduct and in chicken OEC (**chapter 3.2**). The result of these studies indicate that *Salmonella* Enteritidis is capable of avoiding an effective inflammatory response when colonizing the chicken oviduct and when invading in chicken OEC through downregulation of flagellar gene expression and in this way suppressing the flagellin-TLR5 activation pathway. Further studies are needed to identify the signaling and sensing mechanisms involved in the downregulation of flagella expression by *Salmonella* Enteritidis in the environment of the chicken oviduct. This information could be important for future vaccine development.

Current commercial live vaccines contain strains harboring undefined mutations in one or more genes on the chromosome. Strains harboring point mutations might, however, revert to a virulent phenotype and are thus considered to be unsafe. Future live vaccines should therefore contain fully defined strains carrying (multiple) gene deletions for purposes of safety. Most experimental vaccines contain strains deleted for genes important for metabolism or virulence. Numerous experimental vaccines were already tested in various animal hosts, including chickens, but data on the protection of these live vaccines against egg contamination are scarce. A vaccine strain used for the prevention of (vertical) egg contamination of *Salmonella* Enteritidis ideally induces local immunity in the reproductive tract. From a public health point of view, it may not persist here and preferably does not survive in egg white. A logical approach is to eliminate genes playing a role in egg white survival. In the third chapter (**chapter 3.3**), defined mutants in MDR transporters and the TolC outer membrane channel were used as vaccine strains. The TolC outer membrane channel is used by MDR transporters (eg *acrAB, acrEF, mdtABC*) to export host antibacterial compounds and bacterial molecules such as siderophores, and is involved in survival in harmful environments, including egg white. The *ΔtolC* and ∆*acrABacrEFmdtABC* vaccine strains can no longer survive in egg white, thereby eliminating the risk of human exposure through eggs. These genes were never associated with protective immunity in chickens, allowing wild type-like antigen presentation. Data from this chapter indicate that *Salmonella* Enteritidis *ΔtolC* and *ΔacrABacrEFmdtABC* strains are safe vaccines that can induce protection against internal organ colonization after intravenous inoculation of a *Salmonella* Enteritidis challenge strain. The vaccine strains were able to completely prevent egg contamination with *Salmonella* Enteritidis in a 6 months *in vivo* challenge trial.

In conclusion, a number of control measures were being used to avoid *Salmonella* infections in the poultry industry. In spite of these measures, new (monophasic) variants arise. Current commercial available vaccines are able to protect against these upcoming variants. It is important however to keep evaluating the protection offered by current commercial vaccines against new upcoming variants in order to respond as quickly as possible to epidemiological changes. At the same time it is important to guarantee the safety of vaccine strains by deletion

of whole gene(s). The *Salmonella* Enteritidis *ΔtolC* and *ΔacrABacrEFmdtABC* vaccine strains are safe and could be used to prevent egg contamination.

Samenvatting

Samenvatting

Sinds 1990 is *Salmonella enterica* subspecies *enterica* serovar Enteritidis een pandemisch pathogeen, aanwezig in landen met industriële pluimveeproductie. Inname van deze voedsel-geassocieerde pathogeen door de mens veroorzaakt gastro-enteritis en wordt gelinkt aan besmette eieren en ei-producten. Salmonellose veroorzaakt door *Salmonella* enteritidis in kippen leidt echter niet tot klinische symptomen maar zorgt voor zware economische verliezen. Zodoende is er voortdurende interesse om mogelijke manieren te vinden om leghennen te beschermen tegen infectie met *Salmonella*. De controle van deze *Salmonella* infecties in pluimveebedrijven begint met een goed management en strikte veiligheidsmaatregelen. Bij leghennen is vaccinatie een zeer belangrijke maatregel om besmetting te voorkomen. Vaccinatie tegen *Salmonella* Enteritidis gebeurt wereldwijd. Studies rapporteren dat het aantal gevallen van humane salmonellose significant gedaald is na het implementeren van vaccinatieprogramma's in commerciële leghennen. Hoewel er grote vorderingen gemaakt zijn, duiken er toch atypische pathogene *Salmonella* stammen op. Bij het begin van dit doctoraat tonen studies uit verschillende landen het snel opkomen van een monofasische variant van *Salmonella* Typhimurium, ie 4,12:i:- aan. Humane salmonellose veroorzaakt door deze monofasische variant wordt gelinkt aan een aantal oorzakelijke bronnen, voornamelijk varkens. Stammen van dit serotype worden ook teruggevonden in vleeskippen en recentelijk ook in leghennen. Dit toont aan dat besmetting door de monofasische variant een belangrijke bedreiging vormt voor mensen, niet enkel via varkensvlees, maar ook via producten afkomstig van kippen. Bijgevolg werd deze stam geïncludeerd in acties die de controle en detectie van *Salmonella* serovars, gevaarlijk voor de volksgezondheid, omschrijven.

De werkzaamheid van de commerciële levende vaccines AviPro® *Salmonella* VacE en VacT voor de bescherming van leghennen tegen oviduct kolonisatie door *Salmonella* Enteritidis werd reeds beschreven, maar geen enkele data werden reeds gepubliceerd over het potentieel beschermend effect van deze vaccins tegen de opkomende monofasische variant op gebied van lever, milt en caecum kolonisatie. Daarom werden in een eerste studie van deze thesis twee korte-

termijn (2 weken), met een hoge en lage orale toediening van de monofasische variant, en 1 langere termijn studie (6 weken) opgezet om na te gaan of de *Salmonella* Typhimurium stam Nal2/Rif9/Rtt, die aanwezig is in de commercieel beschikbare levende vaccins AviPro® *Salmonella* Duo en AviPro® *Salmonella* VacT ook bescherming biedt tegen deze monofasische variant (**hoofdstuk 3.1**). Orale toediening van het vaccin op dag 1 reduceerde de kolonisatie met de monofasische *Salmonella* Typhimurium variant 4,12,:i:- na toediening ervan op dag twee. De autochtone intestinale microbiota van eendagskuikens is nog niet volledig matuur en ook het immuunsysteem is nog niet volledig ontwikkeld. Dit *Salmonella* 4,12,:i:- serotype is Typhimurium-achtig en kan, zoals aangetoond in dit hoofdstuk, ook gebruikt worden voor bescherming in de vroege levensfase door het gebruik van levende *Salmonella* Typhimurium vaccins. Dit is van belang voor leghennen alsook voor vleeskippen en kan deel uitmaken van een controleprogramma tegen de opkomende monofasische varianten.

Deze monofasische *Salmonella* Typhimurium 1,4,5,12,:i:- varianten ontbreken een fljB-gecodeerde 2de fase antigen. Het ontbreken van deze flagellen zou de virulentiekarakteristieken van *Salmonella* kunnen veranderen, maar de precieze rol van deze flagellen in de pathogenese van *Salmonella* infecties bij de kip is niet volledig duidelijk. Er was slechts zeer weinig gekend over de rol van flagelline tijdens oviduct kolonisatie. Flagelline interageert met pathogene herkenningsreceptoren die aanwezig zijn op epitheelcellen van de oviduct bij leghennen. Binding van flagelline met deze patronen leidt normaalgezien tot een sterke immuunrespons en met ontsteking en weefselschade als gevolg. *Salmonella* Enteritidis is echter in staat om de oviduct te koloniseren zonder een sterke immunologische reactie op te wekken. We hebben kunnen aantonen dat de expressie van flagellen bij *Salmonella* Enteritidis neergereguleerd is na kolonisatie van de oviduct, alsook in de epitheelcellen van de oviduct (**hoofdstuk 3.2**). De studies in hoofdstuk 3.2 tonen aan dat *Salmonella* Enteritidis in staat is om een effectieve immuunrespons van de gastheer te vermijden terwijl hij de oviduct koloniseert door het down reguleren van flagelline expressie. Verdere studies zijn nodig om de signaalmechanismen te identificeren die betrokken zijn in deze downregulatie van flagel door *Salmonella* Enteritidis in de omgeving van de oviduct. Deze informatie kan van belang zijn voor toekomstig vaccin onderzoek.

De huidige commerciële levende vaccins bevatten stammen die ongedefinieerde mutaties bevatten in 1 of meerdere genen op het chromosoom. Stammen met dit soort mutaties zouden echter kunnen terugkeren naar een virulent fenotype en worden dus beschouwd als onveilig. Toekomstige levende vaccins zouden dus volledig gedefinieerde stammen moeten bevatten die enkele of meerdere, volledige genen ontbreken. De meeste experimentele vaccins bevatten stammen die genen ontbreken die belangrijk voor het metabolisme of virulentie. Verschillende experimentele vaccins werd reeds getest in een aantal diersoorten, waaronder kippen, maar data over de bescherming van levende vaccins tegen ei besmetting zijn zeer zeldzaam. Een vaccin stam dat wordt gebruik voor de bescherming van verticale ei besmetting door *Salmonella* Enteritidis induceert idealiter een lokale immuunrespons in de reproductieve tractus. Vanuit het oogpunt van de volksgezondheid, mag het niet persisteren en bij voorkeur niet overleven in eiwit. Een logische aanpak is dus om genen te elimineren die belangrijk zijn voor eiwitoverleving. In het derde hoofdstuk (**hoofdstuk 3**) worden gedefinieerde mutanten voor MDR transporters gebruikt als vaccin stammen. Het tolc buitenste membraankanaal wordt gebruikt door MDR transporters (zoals acrAB, acrF en mdtABC) om antibacteriële componenten en bacteriële molecules te exporteren en is betrokken in de overleving in eiwit. De *Salmonella* Enteritidis *ΔtolC* en *ΔacrABacrEFmdtABC* stammen kunnen niet langer overleven in eiwit, hierbij wordt het risico op humane contaminatie door de vaccin stam via eieren geëlimineerd. De vaccin stammen waren in staat om ei besmetting met *Salmonella* te vermijden in een 6 maand *in vivo* proef.

Samengevat, verschillende maatregelen werden gebruikt om *Salmonella* infecties in de pluimvee industrie te controleren en te vermijden. Ondanks deze maatregelen duiken nieuwe (monofasische) varianten op. De huidige commerciële vaccins bieden bescherming tegen deze opkomende (monofasische) varianten. Het is belangrijk om continu te evalueren als de huidige vaccins bescherming bieden tegen nieuwe, opkomende varianten om zo snel mogelijk in te kunnen spelen op eventuele epidemiologische veranderingen. Tegelijkertijd is het belangrijk om de veiligheid van vaccinstammen te garanderen door het verwijderen van (een) volledig(e) gen(en). De *Salmonella* Enteritidis *ΔtolC* and *ΔacrABacrEFmdtABC* vaccin stammen zijn veilige stammen die zouden kunnen gebruikt worden om ei besmetting te voorkomen.

Curriculum Vitae

Curriculum Vitae

Sofie Kilroy werd op 20 januari 1987 geboren te Aalst. Na haar middelbare studies Latijn-Wetenschappen aan het Sint-Jozefscollege te Aalst, startte ze in 2005 met de studies 'Biomedische Wetenschappen' aan de Universiteit van Gent. In 2011 behaalde ze haar diploma met grote onderscheiding. Diezelfde zomer nog startte ze haar doctoraatsonderzoek aan de vakgroep Pathologie, Bacteriologie en Pluimveeziekten met Prof. Dr. Ir. Van Immerseel en Prof. Dr. Ducatelle als promotoren. In december 2012 behaalde zij een specialisatiebeurs toegekend door het Instituut voor de Aanmoediging van Innovatie door Wetenschap en Technologie in Vlaanderen (IWT). Deze specialisatiebeurs heeft geleid tot het ontwikkelen van een nieuw vaccin voor de preventie van *Salmonella* besmetting in eieren bij leghennen. In 2016 werd een patentaanvraag ingediend.

Gedurende dit onderzoek verwierf zei een beurs waardoor ze de resultaten van het nieuw ontwikkelde vaccin kon presenteren op het XXV World's Poultry Congress in Beijing, China. Aansluitend werd haar een culturele reis in China aangeboden.

Sofie Kilroy is auteur of medeauteur van meerdere wetenschappelijke publicaties in internationale tijdschriften. Zij nam deel aan congressen en presenteerde de resultaten van haar onderzoek in de vorm van posters en voordrachten.

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Dankwoord

Dankwoord

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