Universitat Autònoma de Barcelona

Immune and protective role of vaccines against Salmonella Enteritidis and Salmonella Typhimurium in laying hens

Lourdes Cecilia Vañó Sempere

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PhD Thesis

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Immune and protective role of vaccines against *Salmonella* Enteritidis and *Salmonella* Typhimurium in laying hens

Tesi doctoral presentada per **Lourdes Cecilia Vañó Sempere** per accedir al grau de Doctora en el marc del programa de doctorat en Medicina i Sanitat Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona, sota la direcció del Doctor **Ignacio Badiola Sáiz** i la tutoria del Doctor **Joaquim Segalés Coma**.

Bellaterra, 2020

El Doctor **Ignacio Badiola Sáiz**, investigador de l'Institut de Recerca i Tecnologia Agroalimentàries - Centre de Recerca en Sanitat Animal (IRTA-CReSA) i el Doctor **Joaquim Segalés Coma**, professor titular del Departament de Sanitat i d'Anatomia Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona i investigador adscrit a l'IRTA-CReSA,

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Bellaterra (Barcelona), 17 de febrer de 2020

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Director	Tutor	Doctoranda
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Il·lustració de la portada: Mere Sempere

Disseny de la portada: Ferran Sempere

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List of abbreviations

AF	Alexa Fluor
APC	allophycocyanin
bp	base pairs
BPW	buffered peptone water
CD3+	CD3 T lymphocytes
CD4+	CD4 T lymphocytes
CD8+	CD8 T lymphocytes
cfu	colony forming units
CpG	cytosine linked to a guanine by a phosphate bond
CT	caecal tonsil
DAPI	4′,6-diamidino-2-phenylindole
DC	dendritic cell
DNA	deoxyribonucleic acid
dpi	days post-infection
dpv	days post-vaccination
dsRNA	double-stranded RNA
ECDC	European Centre for Disease Prevention and Control
EFSA	European Food Safety Authority
ELISA	enzyme-linked immunosorbent assay
EU	European Union
FBS	fetal bovine serum
FITC	fluorescein
FSC	forward scatter
FSC-A	forward scatter area
FSC-H	forward scatter height
g	gravitational force equivalent
GALT	gut-associated lymphoid tissue
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
h	hour
HDP	host defense peptide
IEL	intra-epithelial lymphocytes
IFNγ	interferon gamma
Ig	immunoglobulin
IHF	immunohistofluorescence
IL	interleukin
kDa	kilodalton
LPS	lipopolysaccharide
M	microfold
MHC	major histocompatibility complex
min	minute
mRNA	messenger RNA
MSRV	semi-solid Rappaport-Vassiliadis agar

Mφ	macrophage
na	not applicable
NADPH	nicotinamide adenine dinucleotide phosphate
NCP	National Control Programmes
NK	natural killer
OCT	optimal cutting temperature
OD	optical density
p	probability value
PAMP	pathogen-associated molecular pattern
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PCT	percent change
PE	R-phycoerythrin
PP	Peyer's patches
PRR	pattern recognition receptor
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
RT-qPCR	reverse transcriptase quantitative real-time PCR
RV	Rappaport-Vassiliadis enrichment broth
S.	*Salmonella*
S/P	sample-to-positive ratio
SCV	*Salmonella*-containing vacuole
SD	standard deviation
SIF	*Salmonella*-induced filaments
SPF	specific pathogen free
SPI	*Salmonella* pathogenicity islands
SSC	side scatter
ssRNA	single-stranded RNA
T3SS	type III secretion system
TCR	T cell receptor
TLR	toll-like receptor
TNFα	tumor necrosis factor alpha
XLT4	xylose lysine tergitol 4

Abstract

Salmonellosis is a zoonosis caused by non-adapted bacterial strains of the genus *Salmonella. Salmonella* is commonly found in the intestines of healthy birds and mammals and can enter the food chain through contaminated meat, eggs and their products. Consumption of food or water contaminated with *Salmonella* causes gastroenteritis in humans. The last report from the European Food Safety Authority indicates that this bacterium was the second cause of human zoonosis in the European Union in 2018. Particularly, *Salmonella* caused one third of all foodborne outbreaks (30.7%). From these outbreaks almost half were caused by eggs and egg products (45.6%), and most (70%) were caused by only two serovars: *Salmonella* Enteritidis and *Salmonella* Typhimurium.

In 2003 the EU started to implement the *Salmonella* National Control Programmes in all member states to reduce *Salmonella* prevalence in the poultry and pig industry. These plans consisted in measures of biosafety and additional tools like vaccination against *Salmonella*, which achieved an important reduction in prevalence and the consequent reduction of reported cases of human salmonellosis.

Non-adapted serovars do not cause clinical disease in hens older than 3 days. Therefore, the main objective of vaccines against these *Salmonella* serovars in poultry is to increase resistance of animals against infection in order to reduce the contamination of poultry derived products.

The main objective of this thesis was to determine the protective function of a live attenuated *Salmonella* Typhimurium strain (alone or combined with a *Salmonella* Enteritidis strain) against field strains infections and to evaluate the associated immune response. Three studies were carried out at three different production times in hens.

In the first study the objective was to determine the efficacy of a first vaccine dose at day-old after challenge at 14 days comparing oral and spray administration. The oral vaccination partially protected and reduced colonisation in internal organ, indicating the need of additional vaccine boosters to develop a stronger immunity against *Salmonella*. The protection was not correlated with humoral or cellular immune response. The spray administration failed, and animals were not protected, indicating the importance of the administration route.

In the second study, the efficacy of the vaccine was tested after 2 vaccine doses (challenged at 16 weeks of life) or 3 vaccine doses (challenged at 35 weeks of life). Both vaccine groups had reduction of *Salmonella* excretion and colonisation of internal organs. This adaptive protective immune response was not correlated with levels of antibodies (which were especially higher in non-vaccinated animals in the intestine), but was correlated with a general increase in CD3+ T cell population in the intestine and a IFNγ up-regulation, indicating a possible drift to a protective Th1 immune response.

In the third study, the efficacy of vaccination during rearing with a combined *Salmonella* Enteritidis and *Salmonella* Typhimurium vaccine was determined at the end of the laying period. Vaccinated animals had reduced rates of *Salmonella* Enteritidis and *Salmonella* Typhimurium excretion in cloacal swabs and were protected against internal organ colonisation. The protection was not related with humoral response but with a cellular response in the intestine including the infiltration of macrophages, CD4+ and CD8+ T cells.

The results extracted from this thesis indicate that the tested vaccines could be a useful tool to decrease vertical transmission to eggs (reducing infection of internal organs) and horizontal transmission to eggs and poultry products (reducing excretion of bacteria) to ultimately minimise the risk of salmonellosis in humans.

Resum

La salmonel·losi és una zoonosi causada per soques no adaptades de bacteris del gènere *Salmonella*. La *Salmonella* es troba habitualment a l'intestí d'ocells i mamífers sans i pot entrar a la cadena alimentària mitjançant la contaminació de carn, ous i els seus productes derivats. El consum d'aliments o aigua infectada amb *Salmonella* causa gastroenteritis en humans. L'últim informe de l'European Food Safety Authority indica que aquest bacteri va ser la segona causa de zoonosi en humans a la Unió Europea el 2018. En particular, *Salmonella* va causar un terç de tots els brots alimentaris (30,7%). Gairebé la meitat d'aquests brots van ser causats per ous i productes derivats (45,6%), i la majoria (70%) van ser causats només per dues serovarietats: *Salmonella* Enteritidis i *Salmonella* Typhimurium.

L'any 2003 la UE va començar a implementar els Programes Nacionals de Control de *Salmonella* a tots els estats membres per reduir la prevalença de *Salmonella* a la indústria avícola i porcina. Aquests plans van consistir en mesures de bioseguretat i eines addicionals com la vacunació contra *Salmonella*, i van aconseguir una reducció important de la prevalença i la conseqüent reducció dels casos reportats de salmonel·losi humana.

Les serovarietats no adaptades de *Salmonella* no causen malaltia clínica en gallines majors de 3 dies. Per tant, l'objectiu principal de les vacunes contra aquestes serovarietats en aus és augmentar la resistència dels animals contra la infecció per tal de reduir la contaminació dels productes derivats.

L'objectiu principal d'aquesta tesi va ser determinar la funció protectora d'una soca viva atenuada de *Salmonella* Typhimurium (sola o combinada amb una soca de *Salmonella* Enteritidis) contra les infeccions de soques de camp i avaluar la resposta immune associada. Es van realitzar tres estudis en tres èpoques de producció diferents en gallines.

En el primer estudi, l'objectiu va ser determinar l'eficàcia d'una primera dosi de vacuna a dia de vida comparant l'administració oral i via aerosol, després d'una infecció als 14 dies de vida. Els animals vacunats via oral van estar parcialment protegits i es va reduir la colonització en òrgans interns, fet que indica la necessitat d'utilitzar dosis addicionals de vacuna per desenvolupar una immunitat més forta contra la *Salmonella*. La protecció no es va correlacionar amb la resposta immune humoral o cel·lular. L'administració de vacuna via aerosol va fallar, indicant la importància de la ruta d'administració.

En el segon estudi, es va provar l'eficàcia de la vacuna després de 2 dosis (amb infecció experimental a les 16 setmanes de vida) o 3 dosis (infecció experimental a les 35 setmanes de vida). Els dos grups vacunals van reduir l'excreció de *Salmonella* i la colonització d'òrgans interns. Aquesta resposta immune adaptativa protectora no es va correlacionar amb els nivells d'anticossos (que van ser més alts a l'intestí d'animals no vacunats), però es correlacionà amb un augment general de la població de cèl·lules T CD3+ a l'intestí i amb l'expressió d'IFNγ, fet que indica una possible deriva cap a una resposta immune de protecció Th1.

Al tercer estudi, es va determinar l'eficàcia de la vacunació durant la cria amb una vacuna combinada de *Salmonella* Enteritidis i *Salmonella* Typhimurium al final del període de posta. Els animals vacunats van reduir l'excreció de *Salmonella* Enteritidis i *Salmonella* Typhimurium en femtes i van estar protegits contra la colonització d'òrgans interns. La protecció no es va relacionar amb la resposta humoral, però si amb una resposta cel·lular a l'intestí incloent la infiltració de macròfags, cèl·lules T CD4+ i CD8+.

Els resultats extrets d'aquesta tesi indiquen que les vacunes estudiades podrien ser una eina útil per disminuir la transmissió vertical als ous (reduint la infecció d'òrgans interns) i la transmissió horitzontal a ous i productes avícoles (reduint l'excreció) per atenuar el risc de salmonel·losi en humans.

Resumen

La salmonelosis es una zoonosis causada por cepas bacterianas no adaptadas del género *Salmonella*. La *Salmonella* se encuentra comúnmente en el intestino de aves y mamíferos sanos y puede entrar a la cadena alimentaria a través de la contaminación de carne, huevos y sus productos derivados. El consumo de alimentos o agua contaminada con *Salmonella* causa gastroenteritis en humanos. El último informe de la European Food Safety Authority indica que esta bacteria fue la segunda causa de zoonosis en humanos en la Unión Europea en 2018. En particular, *Salmonella* causó un tercio de todos los brotes transmitidos por alimentos (30.7%). De estos brotes, casi la mitad fueron causados por huevos y sus derivados (45,6%), y la mayoría (70%) fueron causados por solo dos serovariedades: *Salmonella* Enteritidis y *Salmonella* Typhimurium.

El año 2003, la Unión Europea comenzó a implementar los Programas Nacionales de Control de *Salmonella* en todos los estados miembros para reducir la prevalencia de *Salmonella* en la industria avícola y porcina. Estos planes consistieron en medidas de bioseguridad y herramientas adicionales como la vacunación contra *Salmonella*, y lograron una reducción importante en la prevalencia y la consiguiente reducción de los casos reportados de salmonelosis humana.

Las serovariedades no adaptadas de *Salmonella* no causan enfermedad clínica en gallinas mayores de 3 días. Por lo tanto, el objetivo principal de las vacunas contra estos serotipos en aves es aumentar la resistencia de los animales contra la infección para reducir la contaminación de los productos derivados.

El objetivo principal de esta tesis fue determinar la función protectora de una cepa viva atenuada de *Salmonella* Typhimurium (sola o combinada con una cepa de *Salmonella* Enteritidis) contra las infecciones por cepas de campo y evaluar la respuesta inmune asociada. Se realizaron tres estudios en tres tiempos de producción diferentes en gallinas.

En el primer estudio, el objetivo era determinar la eficacia de una primera dosis de vacuna a día de vida comparando la administración oral y vía aerosol, tras una posterior infección a los 14 días de vida. Los animales vacunados estuvieron parcialmente protegidos y se redujo la colonización en órganos internos, lo que indica la necesidad de utilizar dosis adicionales de vacuna para desarrollar una inmunidad más fuerte contra la *Salmonella*. La protección no se correlacionó con la respuesta inmune humoral o

celular. La administración de vacuna vía aerosol falló, indicando la importancia de la ruta de administración.

En el segundo estudio, se probó la eficacia de la vacuna después de 2 dosis (con infección experimental a las 16 semanas de vida) o 3 dosis (infección experimental a las 35 semanas de vida). Ambos grupos vacunales redujeron la excreción de *Salmonella* y la colonización de órganos internos. Esta respuesta inmune adaptativa protectora no se correlacionó con los niveles de anticuerpos (que fueron más altos en el intestino de animales no vacunados), pero se correlacionó con un aumento general de la población de células T CD3+ en intestino y con la expresión de IFNγ, lo que indica una posible deriva hacia una respuesta inmune de protección Th1.

En el tercer estudio, se determinó la eficacia de la vacunación durante la cría con una vacuna combinada de *Salmonella* Enteritidis y *Salmonella* Typhimurium al final del periodo de puesta. Los animales vacunados redujeron la excreción de *Salmonella* Enteritidis y *Salmonella* Typhimurium en heces y estuvieron protegidos contra la colonización de órganos internos. La protección no se relacionó con la respuesta humoral, pero si con una respuesta celular en el intestino incluyendo la infiltración de macrófagos, células T CD4+ y CD8+.

Los resultados extraídos de esta tesis indican que las vacunas estudiadas podrían ser una herramienta útil para disminuir la transmisión vertical a los huevos (reduciendo la infección de órganos internos) y la transmisión horizontal a huevos y productos avícolas (reduciendo la excreción) para atenuar el riesgo de salmonelosis en humanos.

1. GENERAL INTRODUCTION

1.1 A BRIEF OVERVIEW: ROLE OF *Salmonella* AS A ZOONOTIC AGENT

1.1.1 Zoonotic diseases

Zoonotic diseases are caused by infections that spread between animals and people. Foodborne zoonotic diseases are caused by consumption of food or water contaminated by pathogenic microorganisms such as bacteria, viruses, fungi and parasites (EFSA, 2019).

Bacteria from genus *Salmonella* cause a foodborne zoonotic disease in humans called salmonellosis. Generally, it is originated through the faecal-oral route by the consumption of contaminated food or water. *Salmonella* bacteria can cause in humans two clinical manifestations, depending on if the serovar is strictly adapted to a narrow host range or is non-adapted and has a broad range of animal reservoirs (Gal-Mor et al., 2014).

Serovars adapted to humans (also referred as typhoidal serovars) cause systemic infections known as typhoid fever. The serovar causing typhoid fever is *Salmonella enterica* subsp. *enterica* serovar Typhi. The affection is often life-threatening and classical symptoms include fever, hepatosplenomegaly, abdominal pain, and sometimes nausea, diarrhoea and headache (Buckle et al., 2012). Other serovars can cause an affection known as paratyphoid fever, which is a mild form of typhoid fever (*Salmonella enterica* subsp. *enterica* serovar Paratyphi A, *Salmonella enterica* subsp. *enterica* serovar Paratyphi B, *Salmonella enterica* subsp. *enterica* serovar Paratyphi C and *Salmonella enterica* subsp. *enterica* serovar Sendai) (Baumler and Fang, 2013). Without effective treatment, typhoid fever has a case-fatality rate of 10-30%. The incidence of typhoid fever is higher in areas with poor hygiene, and therefore typhoid and paratyphoid fever remain important public health problems in low- and middle-income countries (Buckle et al., 2012). The latest studies estimated that there were 14,3 million cases of typhoid and paratyphoid fever globally in 2017, with 135,900 deaths (Stanaway et al., 2019).

Non-adapted serovars (also referred as non-typhoidal serovars) like *Salmonella enterica* subsp. *enterica* serovar Enteritidis or *Salmonella enterica* subsp. *enterica* serovar Typhimurium cause non-typhoidal gastroenteritis in healthy humans, but can be a risk in immunocompromised individuals (such as infants, older people or oncologic patinets) (Pegues et al., 2006). Normally, the origin of these serovars is animal-derived food like eggs, poultry meat, pork meat and dairy products, although in some cases

transmission can result from vegetables, nuts, spices or even from pets such as dogs and cats (Gal-Mor et al., 2014).

The notification of non-typhoidal salmonellosis in humans is mandatory in most member states of the European Union except five where reporting is based on a voluntary system (Belgium, France, Luxembourg, and the Netherlands) or other systems (the United Kingdom). The notification is also mandatory in Iceland, Norway and Switzerland (EFSA, 2019).

1.1.2 The *Salmonella* status in the European Union

Non-typhoidal salmonellosis was the second commonest reported foodborne zoonotic disease in the European Union (EU) in 2018, right after campylobacteriosis, with 91,857 confirmed cases (Figure 1-1). The EU trend for confirmed human salmonellosis cases decreased significantly from 2008 to 2018, but during the last 5 years (2014-2018) this trend has remained constant. The same trend was observed in the prevalence of *Salmonella* spp. in poultry flocks (Figure 1-2) (EFSA, 2019; Koutsoumanis et al., 2019). *Salmonella* caused the highest number of foodborne outbreaks in 2018 with 1,581 notifications (30.7% of total outbreaks). Almost half of the cases of salmonellosis required hospitalisation (41.7%) and the case fatality for this disease was of 0.19%. Interestingly enough, eggs and egg products were the food vehicle that caused the highest number of *Salmonella* outbreaks in 2018, like in the previous years (Figure 1-3) (EFSA, 2019).

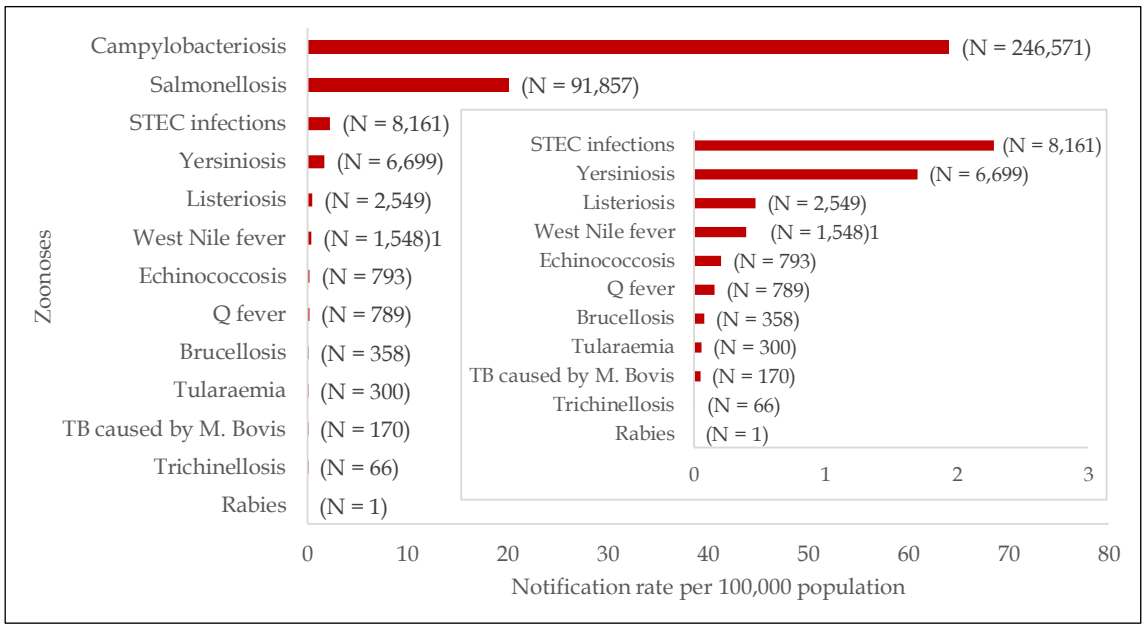

Figure 1-1. Reported numbers and notification rates of confirmed human zoonoses in the EU, 2018. The total number of confirmed cases is indicated between parentheses at the end of each bar. [1]West Nile fever virus used the total number of cases. Source: modified from EFSA, 2019.

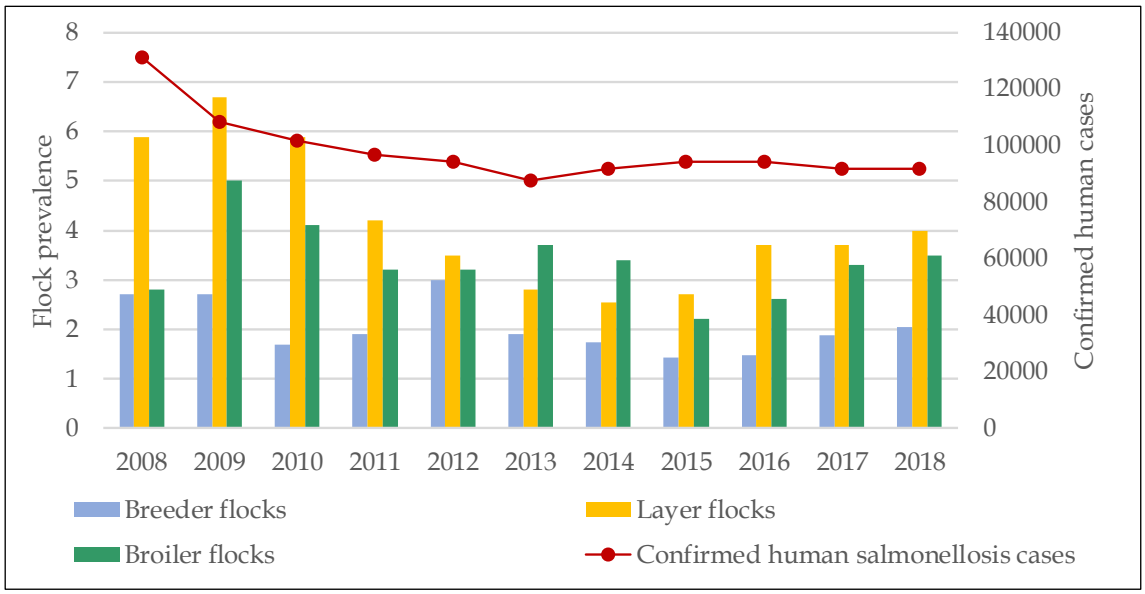

Figure 1-2. Evolution of the prevalence of Salmonella spp. in poultry populations and the number of confirmed human cases of salmonellosis in the EU, 2008–2018. Source: modified from EFSA, 2019; Koutsoumanis et al., 2019.

The five most commonly reported serovars in human cases of salmonellosis in 2018 were: *Salmonella* Enteritidis (49.9% of total cases), *Salmonella* Typhimurium (13.0%), monophasic *Salmonella* Typhimurium (8.1%), *Salmonella* Infantis (2.3%) and *Salmonella* Newport (1.4%) (EFSA, 2019). There is a relation between serovars detected in most important animal species and serovars causing disease in humans (Figure 1-4) (Gast, 2007; EFSA and ECDC, 2018). Furthermore, some pairs of serovars and food matrices like *Salmonella* Enteritidis and table eggs are more frequently associated with outbreaks than others because of consumer habits and climatic factors. For instance, raw pooled eggs are commonly used as an ingredient for several food products; and eggs are not required to be refrigerated in the shops, so temperature can fluctuate in ranges that allow microorganisms to grow (Koutsoumanis et al., 2019).

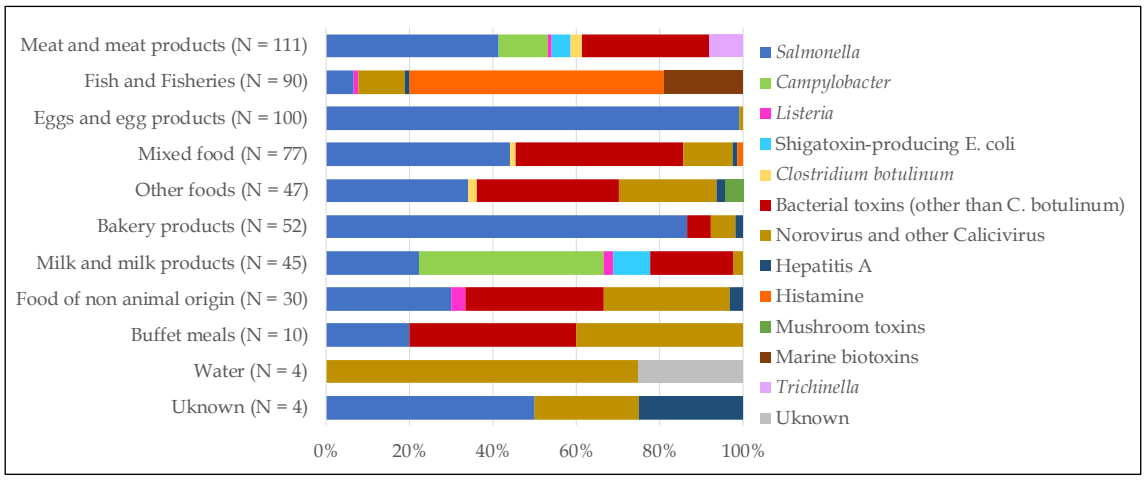

Figure 1-3. Frequency distribution of causative agents associated with strong evidence foodborne and waterborne outbreaks, by food vehicle, in reporting Member States, EU, 2017. Source: modified from EFSA and ECDC, 2018.

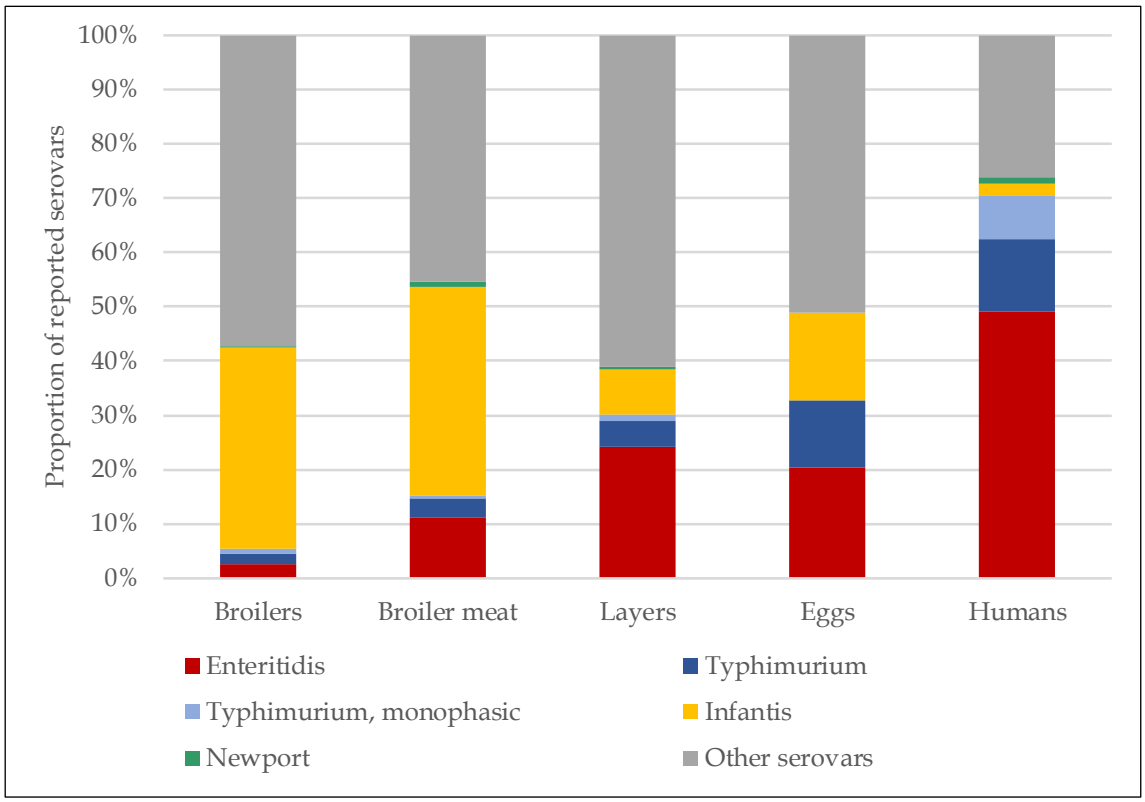

Figure 1-4. Distribution of the top-five and other or unspecified Salmonella serovars isolated in broilers, broiler meat, layers, eggs and in confirmed cases of human salmonellosis in the EU, 2017. Source: modified from EFSA and ECDC, 2018.

1.1.3 The *Salmonella* National Control Programmes

To protect human health and to avoid economic losses in the food industry, the EU started to implement the *Salmonella* National Control Programmes (NCP) in all member states in 2003 with the main goal of reducing *Salmonella* prevalence in the primary sector. The basic regulation regarding the control of *Salmonella* is the Regulation (European Commission) No 2160/2003 to ensure that measures are taken in the poultry and pig industry to detect and control *Salmonella* at all relevant stages of production, processing, and distribution, to reduce its prevalence (Table 1-1) (European Commission, 2003). These measures consisted of strict biosecurity procedures and additional actions like vaccination programmes in animals, good hygiene, good husbandry and welfare. To implement an effective biosecurity programme, an integrated approach is essential. For example, effective vaccines for *Salmonella* control are available but cannot be used as a single measure against *Salmonella*. Their efficacy is improved by an effective strategy of cleaning and disinfection of farms to reduce infection pressure and allow the vaccine to work more efficiently (Lister, 2008). Also, control measures should be implemented considering the properties of each infectious agent. For instance, to avoid the vertical transmission of some *Salmonella* serotypes, the poultry breeding farms need to focus on

egg and hatchery hygiene; and to avoid the spread of *Salmonella* via droppings and litter in broiler and layer flocks, the cleaning and disinfection of pens and equipment is a critical point (Lister, 2008). A detailed list of recommendations on prevention, detection and control of *Salmonella* in poultry has been published by the World Organisation for Animal Health (World Organisation for Animal Health (OIE), 2019a, 2019b).

Table 1-1. General requirements for national control programmes of Salmonella and established targets for the reduction of prevalence in poultry. Source: modified from EFSA and ECDC, 2018.

Target serovars	**Animal population**	**Stage of food chain**	**Prevalence target**
S. Enteritidis, *S*. Typhimurium, *S*. Infantis, *S*. Virchow, *S*. Hadar	Adult breeding hens (*Gallus gallus*) (European Commission, 2010)	Primary production	≤ 1%
S. Enteritidis, *S*. Typhimurium	Adult laying hens (*Gallus gallus*) (European Commission, 2011)	Primary production	≤ 2%[a]
	Broilers (*Gallus gallus*) (European Commission, 2012a)	Primary production	≤ 1%
	Turkeys (*Meleagris gallopavo*) (European Commission, 2012b)	Primary production	≤ 1%

[a]Prevalence reduction calculated according to the prevalence in the preceding year.

1.1.4 The use of vaccines for the control of *Salmonella*

The use of vaccines as a specific method for the control of *Salmonella* in poultry is regulated in the framework of the NCP (European Commission, 2006). Under this regulation, vaccines should comply with specific requirements. Firstly, live *Salmonella* vaccines should be able to differentiate from wild-type strains. Secondly, live vaccines shall not be used in laying hens during production unless the safety has been demonstrated and there is an authorisation for it. And finally, vaccination programmes against *Salmonella* Enteritidis shall be applied at least during rearing to all laying hens if the Member State do not demonstrate a prevalence below 10%. The competent authority may derogate this regulation in a holding if the preventive measures taken are satisfactory and the absence of *Salmonella* Enteritidis has been demonstrated in the previous 12 months.

1.2 THE GENUS *Salmonella*

1.2.1 History of *Salmonella*

At the end of the 19th century, when disease transmission was still little understood, the physician and epidemiologist William Budd hypothesized that Typhoid fever was caused by a microorganism and, therefore, transmissible by water, food, or human carriers (Budd, 1918; Moorhead, 2002). The *Salmonella* bacillus was first identified by German pathologist Karl Eberth in 1880 in the abdominal lymph nodes and the spleen of a patient who died from Typhoid fever after a long research on the aetiological agent of the disease (Barnett, 2016). The first isolation of a *Salmonella* bacteria was in 1884 by the bacteriologist Georg Gaffky (Barnett, 2016). Later, in 1885, a research group of the United States Department of Agriculture under the administration of the veterinary pathologist Daniel Elmer Salmon, isolated a bacillus from the intestine of pigs diagnosed with hog cholera (Salmon, 1885). This microorganism was first named cholera bacillus. The name of the genus *Salmonella* came afterward, and was first used by Joseph Lignières in 1900 in honour to Salmon's group work: *"le microbe du hog-cholera de Salmon"* (Salmonella Subcommittee of the Nomenclature Committee of the International Society for Microbiology, 1934). Since then, *Salmonella* has been considered a relevant zoonotic agent for public health, both in its typhoidal or non-typhoidal form (Gal-Mor et al., 2014).

1.2.2 Taxonomy and description

Bacteria from *Salmonella* genus are non-sporulated Gram-negative bacilli (0.7-1.5 x 2.0-5.0 μm) that belong to the *Enterobacteriaceae* family. *Salmonella* grows in colonies of 2-4 mm in diameter. They are usually motile by peritrichous flagella, facultative anaerobic, and facultative intracellular. Most salmonellae are aerogenic, ferment glucose but not lactose, utilise citrate, and produce hydrogen sulphide (Popoff and Le Minor, 2005). In Figure 1-5 there is an example of *Salmonella* spp. and *Escherichia coli* growth in different culture media plates.

The genus *Salmonella* is composed of two species: *Salmonella bongori* (subsp. V) and *Salmonella enterica*. *Salmonella enterica* comprises six subspecies: *Salmonella enterica* subsp. *enterica* (I), *Salmonella enterica* subsp. *salamae* (II), *Salmonella enterica* subsp. *arizonae* (IIIa), *Salmonella enterica* subsp. *diarizonae* (IIIb), *Salmonella enterica* subsp. *houtenae* (IV), and *Salmonella enterica* subsp. *indica* (VI) (Le Minor and Popoff, 1987; Reeves et al., 1989; Grimont and Weill, 2007).

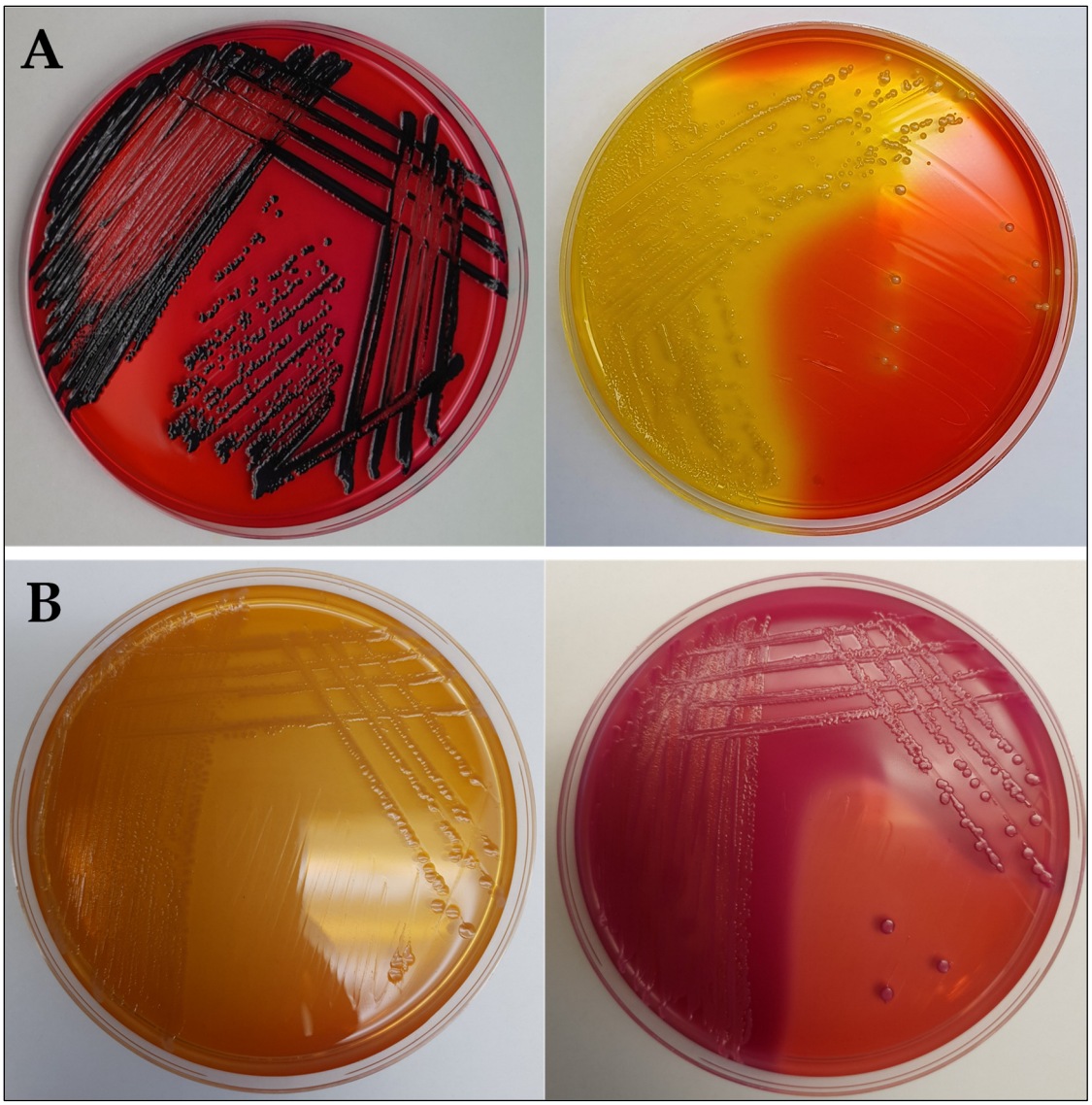

Figure 1-5. Example of Salmonella Typhimurium (left plate) and Escherichia coli (right plate) after 24 h growth at 37 °C in aerobic conditions in selective differential media. (A) XLT4 agar: Salmonella Typhimurium produces black-centred colonies due to the production of hydrogen sulphide; Escherichia coli produces yellow colonies and ferments the lactose in the formulation, causing a pH drop which shifts the colour of the medium from red to yellow. (B) MacConkey agar: Salmonella Typhimurium does not ferment lactose and produces colourless colonies without changing the colour of the medium; Escherichia coli ferments lactose and produces red or pink colonies in a red medium due to the production of acid from lactose.

Salmonella bongori (subsp. V) is present in ectotherms but has lately been identified in humans and birds (Foti et al., 2009). *Salmonella enterica* is commonly found in the intestines of healthy animals. *Salmonella enterica* subsp. I is adapted to endotherms and is the origin of most cases of human salmonellosis (EFSA and ECDC, 2018). *Salmonella enterica* subsp. II, III and IV are commonly found in reptiles and ectotherms (Schroter et al., 2004; Editorial Team et al., 2008).

Figure 1-6 shows an overview of the current classification of *Salmonella* genus (Achtman et al., 2012).

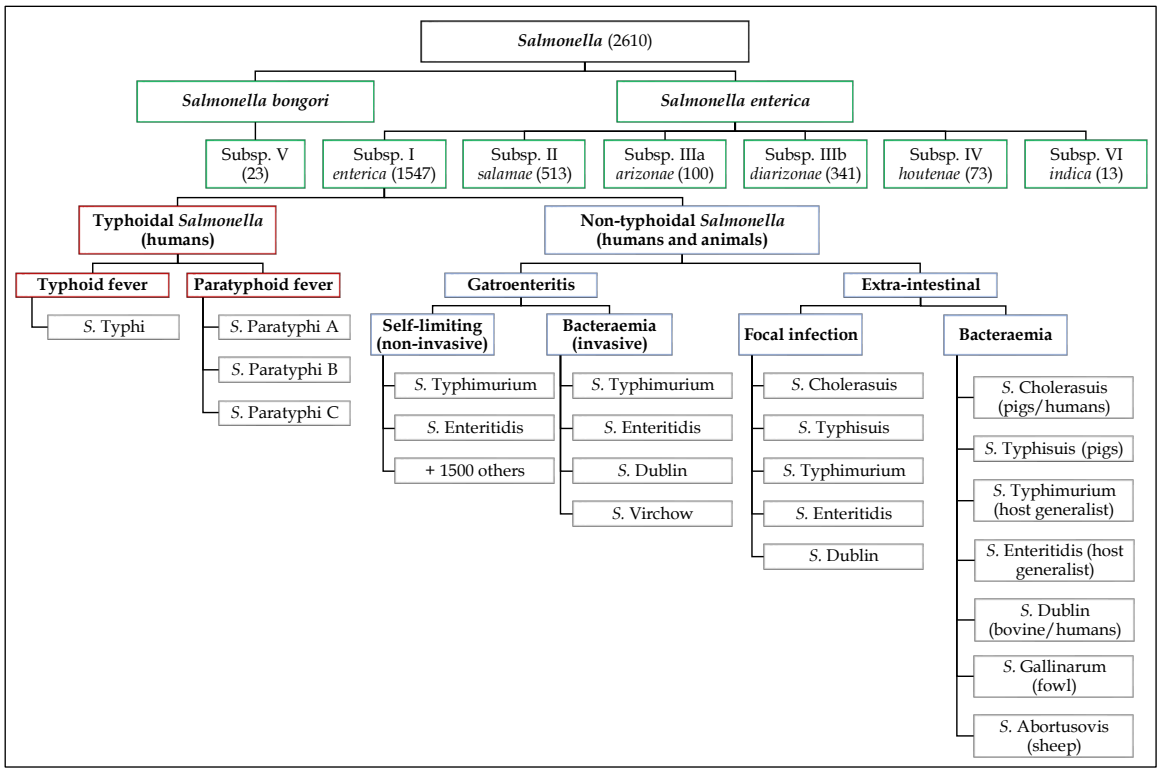

Figure 1-6. Classification of Salmonella genus. Numbers in brackets indicate the total number of serovars included in each subspecies. Source: modified from Achtman et al., 2012.

1.2.3 Serotyping of *Salmonella* genus

The taxonomic group of *Salmonella* contains more than 2600 serovars (also called serotypes) (Guibourdenche et al., 2010). Names are used to designate *Salmonella enterica* subsp. *enterica* (I) serovars, and serovars of other subspecies of *Salmonella enterica* and those of *Salmonella bongori* are designated only by their antigenic formula. Therefore, to designate *Salmonella enterica* subsp. *enterica* (I) serovars and to emphasize that they are not separate species, the serovar name is not italicized and the first letter is capitalized. At the first citation of a serovar the genus name is given followed by the word "serovar" or the abbreviation "ser." and then the serovar name (for example: *Salmonella enterica* subsp. *enterica* serovar/ser. Typhimurium). Subsequently, the name may be written with the genus followed directly by the serovar name (Brenner et al., 2000).

Each *Salmonella* serovar is recognized by a unique antigenic formula (designated with the White-Kaufmann-Le Minor scheme) that is composed of numbers and letters given to the different antigens in the bacteria: O (somatic), Vi (capsular), and first and second phases of H (flagellar) antigens. The formulas with major O antigenic factors in common are included into an O group and arranged alphabetically by the first phase of the H antigen within the group. Factors associated with phage conversion are underlined

(Popoff and Le Minor, 2005). The specificities of O factors are determined by the composition and structure of the lipopolysaccharide (LPS). The Vi antigen is a surface antigen found only in some serovars. Serovars can also be subdivided in biovars (different sugar fermentation patterns), phagovars (different sensitivity of cultures to bacteriophages), or can be subdivided based on different sensitivity to bacteriocins or antibiotics (Grimont and Weill, 2007).

In Table 1-2 there are examples of the antigenic formulae of some serovars based on the White-Kaufmann-Le Minor scheme.

Table 1-2. Examples of some serovars for Salmonella enterica subsp. enterica with their antigenic formula based on the White-Kaufmann-Le Minor scheme. Source: Grimont and Weill, 2007.

Serovar	**Antigenic formula**				
	O Group	**Somatic (O) antigen[ab]**	**Flagellar (H) antigen[c]**		
			Phase 1	**Phase 2**	**Other[d]**
Typhi	9 (D_1)	9,12[Vi]	d		[j],[z_{66}]
Enteritidis	9 (D_1)	<u>1</u>,9,12	g,m		
Gallinarum	9 (D_1)	<u>1</u>,9,12			
Infantis	7 (C_1)	6,7,<u>14</u>	r	1,5	[R1...],[z_{37}],[z_{45}],[z_{49}]
Typhimurium	4 (B)	<u>1</u>,4,[5], 12	i	1,2	

[a]Underlined O factors are determined by phage conversion. [b]O or H factors in square brackets may be present or absent without relation to phage conversion. [c]A diagonal line indicates that the antigen is not present in that serovar. [d]R phases and third phases, uncommon.

1.2.4 Host adaptation of *Salmonella* serovars

Salmonella serovars that affect mammals and poultry come from *Salmonella enterica* subsp. *enterica* (I) and can be strictly adapted to one host or can be non-adapted (ubiquitous) (Baumler and Fang, 2013).

Adapted serovars are characterised for having a narrow host range and are not normally pathogenic for other species. This serovars do not cause gastroenteritis in their hosts, and instead they are associated with disseminated septicaemia. An example of adapted serovars in humans are serovar Typhi or Paratyphi A (as already mentioned in Section 1.1.1), which are transmitted from person to person without an intermediate host, through contaminated water or food. Other serovars are adapted to one animal species: serovar Abortusovi to sheep or serovar Gallinarum to poultry (Popoff and Le Minor, 2005).

Non-adapted serovars (such as serovar Enteritidis or Typhimurium) are the main responsible for foodborne infections and for that reason the present PhD thesis will focus on those serovars. These serovars enter the food chain through different sources: replacement animals, humans, domestic and wild animals and birds, insects, contaminated equipment or water. Once introduced into a poultry unit, *Salmonella* strains can further spread within and between holdings through movements of people, vehicles and equipment, and finally spread to humans causing infections (Foley et al., 2011).

1.2.5 *Salmonella* virulence factors

The genus *Salmonella* owns a variety of factors involved in invasion and survival in the host (Ibarra and Steele-Mortimer, 2009).

1.2.5.1 *Salmonella* Pathogenicity Islands (SPI)

The chromosome of *S. enterica* contains a group of virulence gene clusters called *Salmonella* pathogenicity islands (SPI). So far, a total of five major SPIs have been identified (Shivaprasad et al., 2013). In general, SPI-1 is required for invasion of host cells, SPI-2 is required for replication of intracellular bacteria, SPI-3 is involved in gut colonisation and intracellular survival in low-magnesium environments (Blanc-Potard et al., 1999), SPI-4 can be linked with intramacrophage survival, adhesion and apoptosis (Baumler et al., 1994; Gerlach et al., 2007), and SPI-5 seems to mediate enteric pathogenicity (Wood et al., 1998).

SPI-1 and SPI-2 are the two most important and well-studied SPIs of *Salmonella* and code for proteins forming the type III secretion system (T3SS) which are used by the pathogen to deliver into host cells virulence proteins that interfere with their signalling pathways (Jones et al., 2007; Rychlik et al., 2009). The SPI-1 encoded T3SS specifically allows the transport of bacterial proteins into the host cell cytosol and results in the uptake of *Salmonella*, even by non-phagocytic cells of the intestinal epithelium (Jones et al., 1994; Kaniga et al., 1995). SPI-2 encoded T3SS is required for the transport of *Salmonella* proteins across the phagosome membrane and increases survival and proliferation of bacteria inside the phagocytic cells (Cirillo et al., 1998; Hensel et al., 1998).

1.2.5.2 Surface structures of *Salmonella*

The polysaccharides on the surface of *Salmonella* are the outermost components of the bacterial cell, therefore they are of great importance for bacterial survival in the hostile environment (Rycroft, 2013).

The outer envelope of Gram-negative bacteria is formed by three layers: the cytoplasmic membrane (inner membrane), the peptidoglycan and the outer membrane (Rycroft, 2013). The cytoplasmic membrane and the outer membrane are two lipid bilayers that are separated by the periplasmic space. The two membranes are connected at various points to facilitate the transport of hydrophobic components such as LPS from the cytoplasmic membrane to the outer membrane and may be the site of synthesis of some outer membrane proteins. Periplasmic space contains peptidoglycan and also numerous soluble proteins that have different functions: catabolic, such as alkaline phosphatase which helps transport solutes trough the cytoplasmic membrane; binding proteins, which assist transport of nutrients such as amino acids, ions and sugars; and finally proteins which modify harmful substances such as antibiotics (for example β-lactamase) (Rycroft, 2013).

The cytoplasmic membrane is composed of phospholipids and proteins. Among its functions is the transport nutrients, the site of oxidative phosphorylation and the synthesis of phospholipid, peptidoglycan units and LPS. The cytoplasmic membrane is also the site of anchorage of the DNA during replication and intervenes in the partitioning of daughter cells at cell division (Rycroft, 2013).

The peptidoglycan is a thin layer composed of alternating residues of *N*-acetyl muramic acid and *N*-acetyl glucosamine forming long glycan chains, which are covalently cross-linked by peptide bridges. This layer confers rigidity to the bacterial cell and stabilises it against osmotic lysis (Rycroft, 2013).

The outer membrane is a highly complex lipid bilayer membrane, composed primarily of phospholipid, protein, LPS and lipoprotein. LPS in found in the outer leaflet and lipoprotein in the inner, where it anchors the outer membrane to the peptidoglycan. Enterobacterial common antigen is present in a small quantity (Rycroft, 2013).

In Figure 1-7 there is a representation of the *Salmonella* cell wall structure and its main surface antigens (Minamino and Imada, 2015; Radkov et al., 2018).

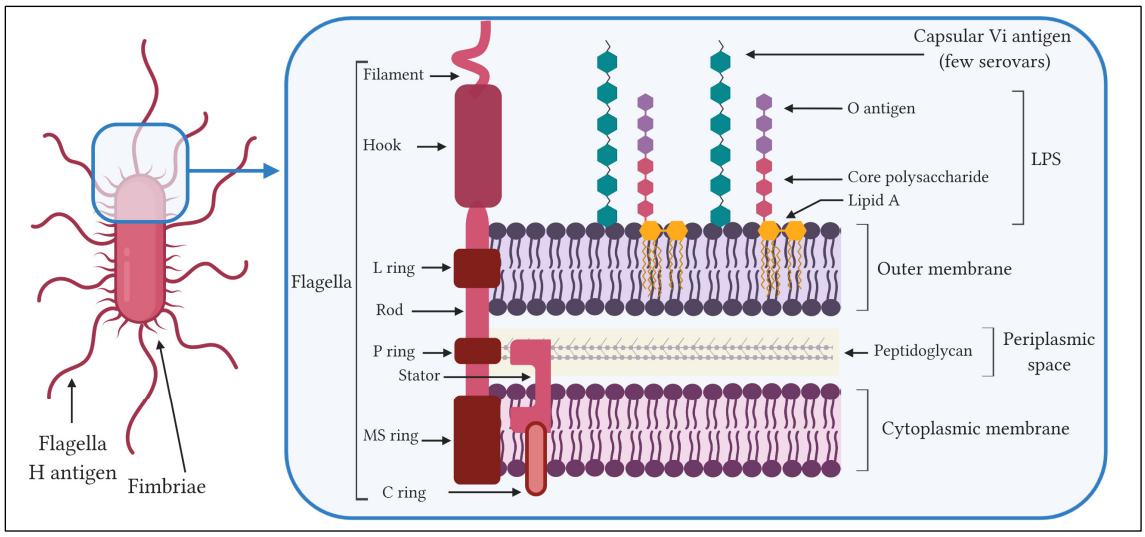

Figure 1-7. Structure of the Salmonella spp. cell wall and the main surface antigens. Source: modified from Minamino and Imada, 2015; Radkov et al., 2018. Created with BioRender.com.

1.2.5.3 Capsular antigen

The Vi antigen is the only true capsular polysaccharide produced by *Salmonella* spp. It is only produced by few strains of *Salmonella* Typhi, *Salmonella* Paratyphi C and *Salmonella* Dublin. It is external to the outer membrane. It was named Vi capsular antigen because of its association with virulence and it is identified in the antigenic formula by the letters Vi. This antigen is common in many other Gram-negative bacteria like *Pasteurella* spp. or *Klebsiella* (Rycroft, 2013). Among the functions of this antigen are the prevention of *Salmonella* LPS recognition by TLR4 and the reduction of opsonisation and expression of interleukin-8 (IL-8) (Raffatellu et al., 2005; Wilson et al., 2008).

1.2.5.4 Lipopolisaccharide (LPS)

As mentioned before, the LPS is the most external molecule in the surface of Gram-negative bacteria, and it is also the main antigen. LPS is a pathogen-associated molecular pattern (PAMP) recognised by the Toll-like receptor 4 (TLR4) causing inflammatory response in host cells and the activation of the innate immune system (Leveque et al., 2003; Akira and Takeda, 2004; Rycroft, 2013).

LPS is an amphipathic molecule formed by three regions: the lipid A, the core oligosaccharide and the O side-chain repeating oligosaccharide. Colonies of wild strains of *Salmonella* usually have a smooth appearance associated with the presence of a full O side-chain, and their LPS is considered to be in the S form. Mutants that lose the O side-chain produce irregular-edged colonies known as rough mutants, and their LPS is considered to be in the R form (Rycroft, 2013).

The O side-chain is a highly variable region of the LPS. This region is hydrophilic and reaches out to the microenvironment of the bacterial cell. It is a repeated tetra- or pentasaccharide, characterised by the inclusion of deoxy- and dideoxyhexoses. At present, its structure is highly important for serovar classification because the system used to differentiate the *Salmonella* serovars is partially based on the structure of the O side-chain polysaccharide. The different antigens of the O side-chain (determined by the component sugars) are designated by a number, and are included in the antigenic formula identified with the letter O (Rycroft, 2013).

LPS has influence in the pathogenicity of *Salmonella* strains. Loss of the O antigen is associated with reduced virulence, and the presence of the antigen is required for colonisation, invasion and survival of *Salmonella* in host cells (Nevola et al., 1987; Murray et al., 2006; Kong et al., 2011). Studies were done in mice to determine if the difference in the pathogenicity of *Salmonella* strains were due to the O side-chain composition. A strain of *Salmonella* Typhimurium (antigen O-4,12) was altered to carry the O antigen of *Salmonella* Enteritidis (antigen O-9,12) or *Salmonella* Montevideo (antigen O-6,7). The study showed that the strain carrying the O-6,7 antigen was least virulent, the strain carrying the O-9,12 antigen was of intermediate virulence, and the parent carrying the O-4,12 was the most virulent strain (Valtonen, 1970).

1.2.5.5 Fimbriae

Fimbriae are filamentous surface proteins expressed by many bacteria. The main structure of fimbriae is a rod composed of subunit proteins called fimbrins ranging between 15 and 25 kDa (Jones, 2013). Thirteen major fimbria subunits of *Salmonella* Enteritidis have been found to play a role in adherence and colonization of the bacteria in the chicken gut (Clayton et al., 2008). However, multiple additional factors seem to be involved in initial adhesion processes (Bäumler et al., 1996).

1.2.5.6 Flagella

Flagella are one of the main antigens of surface of bacteria and are recognized by the host immune system. They are identified in the antigenic formula by the H letter. Most of the *Salmonella* serovars possess about 5 to 10 randomly positioned flagella that are responsible for bacteria locomotion (Jones, 2013). The flagellar filaments are composed of subunits of flagellin protein (Szekely and Simon, 1983). Flagellin is a PAMP recognised by the TLR5. TLR5 recognises flagellin using an extracellular domain and

activates different pathways to produce proinflammatory cytokines in an intend to clear the pathogen (Hayashi et al., 2001; Akira and Takeda, 2004; Iqbal et al., 2005).

The expression of flagella varies among *Salmonella* serovars. In those serovars that express flagella, the antigenic formulae identifies two different antigens: H1 (flagellar phase 1) and H2 (flagellar phase 2) (Andrewes, 1922; Lederberg and Iino, 1956). Individual bacteria can express only one of the different flagellar filament proteins (FljB or FliC) at any time (Szekely and Simon, 1983). The variation (or switch) of expression of the flagellar protein is known as phase variation (Silverman et al., 1979; Szekely and Simon, 1983). The alternative expression of different flagellar antigens results in the oscillation of phenotype and allows *Salmonella* to minimize the host immune response (Van Asten and Van Dijk, 2005). This transition occurs from 10^{-3} to 10^{-5} frequencies per bacterium and per generation (Stocker, 1949). Expression of flagella is reduced in non-motile avian adapted serovars (*Salmonella* Gallinarum and *Salmonella* Pullorum) and can invade systemically more easily by avoiding proinflammatory responses in the gut (Kaiser et al., 2000; Iqbal et al., 2005; Chappell et al., 2009).

The molecular mechanism mediating phase variation occurs by a site-specific DNA inversion event in the chromosome (Figure 1-8). The promoter for the FljB flagellin protein is flanked by a recombination mechanism that mediates its reversible inversion. In one orientation the *fljB* promoter directs transcription of the *fljB* operon and FljB flagellin is produced. The *fljA* gene is cotranscribed with *fljB* and encodes a transcriptional inhibitor of the unlinked *fliC* gene. In the alternate orientation, the *fljBA* operon is not expressed and transcription of the *fliC* gene happens (Bonifield and Hughes, 2003).

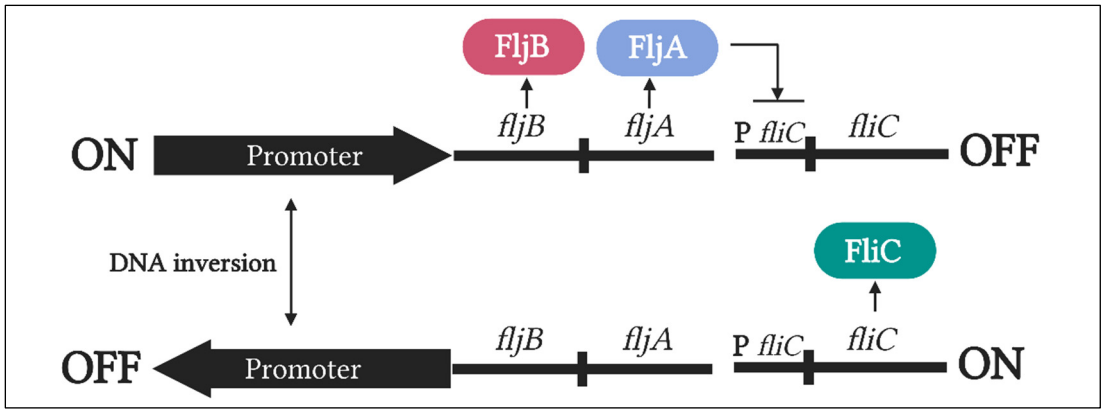

Figure 1-8. Schematic representation of flagellar phase variation in Salmonella enterica. Source: modified from Bonifield and Huges, 2003. Created with BioRender.com.

1.3 THE AVIAN ENTERIC IMMUNE SYSTEM

Besides obtaining the nutrients that live organisms need to survive, the gut is a major site for the symbiosis with several microorganisms that participate on diverse host physiological functions. For this reason, an effective intestinal immune response is vital to prevent the negative interference of some microorganisms with animal homeostasis. The intestinal mucosa of the chickens contains elements from both innate and adaptive immunity responsible for protecting them from pathogens (Smith et al., 2013).

Unlike mammals, chickens lack encapsulated lymph nodes, so the presence of the immune system in the intestine is in the form of diffuse lymphoid tissue (Oláh et al., 2013). The lymphoid tissue that is present in the intestinal tract is called gut-associated lymphoid tissue (GALT) and is scattered throughout its length. The GALT can be found as isolated lymphoid cells in the epithelium and lamina propria or it can form defined lymphoid structures located at strategic sites. Examples of defined lymphoid structures are Peyer's patches (PP), Meckel's diverticulum, caecal tonsils (CT), and bursa of Fabricius (Oláh et al., 2013; Smith et al., 2013). In Figure 1-9 there is a representation of the immune cell compartments in the small intestine of chickens both in the regular nutrients-absorbing tissue and in the lymphoid follicles.

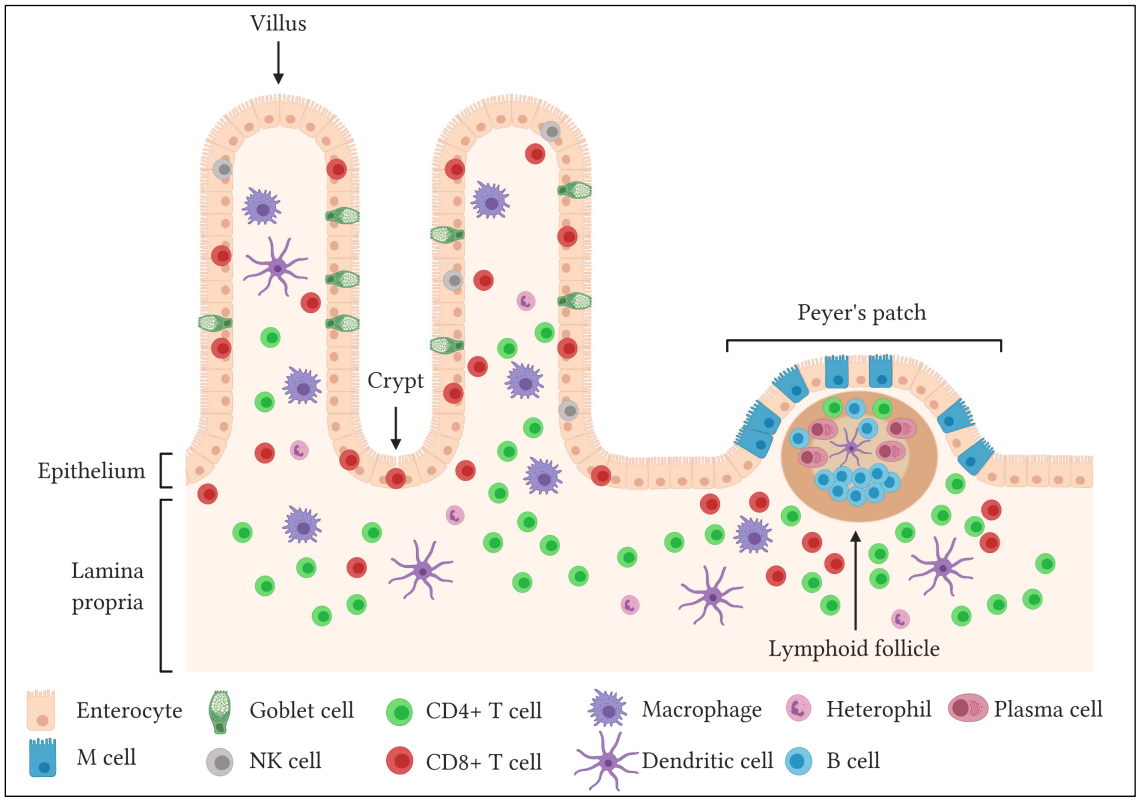

Figure 1-9. Organisation of immune cell compartments in the small intestine of chicken. Source: modified from Smith et al., 2013. Created with BioRender.com

The composition and function of the epithelial layer of enterocytes that forms the intestinal mucosa depends on whether it covers the villi or the lymphoid follicles.

The epithelium that covers the absorptive villi and intestinal glands is formed by a single layer of polarized epithelial cells held by intercellular structures called tight junctions (Hermiston and Gordon, 1995). This epithelium is populated with highly specialised intra-epithelial lymphocytes (IEL). The composition of the IEL includes mainly natural killer (NK) cells and CD8+ T cells expressing both the αβ and γδ form of the T cell receptor (TCR) (Bucy et al., 1988; Vervelde and Jeurissen, 1993; Imhof et al., 2000; Göbel et al., 2001). Underlying this epithelium there are also isolated lymphoid cells residing in the lamina propria (Smith et al., 2013).

On the other hand, the epithelium that covers the lymphoid structures or follicles is highly specialised and is formed by irregular microvilli, is flattened, lacks mucus-producing cells, and also contains microfold (M) cells able to uptake pathogens from the lumen and initiate the immune response (Jeurissen et al., 1999; Foster and Berndt, 2013). The lamina propria that lies below this epithelium hosts a wide range of immune cells (Smith et al., 2013). B and T cells are the most common but there are also heterophils, macrophages, dendritic cells (DC) and natural killer (NK) cells (Smith et al., 2013). The B cells form follicles and the interfollicular space is filled with T cells. In contrast to the IEL population, the main T cell population of the lamina propria express the TCRαβ and is formed by CD4+ T cells, with a less prominent CD8+ population (Bucy et al., 1988; Smith et al., 2013). Most of the B cells in the lamina propria are producers of IgA immunoglobulins (Bienenstock et al., 1973). Macrophages are specially located in the sub-epithelial space (Vervelde and Jeurissen, 1993).

The enterocytes that form the epithelium play a role in the enteric immune system. These multifunctional cells produce and release a variety of biomolecules into the mucosa and lumen that contribute to immunity. A population of specialised enterocytes identified as goblet cells produces mucus that forms a protective layer over the epithelial cells. This mucus is composed of mucin proteins that have been identified in chickens as well as in other species (Lang et al., 2006). The mucus layer is part of the innate immune response and protects against pathogens by creating a barrier, in addition to participating in cell surface signalling (Smirnov et al., 2006; Johansson et al., 2011). Defensins are host defence peptides (HDPs) that are secreted by chicken leucocytes and epithelial cells and are active against a wide range of microorganisms. Only the sub-family of β-defensins

has been isolated in chickens. These molecules have antimicrobial activity against Gram-positive, Gram-negative bacteria (including *Salmonella*), and some fungi (Evans et al., 1995; Sugiarto and Yu, 2004; Lynn et al., 2007). Enterocytes can also recognise potential pathogens by expressing pattern recognition receptors (PRRs), including the TLRs, which can recognise PAMPs on a wide range of microorganisms and trigger innate immune responses (Hayashi et al., 2001; Akira and Takeda, 2004; Keestra et al., 2008). In Table 1-3 there is a summary of avian TLR (He et al., 2006; Brownlie and Allan, 2011; Juul-Madsen et al., 2013; Keestra et al., 2013; Gupta et al., 2015). Another way in which enterocytes can influence the immune response is by expressing major histocompatibility (MHC) class I (all enterocytes can express it) and class II (only induced enterocytes can express it) molecules. Hence, enterocytes can interact with T cells, linking innate and adaptive immune response, but this field needs more in-depth studies in chickens (Kaiserlian et al., 1989; Dimier-Poisson et al., 2004; Smith et al., 2013; Wosen et al., 2018).

Table 1-3. Avian toll-like receptors (TLRs). Source: He et al., 2006; Brownlie and Allan, 2011; Luuj-Madsen et al., 2013; Keestra et al., 2013; Gupta et al., 2015.

TLR	**Alternative names**[a]	**Agonist**	**Origin of the Agonist**	**Location of receptor**
TLR1	TLR6 TLR10 TLR16	Lipoproteins	Gram + bacteria	Cell membrane
TLR2	na	Peptidoglycan Lipoproteins	Gram + bacteria	Cell membrane
TLR3	na	dsRNA	Viruses	Intracellular
TLR4	na	LPS	Gram − bacteria	Cell membrane
TLR5	na	Flagellin	Gram − bacteria	Cell membrane
TLR7	na	ssRNA	Viruses	Intracellular
TLR15	na	Proteases	Bacteria and fungi	Cell membrane
TLR21	TLR9	Unmethylated CpG DNA	Bacteria and viruses	Intracellular

[a]na: not available.

1.4 THE AVIAN IMMUNE RESPONSE TO *Salmonella*

The immune response to *Salmonella* infections in chickens involves the interaction of many elements of the organism, including the innate and the adaptive arms of the immune system (Wigley, 2014).

In Figure 1-10 there is a schematic representation of the interactions between host innate and adaptive immune system and non-adapted *Salmonella* bacteria.

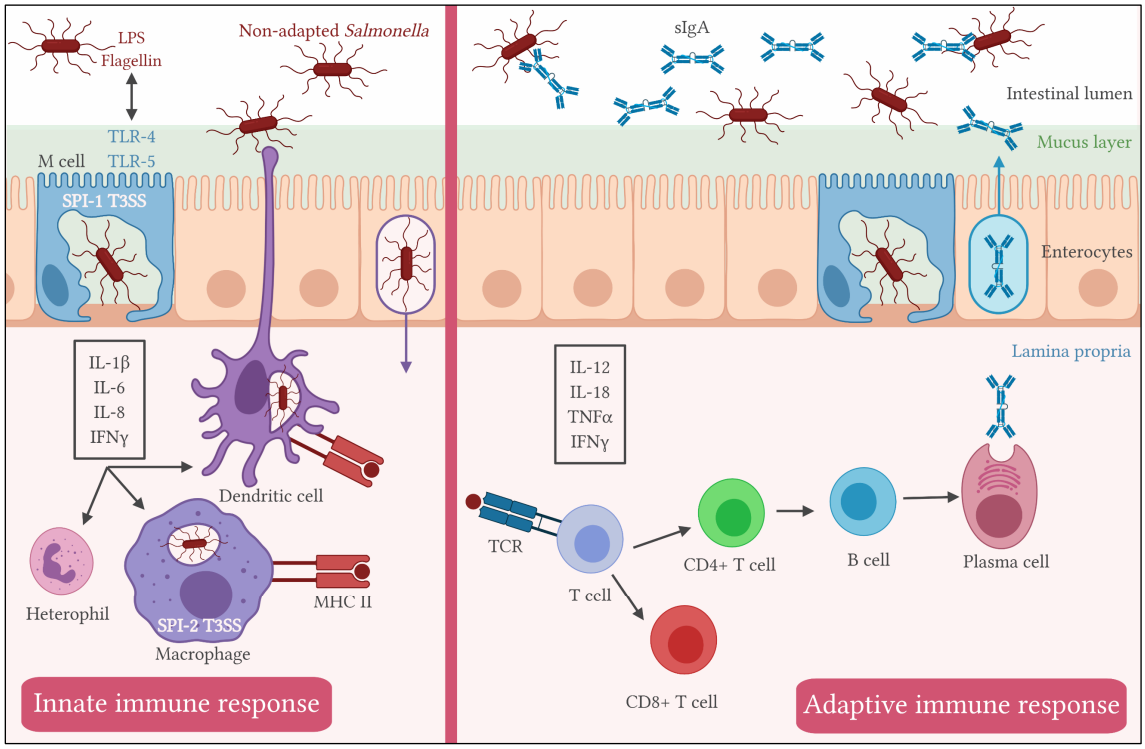

Figure 1-10. Innate and adaptive immune response against Salmonella in the chicken intestine. Created with BioRender.com.

1.4.1 Innate immune response

Effective immunity against *Salmonella* requires the activation of the innate immune system. Epithelial cells, macrophages and dendritic cells are activated by recognition of conserved PAMPs such as LPS, flagellin, or unmethylated CpG motifs in DNA, recognised by receptors TLR4, TLR5 and TLR21, respectively (Barrow et al., 2012).

The initial contact of *Salmonella* with the host intestinal cells stimulates the production of proinflammatory cytokines and chemokines such as IL-1β and IL-6, followed by an up-regulation of IFNγ (Barrow et al., 2012). Infection of chickens with *Salmonella* Enteritidis and *Salmonella* Typhimurium is accompanied by up-regulation of cytokines that attract granulocytes and macrophages to the caecum, such as CXCLi1 and CXCLi2 (chemokines that are similar to human IL-8) (Berndt et al., 2007; Cheeseman et al., 2008).

Heterophils are considered essential in the initial inflammatory response against *Salmonella* and can influence the unspecific uptake of mucosal or luminal pathogens and the production of toxic compounds with antimicrobial activity (Kogut et al., 2001; Van Dijk et al., 2009). Heterophil influx has been correlated with resistance against *Salmonella*

infection and with the up-regulation of proinflammatory cytokines (Ferro et al., 2004; Kogut et al., 2005; Swaggerty et al., 2005, 2006).

Macrophages are other cellular population considered essential in the innate immune response against *Salmonella*, and in stimulating adaptive immune mechanisms (Wigley et al., 2006). Macrophages can recognise, phagocyte and kill *Salmonella* bacteria. However, the ability of *Salmonella* to survive inside them is the key for the progression of the infection (McGhie et al., 2009). The peak of macrophages in the caecal mucosa is reached shortly after infection, but few are colocalised with *Salmonella* bacteria (Berndt and Methner, 2004; Berndt et al., 2007). Avian macrophages express different receptors and proinflammatory cytokines, including IFNγ, which is considered an essential macrophage-stimulating mediator that reduces *Salmonella* survival. These cytokines are also responsible for granulocytes influx and activation of macrophages and lymphocytes (Okamura et al., 2005; Wigley et al., 2006).

1.4.2 Adaptive humoral immune response

A previous infection with *Salmonella* strains leads to a more rapid clearance of a secondary challenge, indicating that adaptive immune responses are involved in clearance of enteric *Salmonella* in chickens (Beal et al., 2004a). A strong B cell reaction is induced after primary and secondary infection, although the antibody production is not always correlated with protection and an effective bacterial clearance (Beal et al., 2004a, 2006; Methner, 2018; Theuß et al., 2018).

Chickens express only three immunoglobulin (Ig) isotypes: IgM, IgA and IgY. From a functional perspective, avian IgM and IgA share many features in common with their mammalian counterparts. Thus, avian IgM is the predominant B cell surface Ig and is the first antibody observed following exposure to antigen. Similarly, avian IgA is the predominant Ig isotype in secretions. IgY is the avian equivalent to mammalian IgG, and is the predominant isotype in secondary antibody responses (Tizard, 2002; Ratcliffe and Härtle, 2013).

The dynamics of the antibody production after *Salmonella* infection is affected by the age of the chickens. The production of *Salmonella*-specific antibodies in serum of animals infected at 10 days of age or less is delayed until 2 weeks after infection (Beal et al., 2005). The production of antibodies in animals infected at 6 weeks starts around one week after infection (Beal et al., 2004a, 2004b; Hassan et al., 2006). Aging also affects the capacity of

producing antibodies, as animals that were 1 year old developed a weaker humoral response compared with younger ones of 20 weeks old (Humphrey et al., 1991).

The creation of B cell-deficient chickens by surgery at 17 days of embryonic development instead of hormonal or chemical methods (which can also affect other cell populations apart from B cells) demonstrated that B cells are not essential for clearance of either primary or secondary *Salmonella* Typhimurium infection (Beal et al., 2006). Faecal excretion of *Salmonella* Typhimurium was equal in surgically bursectomised and intact chickens, but was statistically higher in cyclophosphamide bursectomised chickens, suggesting that *Salmonella* clearance depends on a non-B cell but cyclophosphamide-sensitive compartment, such as the thymus (Beal et al., 2006). Despite this, it cannot be ruled out that B cells can contribute in some way to the process.

Infection of chickens with non-adapted serovars is largely restricted to the gut, so the IgA isotype is present in high concentrations in intestinal fluids and bile of infected chickens (Lebacq-Verheyden et al., 1972, 1974; Rose et al., 1981; Beal and Smith, 2007). However, high levels of IgA antibodies do not correlate with an effective resolution of the infection. Some studies have found that greater production of intestinal IgA antibodies are also associated with higher levels of *Salmonella* colonisation in internal organs (Lee et al., 1981; Berthelot-Hérault et al., 2003; Penha Filho et al., 2012).

A useful aspect of serum antibodies for the poultry industry is flock's infection monitoring. Serological tests are not reliable to detect individual animals excreting *Salmonella*, but can be a tool for determining the exposure of a flock to the microorganism (Barrow et al., 2012; Shivaprasad et al., 2013).

1.4.3 Adaptive cellular immune response

Exposure to *Salmonella* in birds triggers a strong T cell response, which has been found to peak at the time of clearance (Barrow et al., 2012). The role of T cells in protection has not been proven, but in the absence of B cell function and with the evidence of immune memory, cell response needs to be considered as important (Smith et al., 2013).

CD4+ T cells, also known as T helper or Th lymphocytes, can be classified according to the type of cytokines they produce (Th1 or Th2), and this bias has influence on the kind of immune response and the protective capacity against some pathogens (Berger, 2000). Th1 cells produce mainly IFNγ and IL-2 and lead to the activation of macrophages and the development of a cell-mediated response. Th2 cells produce IL-4, IL-5 and IL-10 and

lead to the development of humoral response (Wigley and Kaiser, 2005). The effective T cell response for *Salmonella* clearance seems to be biased to Th1 type with dominance of IFNγ production (Wigley and Kaiser, 2005). Th2 response has been associated with induction of humoral immunity and carrier states in animals infected with *Salmonella* Pullorum (Chappell et al., 2009). Most studies about cell-mediated immunity in *Salmonella* infections have been made in mice. Although infection with *Salmonella* Typhimurium in mice causes a typhoid-like infection, Th1 cytokines (mainly IFNγ and IL-12) have been shown to be crucial for protection (Eckmann and Kagnoff, 2001; Raupach and Kaufmann, 2001).

As previously mentioned, the T cell response is considered important for the protection against *Salmonella*. Changes in number, distribution and antigen-specific proliferation are reflected in some studies in young and adult chickens.

The primary infection in young chicks with both attenuated and field strains of *Salmonella* Typhimurium triggered an influx of CD8+ T cells in many organs (blood, caecum, spleen and bursa of Fabricius), which is indicative of cell-mediated immune response (Berndt and Methner, 2001). In another study, day-old chicks responded different to *Salmonella enterica* infection depending on the invasiveness capacity of the serovar. Those serovars with higher invasiveness ability (*Salmonella* Enteritidis) triggered a stronger immune response in the caecum, consisting of higher granulocytes and CD8+ T cell invasion and higher expression of IL-12, IL-18 and TNFα, compared with less invasive serovars (*Salmonella* Infantis) (Berndt et al., 2007).

The primary infection in adult chickens with *Salmonella* increased the production of CD4+ and CD8+ T cells in the ileum and CD8+ T cells in the spleen (Bai et al., 2014). In another study, adult chickens from a line resistant to intestinal infection with *Salmonella* had higher antigen-specific proliferation of splenocytes after contact with *Salmonella* Typhimurium antigen, compared to a susceptible line, implying that genetics can affect cellular immune response and resistance to *Salmonella* (Beal et al., 2005).

Changes in the T cell population also affect the susceptibility to *Salmonella*. When laying hens reach the sexual maturity at the onset of point-of lay, the vulnerability to *Salmonella* infection increases due to a reduction in the T cell compartment. This situation can occur in both vaccinated and unvaccinated animals. In this moment, there is a change in circulating sexual hormones that lead to a drop in several T cell populations, mainly in

CD4+ T cells. This decrease in T cells also affects the lymphoid populations in the reproductive tract tissue, and can increase egg infection with *Salmonella* serovars (Johnston et al., 2012).

1.5 *Salmonella* INFECTION IN CHICKENS

The outcome of infection with *Salmonella enterica* in chickens differs according to age, dose, route of infection, serovar, strain, chicken genotype, and immune status of the host. Challenge with the adapted serovars causes typhoid-like diseases in chickens of any age and with considerable mortality. In contrast, infection with non-adapted serovars results in a long-lived infection of the gut with little systemic infection in chickens older than 2 or 3 days of age. However, non-adapted serovars can cause mortality in young chickens.

1.5.1 Origins of infection

For the poultry industry, it is important to identify risk factors for the primary introduction of *Salmonella* organisms into the farms, because this can have serious effects on human health. The potential sources of *Salmonella* infection are numerous. Birds and other animals can be carriers and become a source of contamination for poultry, humans and the environment (Shivaprasad et al., 2013). In Figure 1-11 there is an overview of all factors that can be involved in the cycle of *Salmonella* infection.

1.5.2 Mechanisms of *Salmonella* infection

Salmonella organisms infect birds via the faecal-oral route. The pathogen resists the low pH of the crop, passes through the proventriculus and gizzard, and reaches the distal ileum and caecum, where the intestinal epithelium is the first physical barrier and starter of the immune response.

Bacteria may be taken up by M cells, be captured in the lumen by phagocytes that penetrate the epithelial monolayer, or may entry into non-phagocytic enterocytes in a slight different way (McGhie et al., 2009).

The engulfment of *Salmonella* by enterocytes may be started by initial binding through virulence factors such as fimbriae and flagella (Misselwitz et al., 2011; Foley et al., 2013). Then, multiple effector proteins delivered by SPI-1 T3SS are involved in membrane ruffling (deformation of the actin cytoskeleton) allowing bacterial internalisation into a phagosomal compartment called *Salmonella*-containing vacuole (SCV) (Chappell et al., 2009; McGhie et al., 2009). After that, SPI-2 T3SS effectors seem to mediate mechanisms

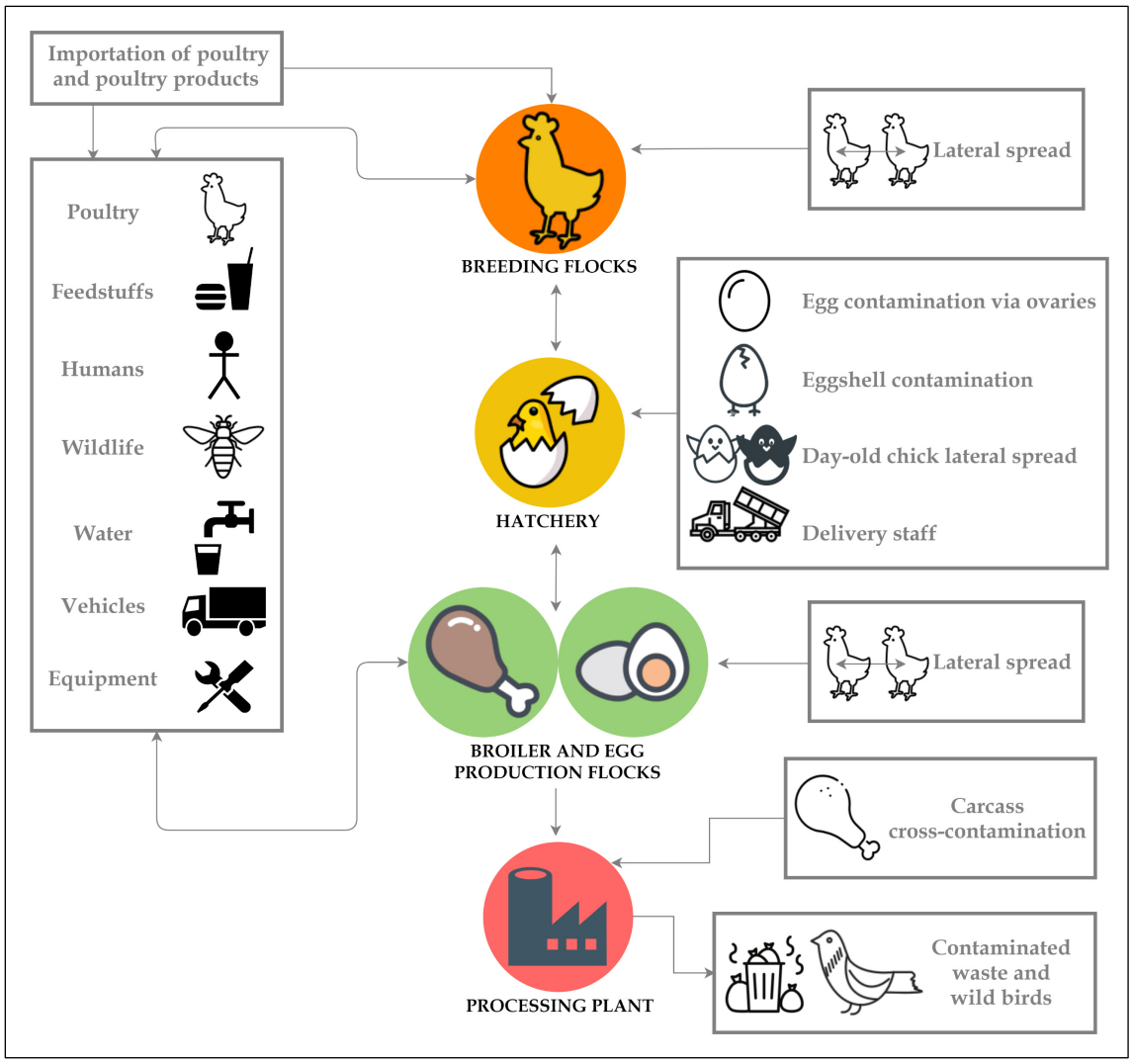

Figure 1-11. Cycle of Salmonella infection. Source: modified from Lister and Barrow, 2008.

that allow *Salmonella* to survive and replicate inside the SCV (Steele-Mortimer, 2008). This process includes the movement of the SCV near to the nucleus, the accumulation of actin around the SCV and the extension of *Salmonella*-induced filaments (SIFs) that radiate from the SCV (Steele-Mortimer, 2008). *Salmonella* avoids lysosomal fusion with SCV by a not-fully understood mechanism that prevents host antibacterial activities and could involve the uncouple of markers from the late endocytic pathway (Steele-Mortimer, 2008). Finally, survival *Salmonella* can exit from epithelial dead cells and can be phagocyted by other immune cells recruited by inflammation or reinvade epithelial cells through basolateral cell wall (Fink and Cookson, 2007; Knodler et al., 2010; Hurley et al., 2014).

Macrophages internalise *Salmonella* by phagocytosis and bacteria are also localised inside a SCV (Gorvel and Méresse, 2001). The mechanism by which *Salmonella* survives and replicates inside macrophages involves the evasion of endosomal fusion with the

NADPH oxidase complex also mediated by SPI-2 T3SS effectors (Gorvel and Méresse, 2001). Bacteria that survive inside intestinal macrophages use them as a vehicle to disseminate to the liver and spleen via the bloodstream (McGhie et al., 2009).

In Figure 1-12 there is a schematic representation of the main steps involved in *Salmonella* infection.

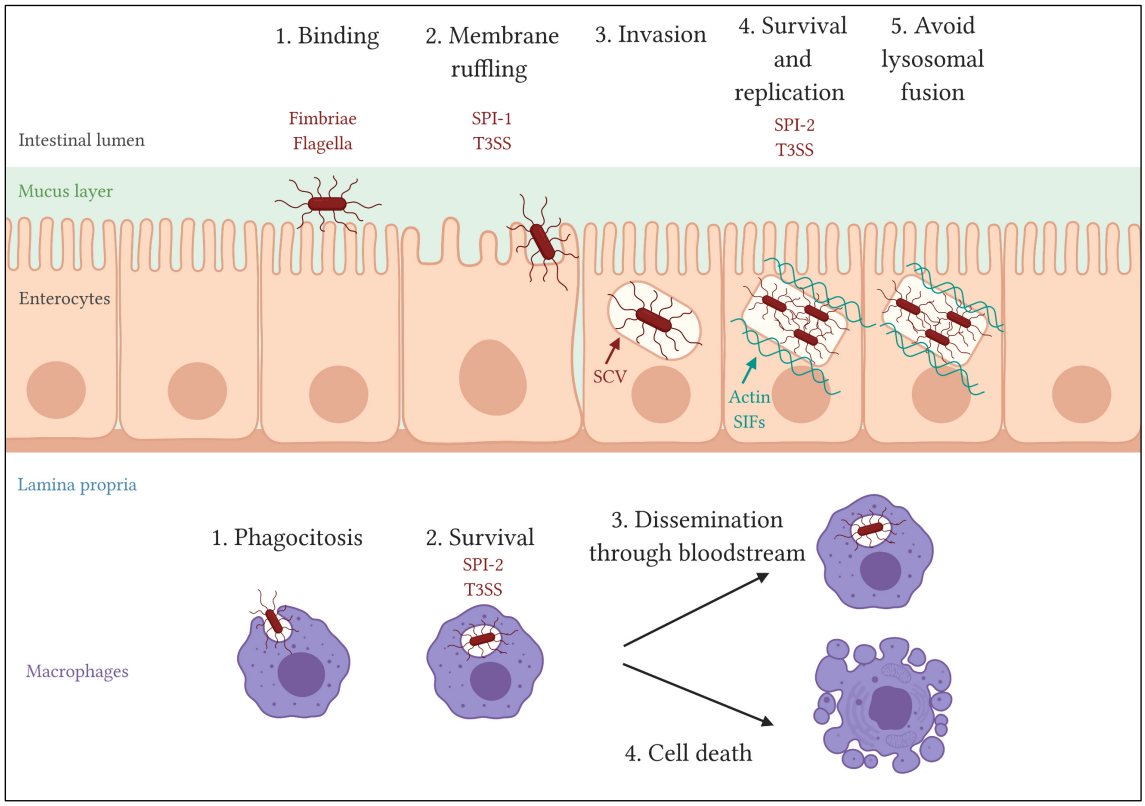

Figure 1-12. Main steps involved in Salmonella infection. Source: modified from McGhie et al., 2009; Hurley et al., 2014. Created with BioRender.com.

1.5.3 Infection with adapted *Salmonella* serovars

Salmonella enterica subsp. *enterica* serovar Gallinarum biovars Pullorum (*Salmonella* Pullorum) and Gallinarum (*Salmonella* Gallinarum) can cause pullorum disease and fowl typhoid in avian species, respectively, and remain a considerable economic problem in countries where inefficient control measures or climate conditions help the environmental spread of the microorganisms (Barrow and Freitas Neto, 2011; Xiong et al., 2018). These serovars are highly adapted to avian species, and so they represent a minor threat to public health (Shivaprasad, 2000).

Salmonella Pullorum is normally non-motile and does not express flagellar antigens, a fact that avoids an inflammatory response in the gut and allows the pathogen to cause systemic infection. Pullorum disease is an acute disease with high mortality that can be

up to 100%, especially in young birds under 3 weeks of age. Normally the first indicator of the disease is an increase in the mortality rate in chicks before and after hatching. Animals that recover from this disease can develop a carrier state and transmit the bacteria vertically to their progeny (most common transmission method) or horizontally within the flock (Wigley et al., 2001; Lister and Barrow, 2008). Clinical signs are inconstant and unspecific: depression, tendency to huddle, respiratory distress, decreased appetite, and white and sticky faeces. Growing birds can be affected by a subacute form with lameness, and affected adult birds can experiment a reduction in fertility, egg production and hatchability (Lister and Barrow, 2008). Due to non-specific signs, the diagnosis needs to be done by isolation of the causal organism. Best control of the disease is made by repeated serological tests and eradication of positive birds to *Salmonella* Pullorum antibodies (Shivaprasad et al., 2013).

Salmonella Gallinarum is also a non-motile and non-flagellated bacillus. This pathogen is the causative agent of fowl typhoid, which is an acute or chronic septicaemic disease that causes variable morbidity and high mortality (up to 50%) and normally affects adult birds, though other age ranges may be susceptible (Barrow and Freitas Neto, 2011). The transmission of the disease is normally horizontal by infected droppings, although egg transmission can occur (Lister and Barrow, 2008). In acute outbreaks, the animals first experience a decrease in food consumption and in egg production. Other clinical signs include depression, ruffled feathers, watery to mucoid yellow diarrhoea and, on some occasions, respiratory distress. Animals that survive after 2 or 3 days of presenting these signs develop a carrier state with a progressive loss of condition. In subacute outbreaks, the flock mortality slightly increases over a long period. Signs in young birds are similar to those of pullorum disease (Lister and Barrow, 2008). Diagnosis and control of the disease can be done in the same way as for *Salmonella* Pullorum (Lister and Barrow, 2008).

The immunobiology behind the systemic salmonellosis is similar for both abovementioned serovars. The *Salmonella* strains invade the intestine via enterocytes and lymphatic tissues without triggering an immune response because of lack of flagella, as mentioned above (Shivaprasad et al., 2013). The lack of inflammatory response includes also the absence of heterophils influx and a down-regulation of chemokines attractant for granulocytes and monocytes (Henderson et al., 1999; Chappell et al., 2009). After that, the systemic infection starts. The bacteria survive in the macrophages and are

transported to spleen, liver and the reproductive tract, where they can survive for many weeks (Wigley et al., 2001). After this, if innate immune response is not able to control bacterial replication, there is a production of high amounts of antibodies and T cell proliferation. If the infection is not cleared by the adaptive response, animals can die or can develop a carrier state, with a suspected modulation towards a Th2-type immune response that leads to an equilibrium between the host and small numbers of bacteria persisting in intracellular niches (Chappell et al., 2009).

1.5.4 Infection with non-adapted *Salmonella* serovars

1.5.4.1 *Salmonella* infection in young chicks

The susceptibility of chickens to systemic and potentially lethal infection of non-adapted *Salmonella* serovars is markedly age-dependent (Smith and Tucker, 1980). Birds younger than 3 days in the absence of an established intestinal microbiota are very susceptible to infection with non-adapted *Salmonella* strains (Foster and Berndt, 2013). The morbidity and mortality vary considerably and deaths are normally less than 10% but can reach up to 100% of the affected animals (Lister and Barrow, 2008). Clinical signs are not specific and are similar to those caused by other *Salmonella* serovars. Affected birds are depressed, and huddle together with eyes closed, ruffled feathers and drooping wings. Diarrhoea is also a common sign. The most representative post-mortem finding is typhlitis, and unabsorbed yolk sac can also be found (Lister and Barrow, 2008). *Salmonella* organisms are mainly found in the yolk sac, caecum, liver and spleen but are practically absent in blood due to intracellular location of the bacteria (Barrow et al., 1987).

1.5.4.2 *Salmonella* infection in adult chickens

Non-adapted serovars do not cause clinical disease in chickens older than 3 days due to a more developed immune system (Beal et al., 2004b). Therefore, the main concern is the colonisation of the gut that leads to infection of poultry derived products that are the main cause of *Salmonella* outbreaks in humans (Foster and Berndt, 2013; Smith et al., 2013).

The contact of intestinal cells with live bacteria or their products (including LPS and flagellin) mediates a proinflammatory cytokine response attracting heterophils and macrophages. Following invasion, *Salmonella* can be transported to internal organs like liver or spleen inside phagocytic cells and can persist for many weeks in the mucosa of

ileum and caecum, until clearance is finally achieved by an adaptive immune response (Jones et al., 2007).

1.5.5 Contamination of eggs by *Salmonella*

Eggs can be contaminated by two routes and it is still not clear which route is most important (Gantois et al., 2009). One route is the penetration of eggshell from *Salmonella* contaminated environment following oviposition, considered as horizontal transmission. The second route happens when *Salmonella* infects the reproductive tract and it is considered the vertical transmission (Messens et al., 2005).

If horizontal transmission is considered, faeces adhered to the shell may contaminate the egg content when the eggs have cracks, when the eggs are opened to prepare food, or faeces can also cross the eggshell pores of intact eggs as they cool before the establishment of the cuticula (Shivaprasad et al., 2013). Extrinsic and intrinsic factors affect the capacity of *Salmonella* to invade intact eggs after hatching. Acting as extrinsic factors there are the bacterial strain, number of organisms, temperature, moisture and storage conditions. The rate of egg contamination increases when *Salmonella* counts are higher in faeces surrounding the eggs (Schoeni et al., 1995). The temperature differential needs attention because when eggs are warmer than the environment, the subsequent cooling causes their content to contract and this negative pressure helps the bacteria pass through the shell pores (Miyamoto et al., 1998). Moisture also promotes bacteria penetration. The presence of water droplets on the egg surface when eggs are removed from refrigeration and placed at room temperature increases the chance of *Salmonella* contamination (Fromm and Margolf, 1958). As intrinsic factors there are the presence of cuticle, shell quality, and membrane properties (Messens et al., 2005). The cuticle is a hydrophobic proteinaceous barrier that overlies the eggshell and the pores and prevents penetration of bacteria, but is less effective early after hatching (immature) and later as the egg ages (dry and broken) (Mayes and Takeballi, 1983; Miyamoto et al., 1998). The shell quality also affects bacterial invasion: some breeds, stressed animals or older animals produce eggs with low shell quality that facilitates contamination (Jones et al., 2002).

Other route is the contamination of intact eggs via vertical transmission as a result of ovaries and oviduct contamination. Bacteria can also access the oviduct through ascending infection from the cloaca. In this route the yolk, yolk membranes, albumen,

shell membranes or eggshell can be directly contaminated during egg formation. When *Salmonella* has infected the ovaries, the egg yolk can be contaminated. *Salmonella* colonising the oviduct can be incorporated into the vitelline membrane, albumen, the eggshell membranes, or the eggshell itself, depending on the area of colonisation (infundibulum, magnum, isthmus, or uterus, respectively). However, it is not clear which is the main way of contamination, despite the studies performed (Gantois et al., 2009; Wales and Davies, 2011).

1.6 VACCINES AGAINST *Salmonella* IN POULTRY PRODUCTION

Immunisation represents one of the most important methods to increase the resistance of chickens against *Salmonella* infection (Mastroeni et al., 2001). In Table 1-4 there is a summary of the main characteristics of *Salmonella* vaccines. Vaccination against host-adapted *Salmonella* serovars induces a strong serovar-specific protective immunity. In contrast, vaccination against non-adapted serovars has achieved variable success levels. The gold standard of protection against *Salmonella* is considered the infection with a fully wild-type strain (Berndt and Methner, 2004; Methner et al., 2011a). In any case, vaccination against most common *Salmonella* serovars is considered a useful tool to reduce the prevalence of *Salmonella* in the poultry industry, which in turn reduces risk for food-borne disease transmission (Van Immerseel et al., 2005).

The effectiveness of a vaccine is evaluated according to the level of systemic and intestinal colonisation after vaccination and experimental infection. Vaccination schemes using a combination of live and inactivated *Salmonella* vaccines have been applied to poultry industry with diverse results in reducing egg contamination and shedding by wild-type *Salmonella* spp. (Aehle and Curtiss, 2016).

The level of protection against *Salmonella* depends mainly on the vaccine mechanism, challenge strain, route of administration of the challenge, infection dose, and the age and genetics of animals (EFSA, 2004). Therefore, it is difficult to compare the efficacy of the vaccines currently available in the market. Different types of vaccines have been tested with different outcomes. Currently, only live attenuated and killed vaccines are registered in EU. But recently new vaccine technologies are being developed including subunit and DNA vaccines to obtain safer, more effective and more stable vaccines for the maintenance of poultry health and welfare. In Table 1-5 there is a summary of different studies testing registered and commercially available vaccines.

Table 1-4. Summary of characteristics of Salmonella vaccines. Source: Barrow, 2007; Barrow and Methner, 2013; Desin et al., 2013; Aehle and Curtiss, 2016.

Criteria for an ideal *Salmonella* vaccine in poultry	
- Protection against intestinal and systemic infection - Attenuated and safe for animals and humans - Cost-effective mass application - Compatibility with other control measures - Cross-protection between different serovars - Long duration of immunity - Easily differentiable from wild-type strains (live attenuated vaccines only) - Not affecting animal productivity	
Live attenuated vaccines	
Advantages	**Disadvantages**
- Stimulation of humoral and cellular immunity - Expression of all *in vivo* antigens - Competitive exclusion with oral administration - Mass application by coarse spray or drinking water - Horizontal transfer between animals	- Long persistence in animals and environment - Possible reversion to virulence - Interference with *Salmonella* tests
Killed vaccines	
Advantages	**Disadvantages**
- Stimulation of humoral immune response - Possible use through egg-laying period - Easy combination of different serovars - Protection of progeny of vaccinated hens - Lack of zoonotic risk	- Lack of stimulation of cellular immunity - Injectable application time-consuming and expensive - Expression of limited antigens from the *in vitro* environment
Subunit vaccines	
Advantages	**Disadvantages**
- Lack of zoonotic risk	- Injectable application time-consuming and expensive - Poorly immunogenic
DNA vaccines	
Advantages	**Disadvantages**
- Stable at high temperatures - Preservation of antigen conformation - Better antigen presentation via MHC - Possibility of *in ovo* vaccination	- Limited protective capacity - Risk of integrating in the host genome

Table 1-5. Overview of studies conducted to test commercial Salmonella vaccines.

Serovar and Vaccine Type[a]	Commercial name/Manufacturer	Vaccine schedule[b]	Infection Serovar and Age	Main results[c]	References
G1: SE LV G2: ST LV G3: SE/ST LV	TAD Salmonella/Lohmann	1d, 6w, 16w	SE 24w	↓ colonisation of spleen and oviduct at 3wpi in all vaccinated groups. ↓ infection of eggs at 1-3wpi in group 3	(Gantois et al., 2006)
G1: ST LV + SB/SK KV	Megan Vac/Lohmann + Lohmann	1d, 2w, 5w, 10w, 18w	Field trial (no experimental infection)	↓ colonisation of caecum and reproductive tract in vaccinated group (breeders). ↓ prevalence in broilers from vaccinated breeders	(Dorea et al., 2010)
G1: ST LV	Avipro/Lohmann	1d	ST low 2d ST high 2d ST seeder 2d	↓ colonisation in caecum and spleen and ↓ excretion with monophasic ST at an early age (colonisation-inhibition)	(Kilroy et al., 2015)
G1: SE LV G2: SE/ST LV G3: SE/ST LV G4: SE/ST LV	Avipro/Lohmann	1d 1d 1d, 6w 1d, 6w, 16w	(group 1 and 2) SE 2d SI 2d (group 3) SE 7w SI 7w (group 4) SE 17w SI 17w	No ↓ of excretion of SI in groups 1 and 2 at 7 dpi. ↓ of colonisation of liver, spleen and caecum by SE and SI in groups 3 and 4. ↓ of colonisation of oviduct by SE in groups 3 and 4	(Eeckhaut et al., 2018)
G1: SE LV G2: SE LV G3: SE LV G4: SE LV G4: SE LV G6: SE LV G7: SE LV	Avipro/Lohmann	1d 7w 16w 1d, 7w 1d, 16w 7w, 16w 1d, 7w, 16w	(group 1) SE 8w, 12w (group 1, 2 and 3) SE 16w (all groups) SE 29w, 55w	↓ excretion in all groups, higher ↓ of colonisation of liver, spleen, caecum and ovary with more vaccine doses and less time between last vaccination and infection. No ↓ of infection of eggs was observed in vaccinated groups	(Huberman et al., 2019)
G1: SE LV	Salmovac/IDT	1d, 2w	SE 8w	No ↓ colonisation in caecum, ↓ colonisation in liver.	(Carvajal et al., 2008)
G1: CE G2: SE LV G3: SE LV + CE	Aviguard/Microbial Developments Salmovac/IDT Salmovac/IDT + Aviguard/MD	1d 1d 1d, 2d	(all groups) SE 3d	↓ colonisation in liver and caecum, higher protection in group 3 (colonisation-inhibition)	(Braukmann et al., 2016)
G1: SE LV G2: SE LV G3: SE LV	Salmovac/IDT Salmovac/IDT Salmovac/IDT	1d, 4w 1d, 5w 1d, 6w	(all groups) SE 8w	Equal ↓ colonisation in liver and caecum in all groups, time of booster vaccination is not crucial	(Methner, 2018)

Table 1-5. Continued.

Serovar and Vaccine Type[a]	Commercial name/Manufacturer	Vaccine schedule[b]	Infection Serovar and Age	Main results[c]	References
G1: SE LV	Salmovac/IDT	2d, 7w, 12w	SE 77w	↓ excretion at first stages of infection at 5 and 7 dpi. No ↓ colonisation in liver, spleen, caecum, ovary and oviduct	(Theuß et al., 2018)
G1: SG LV G2: SG LV + SE KV G3: SE KV	Nobilis SG 9R/MSD Nobilis SG 9R/MSD + NA NA	8w, 18w 8w, 18w, 22w 18w, 22w	(all groups) SE 24w, 27w, 30w	↓ excretion in groups 2 and 3, ↓ eggshell infection in all groups, higher protection in group 2	(Nassar et al., 1994)
G1: SE/ST KV	Salenvac/MSD	1d, 4w	ST low 8w ST high 8w ST seeder 8w	↓ excretion in all groups, ↓ colonisation in liver, spleen and caecum only in seeder challenge group	(Clifton-Hadley et al., 2002)
G1: SE KV G2: SE KV	Salenvac/MSD Salenvac/MSD	1d, 4w 1d, 4w, 18w	(all groups) SE 8w, 17w, 23w, 30w, 59w	↓ excretion in all groups, ↓ colonisation in caecum and gallbladder and no ↓ colonisation in ovary and oviduct, ↓ infection of eggs in all groups	(Woodward et al., 2002)
G1: ST LV	Vaxsafe/Bioproperties	1d, 6w, 12w	Field trial (no experimental infection)	No ↓ prevalence of *Salmonella* in egg belt or floor faeces in vaccinated farm	(Sharma et al., 2018)
G1: SE KV G2: SG LV + KV G3: SE KV + SE KV G4: SE KV G5: SG LV + KV	Layermune/Ceva Cevac SG 9R/Ceva + Layermune Corymune 4K + Corymune 7K/Ceva Layermune/Ceva Cevac SG 9R/Ceva + Layermune	(all groups) 5w, 9w	(all groups) SE 12w	↓ excretion and ↓ colonisation of liver, spleen and caecum in all vaccinated groups. Layers (groups 1, 2 and 3) are more protected with vaccination schedule of groups 1 and 2. Broiler breeders (groups 4 and 5) are more protected with vaccination schedule of group 2	(Filho et al., 2009)
G1: SE LV G2: SE LV G3: SE LV + ST LV G4: SE/ST KV	Salmovac/IDT Avipro/Lohmann Avipro/Lohmann Salenvac/MSD	1d, 2w, 3wbo 1d, 7w, 3wbo 1d, 7w, 3wbo 12w, 16w	(all groups) SE low 17w SE high 17w	↓ colonisation of caecum 10 dpi in vaccinated groups 1 and 4	(Atterbury et al., 2009)
G1: SE LV G2: SE LV + SE KV G3: SE LV + ST LV	Avipro/Lohmann Avipro/Lohmann + Salenvac/MSD Avipro/Lohmann	NA	SE 82w SE 82w SE 83w	No ↓ colonisation of liver, spleen, caecum and follicles in vaccinated groups	(Van De Reep et al., 2018)
G1: SE LV G2: SE LV + SE/ST KV	Salmovac/IDT Salmovac/IDT + Gallimune/Merial	1w, 7w, 13w 1w, 7w, 14w	(all groups) SE 24w, 51w, 71w ST 26w, 54w, 73w	↓ colonisation (quantitatively) of liver and caecum in vaccinated groups	(Springer et al., 2011)

[a]G: group; SB: *S.* Berta; SE: *S.* Enteritidis; SG: *S.* Gallinarum; SI: *S.* Infantis; SK: *S.* Kentucky; SM: *S.* Montevideo; ST: *S.* Typhimurium; SZ: *S.* Zanzibar; LV: live vaccine; KV: killed vaccine; CE: competitive exclusion culture. [b]d: days of age; w: weeks of age; wbo: weeks before onset of lay; NA: not available. [c]Main results are compared with the non-vaccinated control groups. Excretion is referred to cloacal swabs. Colonisation is referred to internal organs. ↓: reduction; wpi: weeks post infection.

1.6.1 Live attenuated vaccines

Live attenuated vaccines have been generated by mutation of genes involved in metabolism and survival in host tissues, with the aim of prolonging generation times and reducing the persistence and spread in the environment, but at the same time without greatly affecting the expression of key virulence determinants, required for inducing an adaptive immune response (Barrow, 2007).

Regardless of the type of mutations, it is crucial that the vaccine strains retain the capacity of invasiveness in order to invade and stimulate internal lymphoid tissues and induce cellular and mucosal immunity (Wang et al., 2013). *Salmonella* vaccines should compensate the lower susceptibility of chickens with a lower level of attenuation, to achieve enough level of invasiveness and accumulate enough antigen in the gut wall. At the same time the vaccine strain needs to be eliminated before slaughter age in broilers, and before onset of lay in layer and breeder chickens (Barrow, 2007).

Different attenuation methods have been used, most of them in licensed vaccines in the EU:

- Metabolic drift mutants. Contain negative mutations in essential enzymes and metabolic control centres of the bacterium (Gantois et al., 2006; Atterbury et al., 2009; Kilroy et al., 2015; Eeckhaut et al., 2018; Van De Reep et al., 2018; Huberman et al., 2019). In the next section this type of vaccines will be discussed more deeply because the vaccines tested in the present thesis were obtained using this method.
- *aroA* mutants. Contain alterations in the synthesis of a key component in the aromatic amino acids biosynthetic pathway (Cooper et al., 1994; Sharma et al., 2018).
- *Cya/crp* mutants. Contain deletions in genes encoding for adenylate cyclase and cyclic adenosine monophosphate receptor protein (Hassan and Curtiss, 1994; Dorea et al., 2010).
- Modification in the lipopolysaccharide. Rough strain of *Salmonella* Gallinarum SG 9R (Smith, 1955; Nassar et al., 1994; Filho et al., 2009).
- Adenine-histidine auxotrophic mutants. Contain metabolic deficiencies that inhibit vaccine strain growth in adenine and histidine deficient medium (Carvajal et al., 2008; Atterbury et al., 2009; Springer et al., 2011; Braukmann et al., 2016; Methner, 2018; Theuß et al., 2018).

1.6.1.1 Metabolic drift mutants

Metabolic drift mutants are a type of live attenuated *Salmonella* vaccines. These strains are generated by spontaneous chromosomal mutations in essential enzymes and metabolic compartments of the bacteria (Linde et al., 1990). These mutations generate attenuated clones with reduced virulence and prolonged generation time (Linde, 1997)

The mutations affect antibiotic binding sites, and as a result of the attenuation, the strains gain additional resistance to some antibiotics (Linde et al., 1990). Some examples of the chromosomal mutations in metabolic compartments and the corresponding resistance to antibiotic are RNA polymerase and resistance to Rifampicin, and Ribosomal protein S12 and resistance to Streptomycin (Hahn, 2000). Additionally, these markers are also useful to differentiate the vaccine strains from the wild type strains.

The mentioned mutations can also confer to the vaccine strains an increased permeability of the cell membrane to antibiotics and detergents, such as an extreme sensitivity to quinolones (Linde et al., 1990; Hahn, 2000).

The statistical probability of back mutation of the metabolic drift mutant vaccines was calculated. The total stability of the strain was calculated as the product of the stabilities of each marker, which is 10^{-8}. For example, the risk of back mutation in strains with three independent mutations would be 10^{-24} (Linde, 1997).

There is a concern about the possible transmission of these resistances to other bacteria *in vivo*. This possibility was studied by means of transduction experiments where transfer of spontaneous mutations from mutated strains to wild-type strains were tested (Linde et al., 1998). The probability of gene transfer had an expected rate of 10^{-10} (Kaper et al., 1994). In this study, the resistance to an antibiotic was transferred as a functional unit together with attenuation markers: prolonged generation time, temperature sensitivity and auxotrophy (Kaper et al., 1994). For that reason, the result of transmission would be a resistant but also attenuated bacteria. Additionally, the appearance of metabolic mutants also occurs spontaneously in the gut, so it is considered a normal biological and evolutionary process (Linde et al., 1998).

1.6.1.2 Competitive exclusion

Competitive exclusion (CE) is a strategy that is based on the early administration of non-pathogenic bacteria to the animals to promote microbial competition and thus reduce colonisation and/or populations of pathogenic bacteria in the gastrointestinal tract

(Callaway et al., 2008; La Ragione and Mead, 2013). Among the mechanisms by which bacteria may inhibit enteropathogen proliferation are: creation of a restrictive physiological environment, competition for enteric receptor sites, competition with the host and other organisms for nutrients, production of antimicrobial compounds, and stimulation of the immune system (La Ragione and Mead, 2013). This phenomenon has been studied concerning live vaccines against intestinal pathogens. *Salmonella* organisms can induce a very rapid form of protection in very young chicks before the maturation of the immune system as a result of the competitive exclusion (Methner et al., 2011b). The protection is not the result of a fast adaptive immune response, although a rapid colonisation with cells from the innate immune system could have a slight influence (Van Immerseel et al., 2002a). Instead, non-virulent bacterial strain administered to chicks soon after hatching has the capacity of inhibit subsequent colonisation by virulent bacteria (Berchieri and Barrow, 1991; Methner et al., 2004; Braukmann et al., 2016). This exclusion capacity loses effect as normal intestinal microbiota develops. These data suggest that it is possible to administer live vaccine strains to newly hatched chicks such that they can colonize the gut before the normal microbiota stablishes, and that this should induce a resistance to colonization by *Salmonella* strains that may be already present in the poultry house or in the hatchery (Barrow, 2007).

Studies have been done to determine the capacity of growth inhibition of mixtures of *Salmonella* serovars against homologous and heterologous serovars. The highest degree of colonisation-inhibition occurred between strains of the same serovar, suggesting that there is a still unknown competition between strains for limiting nutrients, specific receptor sites or interaction between bacteria by signalling or metabolism. Identifying a potential strain able to inhibit a wide range of serovars was not achieved (Methner et al., 2011b).

1.6.2 Killed vaccines

This type of vaccines consists of the whole bacteria inactivated by different methods: glutaraldehyde, heat, formalin or acetone. Killed vaccines are normally administered intramuscularly or subcutaneously and require at least 2 immunisations. The administration of these vaccines also requires adjuvants to booster the immune response like oil or aluminium (Barrow and Methner, 2013).

Different killed vaccines are commercially available:

- Inactivated *Salmonella* strains grown under iron-limiting conditions (Clifton-Hadley et al., 2002; Woodward et al., 2002; Atterbury et al., 2009; Van De Reep et al., 2018).
- Inactivated multiple *Salmonella* strains in oil adjuvant (Filho et al., 2009; Springer et al., 2011)

1.6.3 Subunit vaccines

Subunit vaccines are composed of single or multiple defined antigens, often formed by proteins of the bacterial surface (Desin et al., 2013). Some studies have been performed immunising animals with type I fimbriae, which is involved in adherence, and reduced the colonisation of reproductive organs and the contamination of eggs (De Buck et al., 2005). Salmonella FliC protein from the flagellar filament was also used to immunise animals and the caecum colonisation was reduced (Okamura et al., 2012). Other studies immunised animals with outer membrane proteins of 75.6 and 82.3 kDa and were effective in reducing colonisation of intestinal mucosa and caecum (Khan et al., 2003). Finally, T3SS proteins have been also used as vaccines with a minor effect in reducing colonisation (Desin et al., 2011; Wisner et al., 2011). This type of vaccines is not currently available in the market.

1.6.4 DNA vaccines

DNA vaccines are plasmids that encode antigens whose expression is under the control of a eukaryotic promoter (Haygreen et al., 2005). Attenuated *Salmonella* spp. strains have been tested as a carrier for DNA vaccine against other pathogens like Newcastle disease virus, *Campylobacter* spp. or *Clostridium perfringens*, but not as a *Salmonella* vaccine in itself (Adams et al., 2019; Gao et al., 2019; Wilde et al., 2019). However, *Salmonella* DNA vaccines induce strong systemic and mucosal responses and can induce dual protection against both *Salmonella* and the encoded pathogen in murine models (Park et al., 2019). Nevertheless, *Salmonella* DNA vaccines for poultry are still not registered in Europe (Gao et al., 2019).

1.6.5 Cross-protection in *Salmonella* vaccines

The ability of protect against infection with heterologous serovars is known as cross-protection (Nandre et al., 2015). Nowadays the existence of cross-protection between different *Salmonella* serovars in poultry is not clear. The general consensus is that cross-protection exists but is not as strong as the conferred by the same serovars, and the

duration of the immunity is shorter (Barrow and Methner, 2013). In spite of this scenario, some studies have indicated a certain degree of cross-protection between serovars.

The cross protection of *S.* Gallinarum vaccine (9R vaccine developed against fowl typhoid) against *S.* Enteritidis (a member of the same O-group) was demonstrated some years ago with a reduction of internal organ colonisation and eggs contamination in vaccinated laying hens (Barrow et al., 1991). Shortly after, it was demonstrated that chickens vaccinated with a *Salmonella* Typhimurium vaccine were protected against homologous and heterologous *Salmonella* strains from same (B) and different (C, D and E) O-groups, mainly concerning internal organ colonisation, even though best protection against gut colonisation was conferred by homologous strains and O-group C seemed to be less protected. These results imply that there are antigenic components unique to each serovar that are involved in immune response and protection (Hassan and Curtiss, 1994).

The existence of cross-protection between two of the main serovars causing salmonellosis in humans has also been demonstrated. Oral vaccination of laying hens with *Salmonella* Typhimurium reduced spleen and oviduct colonisation with heterologous challenge with *Salmonella* Enteritidis. But in this study, internal egg contamination was only significantly reduced in the group vaccinated with both strains of *Salmonella* Enteritidis and *Salmonella* Typhimurium and challenged with *Salmonella* Enteritidis, compared with non-vaccinated group or animals vaccinated with only one strain (either *Salmonella* Enteritidis or *Salmonella* Typhimurium). These results can suggest the existence of synergy when vaccinating with various serovars that improve protection (Gantois et al., 2006).

More recently, a study tested the cross-protection of a bivalent *Salmonella* Enteritidis and Typhimurium vaccine against emergent serovar *Salmonella* Infantis. In this study, vaccination with the bivalent vaccine reduced qualitatively liver, spleen and caecum colonisation by *Salmonella* Infantis, but this effect was not seen in reproductive organs like ovary and oviduct, indicating again that there is only partial protection between different serovars (Eeckhaut et al., 2018).

Other strategies intended to induce cross-protective immunity with vaccines have also been performed. A vaccine with the LPS core structure common to a great number of serovars have been tested. These non-commercial vaccine strains are able to *in vitro*

synthesize LPS O antigen but *in vivo* gradually lose LPS components and are exposed to immune system, becoming sensitive to complement and phagocytosis, and generating antibodies against the LPS that confer some level of cross-protection (Aehle and Curtiss, 2016).

A possible solution to mitigate the lack of cross-protection would be to include different serovars in the vaccine formulation, in both live and killed vaccines, with the aim of expanding the range of protection against the largest possible number of serovars.

1.6.6 Regulations for *Salmonella* vaccines in Europe

Salmonella vaccines are immunological veterinary medicinal products and, therefore, they need to meet the requirements of European regulations in order to be authorised. General guidelines exist on the requirements for registration of vaccines, including Directive 2001/82/EC of the European Parliament on the Community code relating to veterinary medicinal products (European Commission, 2001). More concretely, since 2013 the efficacy of new live *Salmonella* Enteritidis and *Salmonella* Typhimurium vaccines for chickens needs to be demonstrated according to European Pharmacopoeia Monograph 04/2013:2520 and 04/2013:2521 respectively to be licensed in the EU. Each Monograph includes the qualitative and quantitative composition of the strain and the tests to be carried out on each vaccine in order to market the product.

As a brief example, the Monograph 04/2013:2521 applies to vaccines intended for the active immunisation of chickens against colonisation by and faecal excretion of *Salmonella* Typhimurium. This guide regulates the tests to perform during the culture and preparation of the *Salmonella* Typhimurium vaccine strain, such as monitoring of growth parameters, confirmation of the purity of the culture, and determination of tests for identification of the vaccine strain by relevant markers.

The monograph also includes all tests to demonstrate the safety of the vaccine strain in the target species as well as other species to which the vaccine could be spread and in the persons handling the vaccine. The safety tests include general safety (to test that the vaccine does not cause signs of disease or death); excretion, duration of excretion and survival in the environment (the results are used to know the length of time of excretion of the vaccine strain); spread of the vaccine strain (the results are used to know the extent to which the vaccine spreads to in-contact non-vaccinated chickens), dissemination and survival of the vaccine strain in vaccinated chickens after each vaccination (the results

are used to know the length of time the vaccine survives in the body and to define a suitable withdrawal period); and increase in virulence (to test that there is no reversion to virulence and to test the presence and stability of the markers that identify the vaccine strain).

The efficacy should be demonstrated with the immunogenicity tests carried out after each recommended vaccination and at the end of the laying period (in the case of layers). The vaccinated chickens and a non-vaccinated control group are challenged after each vaccination with a virulent strain of *Salmonella* Typhimurium to determine if there is a significant reduction in the number of cloacal swabs and internal organs from the vaccinated group positive to the challenge bacteria compared with the control group.

All indicated tests should be carried out in chickens from a flock free from specified pathogens (SPF) and free from antibodies against *Salmonella* spp., except for field trials that should use each category of chickens for which the vaccine strain is intended. The safety tests should be carried out using the bacteria at the least attenuated passage level that will be present in a batch of vaccine and at a titre not less than the maximum expected in one dose of the vaccine. The immunogenicity tests should be carried out using the vaccine at a titre not greater that the minimum expected in one dose of vaccine.

The mentioned guidelines should be followed to comply with the requirements for the test, production, and control of immunological veterinary medicinal products in the EU.

2. HYPOTHESIS AND OBJECTIVES

As previously explained in the introduction, the aim of vaccination is to increase the resistance of birds to *Salmonella* infection by stimulation of the immune system. The use of safe and effective vaccines is, therefore, one of the tools available to ultimately reduce the prevalence of *Salmonella* in the poultry industry and, in this way, reduce salmonellosis in humans, which would be the goal of any control program of *Salmonella*.

Live vaccines are known for generating immune memory in animals and generating a cellular immune response effective in protection against *Salmonella* infection. We used a live *Salmonella* Typhimurium strain attenuated by the principles of the metabolic drift mutants, alone or combined with a *Salmonella* Enteritidis licensed vaccine. The strains used have been demonstrated to be safe, but also that colonise target organs and produce detectable humoral response.

Cross-protection between different *Salmonella* serovars has not been proven to be highly effective, thus the immunisation with the main serovars affecting animals and humans are required. We hypothesised that vaccination with combined *Salmonella* Enteritidis and *Salmonella* Typhimurium strains, instead of reducing effectiveness, can improve the immune status of animals and protect them against a combined challenge inoculation.

The intestine is the first barrier when *Salmonella* invades the chicken organism. However, the evaluation of the immune response at the intestinal level after vaccination and later after experimental infection has not been described extensively in the literature. On the other hand, the in-depth understanding of the immune response against a pathogen is crucial for the strategic development of new vaccines.

Consequently, the general objective of this thesis was to evaluate the mechanisms of protection generated by vaccines against *Salmonella* in poultry.

Derived from this main goal of the thesis, several objectives were defined to frame all executed actions:

1. To evaluate the efficacy of a monovalent *Salmonella* Typhimurium live attenuated vaccine with oral inoculation and spray delivery methods in SPF chicks.
2. To evaluate the efficacy of a monovalent *Salmonella* Typhimurium live attenuated vaccine after two or three vaccine doses in commercial laying hens.
3. To evaluate the efficacy of a bivalent *Salmonella* Enteritidis and *Salmonella* Typhimurium live attenuated vaccine after three vaccine doses at the end of the productive period in SPF laying hens.

4. To evaluate the immune response at the intestinal level after vaccination and infection with field strains of *Salmonella* with different methods: ELISA, immunohistofluorescence, flow cytometry and reverse transcriptase quantitative real-time PCR (RT-qPCR).

3. STUDY I:

Immunogenicity test of *Salmonella* Typhimurium vaccine after first vaccination by oral route and spray route in SPF chicks

3.1 INTRODUCTION

Vaccines are a key factor for the productivity of the modern poultry industry because, in general, they allow to control the main infectious diseases that produce loses due to morbidity and mortality. In the case of *Salmonella*, vaccines reduce the dissemination to humans of this potential zoonotic agent. In addition, vaccines can contribute to the welfare of domestic animals (Cserep, 2008).

Different factors can affect the level of protection conferred by a vaccine, among them the route of administration. There may be differences in the protection of the same vaccine depending on whether it is administered orally or via spray, and even differences in colonization of a challenge strain according to the route of application, finding that application via spray may be more effective with a lower dose (Leach et al., 1999; Atterbury et al., 2010). Live vaccines are usually applied by mass application techniques such as drinking water or spray, although they can also be administered by eye drop or sometimes by injection (Atterbury et al., 2010). Vaccination via drinking water requires simple actions to achieve good results. Main actions include calculation of the required volume of water and best time of vaccination and the use of coloured and stabiliser preparations with the vaccine. The ease of vaccination via drinking water is obvious, because this method is non-stressful for chickens and cost-efficient for the farmer (Iburg, 2003). But the procedure of administration is critical because all animals should drink a full dose. The first vaccination is usually administered to day-old chicks, but at this age the water consumption in not regular, and therefore the acquisition of the vaccine and the consequent protection is not uniform in the batch (Atterbury et al., 2010). A solution for this can be the vaccination by coarse spray. In this method droplets greater than 100 μm are sprayed over the animals, normally using a colour stabiliser to dilute the vaccine. The chicks pick and swallow the coloured droplets from their partners during preening and consequently the vaccine uptake and the level of protection in more uniform than with administration via drinking water (Marangon and Busani, 2007; Atterbury et al., 2010). Currently, few studies have demonstrated the efficacy of a licensed *Salmonella* vaccine in chickens administering the first dose via spray, and supposedly none is registered for this vaccination route in the EU, although it is an interesting solution for the poultry industry (Parker et al., 2011; Howard et al., 2018; Sharma et al., 2018).

In addition to the route of administration of the vaccine, the age of the animals should be considered since the developmental stage of the intestine in young chickens affects the immune response to microorganisms. During the first three to four weeks after hatching, the chicken gut develops until the mature complexity of villus length, defined lamina propria and developed lymphoid tissue (Vervelde and Jeurissen, 1993; Friedman et al., 2003; Smith et al., 2013). Thus, in this young age, birds are highly susceptible to *Salmonella* infection (Desmidt et al., 1997). Furthermore, primary infections with virulent *Salmonella* in very young chicks have been correlated with long periods of bacterial shedding compared with infections in mature birds (Barrow et al., 1988; Immerseel et al., 2004). Therefore, the first vaccination is administered in a period of susceptibility to intestinal infections, but if this first dose manages to protect the animals, it can help to maintain the flock negative to *Salmonella* during all the productive period.

Very young birds are still immunological immature, and the resistance to colonisation after vaccination in this age is achieved thanks to a combination of colonisation-inhibition effect and immune response (Braukmann et al., 2016). A better understanding of the immunological mechanisms of protection is required to allow a more rational approach to vaccination in the chicken. To our knowledge, few studies have analysed the protective capacity of a first dose of *Salmonella* vaccine at day-old, but no study has determined it with the experimental design that demands the European Pharmacopoeia 04/2013:2521 for the immunogenicity test at 14 days after first vaccination.

In this study, the main objective was to compare the protective capacity of a live attenuated vaccine with two different delivery routes in day-old chicks and to study the changes in antibody production, cellular recruitment, and cytokine expression in the intestine. A secondary objective was to study the influence of vaccination in chicks in the capacity of modifying the metabolism of the challenge bacteria after invading the intestine.

3.2 MATERIALS AND METHODS

3.2.1 Animals

Newly hatched day-old SPF chicks (White Leghorn) were used in the study (Valo Biomedia, Salamanca, Spain). The SPF eggs were incubated and hatched at AM Animalia Bianya SL facilities (Girona, Spain) and transported to Clinobs SL facilities (Girona, Spain) for the experimental study. Vaccinated groups were transported to Clinobs SL

facilities on hatching day (before vaccination) and remained there until the end of the study. Non-vaccinated control animals were transported to Clinobs SL on challenge day (before infection) and remained there until the end of the study. During the study, groups were housed separately in pens with wood shavings. Commercial feed and drinking water were provided ad libitum. This study was approved by the Animal Experimentation Ethics Committee of Clinobs SL and by the Government of the Generalitat de Catalunya (Departament de Medi Ambient, procedure No. 10364).

3.2.2 Bacterial strains

The vaccine used was a live attenuated *Salmonella* Typhimurium streptomycin and rifampicin resistant and enrofloxacin highly sensitive strain (see section 1.6.1.1 for more details). Freeze-dried vial was diluted in double-distilled water (Orravan, Barcelona, Spain) and adjusted to a concentration of 1×10^8 cfu per ml. Same vaccine dilution was used for oral and spray vaccination.

The challenge inoculum was prepared with a *Salmonella* Typhimurium field strain (GN-3326) isolated from liver and intestine of adult hens. The strain was plated on Columbia agar + 5% sheep blood (bioMérieux, Marcy-l'Etoile, France) at 37 °C. After overnight incubation, the bacteria were suspended in buffered peptone water (BPW) (VWR, Leuven, Belgium) and adjusted to 1.31×10^7 cfu per ml.

3.2.3 Study design

Healthy chicks (n = 78) were divided into three groups. Group V1 (n = 27) was vaccinated at day-old by oral gavage using a plastic cannula. The vaccine was applied at a dose of 1×10^8 cfu in 1 ml per chick. Group V2 (n = 26) was vaccinated at day-old by coarse spray. The vaccine was applied with a manual sprayer (Goizper, Antzuola, Spain) with a mean droplet size of 175 μm. Chicks were placed inside a plastic box to receive the spray vaccine (26 ml of vaccine for 26 chicks) and remained there for at least 30 minutes to provide a suitable period for the uptake of the vaccine before being transferred to their pen. Control group (n = 25) was kept as non-vaccinated group. At 14 days of age, all the animals were inoculated by oral gavage with the *Salmonella* Typhimurium challenge strain GN-3326 at a dose of 1.31×10^7 cfu in 1 ml. The study design is summarised in Table 3-1.

Meconium samples were collected from day-old chicks to confirm *Salmonella*-free status of the experimental groups. After the vaccination, cloacal swabs were sampled from each

animal at 7 and 14 days post-vaccination (dpv) to detect vaccine strain. After the infection, cloacal swabs were sampled from each animal at 3, 5, 7, 10 and 14 days post-infection (dpi) to detect challenge strain. At 7 and 14 dpi at least 10 animals per group were euthanised by cervical dislocation and samples of blood, bile, liver, spleen, small intestine and caecum were collected. Samples of liver, spleen and caecum were qualitatively examined for detection of challenge strain by culture. Blood and bile were analysed for *Salmonella* antibodies. The small intestine was collected for cells isolation and RNA extraction.

Table 3-1. Study design.

Group	**Vaccine**	**Vaccine delivery route**	**Challenge**	**Euthanasia[a]**
Control (n = 25)	No	na	*S.* Typhimurium (14 days of age) 1.31×10^7 cfu in 1 ml	7 dpi (n = 10) 14 dpi (n = 14)
V1 (n = 27)	*S.* Typhimurium (day-old) 1×10^8 cfu in 1 ml	Oral gavage		7 dpi (n = 10) 14 dpi (n = 17)
V2 (n = 26)	*S.* Typhimurium (day-old) 1×10^8 cfu in 1 ml	Spray		7 dpi (n = 10) 14 dpi (n = 16)

[a]dpi: days post-infection. na: not applicable.

3.2.4 Detection of *Salmonella* spp. strains by culture

Salmonella Typhimurium vaccine and challenge strains were detected qualitatively in swabs and internal organs by culture. First step was non-selective pre-enrichment in BPW (37 ± 1 °C for 18 h). Second step was cultivation in selective enrichment media Rappaport-Vassiliadis (41.5 ± 1 °C for 48 h). Rappaport-Vassiliadis enrichment broth (RV) (Oxoid, Basingstoke, UK) was used for non-motile vaccine strain; and semi-solid Rappaport-Vassiliadis agar (MSRV) (Merck, Darmstadt, Germany) was used for motile challenge strain. Finally, samples were plated in selective isolation agar xylose lysine tergitol 4 (XLT4) (Merck, Darmstad, Germany) (37 ± 1 °C for 24 h) where the presence of typical black colonies resulted in the detection of *Salmonella* spp. strains. In order to ensure that no vaccine strain was detected in post-infection sampling points, doxycycline (30 µg/ml) (Sigma-Aldrich, St. Louis, MO, USA) was added to XLT4 from 3 dpi onwards (vaccine strain is sensitive and challenge strain is resistant to this antibiotic).

3.2.5 Enzyme-linked immunosorbent assay (ELISA)

Commercial indirect ELISA kit (*Flocktype* Salmonella Ab) (QIAGEN, Leipzig, Germany) was used for the detection of antibodies against *Salmonella* Typhimurium in serum and bile. Serum was analysed for the detection of IgY according to the manufacturer's protocol. Bile was analysed for the detection of IgA according to the manufacturer's protocol but changing the dilution of the sample (1:20) and the secondary antibody solution. The secondary antibody solution was prepared with HRP conjugated goat anti-chicken IgA (0.02 μg/ml) (Bethyl, Montgomery, TX, USA) in phosphate-buffered saline (PBS) Tween-20 (0.01%) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 0.5% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA).

The results of ELISA in serum are expressed as S/P (sample-to-positive) ratio, where S/P:

$$S/P = \frac{(Optical\ Density\ (OD)\ sample - OD\ Negative\ Control)}{(OD\ Positive\ Control - OD\ Negative\ Control)}$$

The threshold determined by the manufacturer is indicated in the graph as additional discontinuous lines (negative: $S/P < 0.2$; doubtful: $0.3 > S/P \geq 0.2$; positive: $S/P \geq 0.3$). The results of ELISA in bile are expressed as OD. OD was measured at 450 nm. Results of each individual animal are represented and error bars express mean ± standard deviation (SD).

3.2.6 Flow cytometry

An intestinal segment of the first 10 cm after Meckel's diverticulum was collected where almost one proximal Peyer's patch is located (Holt et al., 2010). The intestine was flushed first with sterile PBS and then with complete RPMI (CRPMI) (Lonza, Walkersville, MD, USA) supplemented with 5% FBS, 10 U/ml penicillin (Gibco, Life Technologies, NY, USA), 10 μg/ml streptomycin (Gibco, Life Technologies, NY, USA), and 2 mM L-Glutamine (Gibco, Life Technologies, NY, USA). Then intestinal segments were placed in a 50 ml tube with 20 ml of CRPMI on ice.

Intestinal cells isolation protocol was adapted from a previously published article with some modifications (Couter and Surana, 2016). Briefly, fat was removed from intestine and this fragment was cut in a tube with 4 ml of RPMI 1640 containing 1% FBS and 1 mg/ml Type I Collagenase (Sigma-Aldrich, St. Louis, MO, USA). This was incubated for 45 min at 37 °C and 200 rpm in an orbital shaker, vortexing briefly every 15 min. Digested

tissue was filtered through a 70 µm cell strainer (Falcon, Corning, NY, USA) and filter was rinsed with 15 ml of RPMI containing 5% FBS. The filtered solution was centrifuged for 15 min at 500 x g at 4 °C. The supernatant was discarded, and pellet was resuspended in 15 ml of RPMI containing 5% FBS. The cell suspension was filtered again through a 40 µm cell strainer (Falcon, Corning, NY, USA) and centrifuged for 15 min at 500 x g at 4 °C. The supernatant was discarded, and pellet containing isolated cells was resuspended in 20 ml CRPMI containing 0.1 mM non-essential amino acids (Gibco, Life Technologies, NY, USA) and 240 U/ml Nystatin (Sigma-Aldrich, St. Louis, MO, USA). Cells were stored over-night at 4 °C with the caps loosely fitted for aeration (approximately 12 hours).

Before staining, viable cell counts were made using the trypan blue exclusion method and 1 x 10^6 viable cells were aliquoted into each well of a polystyrene V-bottom 96-well plate (Falcon, Corning, NY, USA). Cells were stained with the antibodies detailed in Table 3-2. All antibody reagents were purchased from Southern Biotech (Birmingham, AL, USA). Cells were stained at 4 °C in the dark for 60 min, then washed twice with PBS, and finally resuspended in PBS containing 5% FBS. Flow cytometry was performed using a MACSQuant Analyzer 10 (Miltenyi biotec, Bergisch Gladbach, Germany). Five parameters were collected and evaluated using MACSQuantify software version 2.8 (Miltenyi biotec, Bergisch Gladbach, Germany): forward scatter (FSC), side scatter (SSC), FITC (filter 525/50 nm), PE (filter 585/40 nm), and Allophycocyanin (APC) (filter 655-730 nm). Dead cells were removed from the analysis by sequential gating: first by doublet discrimination performed by plotting forward scatter height (FSC-H) vs. forward scatter area (FSC-A) and second by viability dye using propidium iodide staining (5 µg/10^6 cells) (Biolegend, CA, USA). Isotype control was used to determine the level of non-specific binding (Goat anti-mouse IgG1 AF647, 0.2 µg/10^6 cells) (Invitrogen, IL, USA). Results of cell populations are presented as percentage.

Table 3-2. Monoclonal antibodies used for the detection of avian antigens in Flow Cytometry.

Abbreviation	Specificity	Clone	Isotype	Conjugation[a]	Dilution
Mφ	Monocyte/Macrophage	KUL01	Mouse (BALB/c) IgG1κ	FITC	0.5 µg/10^6 cells
MHC II	MHC Class II β-chain	2G11	Mouse (BALB/c) IgG1κ	AF488	0.25 µg/10^6 cells

B cell	Chicken Bu-1a, mainly bursal cells	21-1A4	Mouse (BALB/c) IgG1κ	FITC	1 μg/10^6 cells
CD3+	CD3 T lymphocytes	CT-3	Mouse (BALB/c) IgG1κ	AF647	0.25 μg/10^6 cells
CD4+	CD4 T helper lymphocytes	CT-4	Mouse (BALB/c) IgG1κ	PE	0.05 μg/10^6 cells
CD8+	CD8α T cytotoxic lymphocytes	CT-8	Mouse (BALB/c) IgG1κ	AF647	0.25 μg/10^6 cells

[a]FITC: fluorescein; AF: Alexa Fluor; PE: R-phycoerythrin.

3.2.7 Reverse transcriptase quantitative real-time PCR (RT-qPCR)

The relative gene expression of cytokines and cellular surface proteins at ileal mucosa was determined by RT-qPCR. Ileal mucosa was collected in RNAlater stabilisation solution (Thermo Fisher Scientific, Vilnius, Lithuania) and frozen at −80 °C. RNA was extracted from 15-20 mg of tissue with RNeasy Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol and treated with RNase-free DNase set (QIAGEN, Hilden, Germany). Quality and quantity of RNAs were determined using BioDrop μLITE spectrophotometer (Biodrop, Cambridge, UK). The mRNA expression rates of Interferon gamma (IFNγ), Tumor necrosis factor alpha (TNFα), Interleukin 10 (IL-10), Toll-like receptors 4 and 5 (TLR4 and TLR5) and major histocompatibility complex class II (MHC II) were determined for every individual chicken using the EXPRESS One-Step SYBR GreenER kit (QIAGEN, CA, USA) according to the manufacturer's protocol. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as reference gene. Primers for the IFNγ and TNFα (Carvajal et al., 2008) and IL-10 (Fasina et al., 2008) had been described previously. Primer sequences for GAPDH, TLR4, TLR5 and MHC II were designed using National Center for Biotechnology Information database (Sayers et al., 2019) and Oligo explorer 1.5 software (http://www.genelink.com). Primer sequences are described in Table 3-3. Amplification and detection of specific products were performed using 7500 Fast Real-Time PCR system and 7500 software version 2.3 (Applied Biosystems, CA, USA) with the following conditions: cDNA synthesis at 50 °C for 5 min, followed by initial denaturation at 95 °C for 5 s, and 40 cycles of denaturation at 95 °C for 3 s and annealing/extension at 60 °C for 30 s. Results are expressed as $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001), where ΔΔCT:

$$\Delta\Delta CT = ((CTtarget - CTgapdh)vaccinated - (CTtarget - CTgapdh)control)$$

Statistics were calculated using ΔΔCT values.

Table 3-3. Primer sequences for RT-qPCR.

Target	**Sequence (5′-3′)**		**Product (bp)**
GAPDH	Forward	GGGTGTCAACCATGAGAAATAT	120 bp
	Reverse	CCCTCCACAATGCCAAAGTT	
IFNγ	Forward	CCCGATGAACGACTTGAGAAT	106 bp
	Reverse	AGACTGGCTCCTTTTCCTTTTG	
TNFα	Forward	GCTGTTCTATGACCGCCCAGTT	140 bp
	Reverse	AACAACCAGCTATGCACCCCA	
IL-10	Forward	CATGCTGCTGGGCCTGAA	94 bp
	Reverse	CGTCTCCTTGATCTGCTTGATG	
TLR4	Forward	TCCCTCACACCCATTCCACG	109 bp
	Reverse	ATGGGGAAGGGGCTGAGGA	
TLR5	Forward	TCACACGGCAATAGTAGCAACA	137 bp
	Reverse	TCACACAGTAAGAGAAGCGAT	
MHC II	Forward	CACTACCTGAACGGCACC	156 bp
	Reverse	AATCTCGGCGTTGCTGTTCC	

3.2.8 Comparative analysis of biochemical kinetics profile in challenge strain

Challenge strain isolated from vaccinated and non-vaccinated control animals was characterized by its biochemical kinetics profile by using the organism identification system Vitek 2 Compact (bioMérieux, Marcy-l'Etoile, France). The system uses the temporal changes of different biochemical tests to identify bacteria.

For this analysis, the same 5 animals from each group and 2 *Salmonella* compatible colonies from each animal were chosen from cloacal swabs samples at 3, 5, 7, 10 and 14 dpi. Each colony of the challenge strain was isolated from XLT4 agar onto Columbia agar + 5% sheep blood (bioMérieux, Marcy-l'Etoile, France) and incubated for 24 h at 37 °C. A 0.6 McFarland suspension was prepared by turbidimetric adjustment in 0.45% sterile saline solution for each isolate. Gram-negative organism identification cards (GNI) (bioMérieux, Marcy-l'Etoile, France) were then inoculated and incubated in the Vitek 2 Compact system (bioMérieux, Marcy-l'Etoile, France). These cards contain 47

biochemical tests and one negative control well (Decarboxylase) that are detailed in Table 3-4.

The system performed readings by means of a transmittance optical system that measured turbidity or colour changes every 15 minutes. For each period of 15 minutes, the results were expressed as the percentage of transmittance reduction when compared with the reading at time zero (Percent Change, PCTs). This data was recorded on a text file and transferred to an Excel spreadsheet (Microsoft Office 365, Redmont, WA, USA).

The arithmetical mean of the absolute differences between the PCTs of two different strains was considered the absolute distance between each pair of bacteria.

The relationships between strains were established using a hierarchical cluster analysis depicted by dendrograms.

Table 3-4. Gram-Negative card well contents.

Well[a]	Test	Description	Well[a]	Test	Description
2	APPA	Ala-Fe-Pro-ARYLAMIDASE	33	SAC	SACCAROSE/SUCROSE
3	ADO	ADONITOL	34	dTAG	D-TAGATOSE
4	PyrA	L-Pyrrolidonyl-ARYLAMYDASE	35	dTRE	D-TREHALOSE
5	lARL	L-ARABITOL	36	CIT	CITRATE (SODIUM)
7	dCEL	D-CELLOBIOSE	37	MNT	MALONATE
9	BGAL	BETA-GALACTOSIDASE	39	5KG	5-KETO-D-GLUCONATE
10	H2S	H2S PRODUCTION	40	lLATk	L-LACTATE alkalinisation
11	BNAG	BETA-N-ACETYL-GLUCOSAMINIDASE	41	AGLU	ALPHA-GLUCOSIDASE
12	AGLTp	Glutamyl Arylamidase pNA	42	SUCT	SUCCINATE alkalinization
13	dGLU	D-GLUCOSE	43	NAGA	Beta-N-ACETYL-GALACTOSAMINIDASE
14	GGT	GAMMA-GLUTAMYL-TRANSFERASE	44	AGAL	ALFA-GALACTOSIDASE
15	OFF	FERMENTATION/GLUCOSE	45	PHOS	PHOSPHATASE
17	BGLU	BETA-GLUCOSIDASE	46	GlyA	Glycine ARILAMIDASE
18	dMAL	D-MALTOSE	47	ODC	ORNITHINE DESCARBOXYLASE
19	dMAN	D-MANNITOL	48	LDC	LYSINE DESCARBOXYLASE
20	dMNE	D-MANNOSE	52	0DEC	DECARBOXYLASE BASE
21	BXYL	BETA-XYLOSIDASE	53	lHlSa	L-HISTIDINE assimilation
22	BAlap	BETA-Alanin arylamidase pNA	56	CMT	COURMARATE
23	ProA	L-Proline-ARYLAMIDASE	57	BGUR	BETA-GLUCORONIDASE
26	LIP	LIPASE	58	O129R	O/129 RESISTANCE (comp. vibrio.)
27	PLE	PALATINOSE	59	GGAA	Glu-Gly-Arg-ARYLAMIDASE
29	TyrA	Tirosina ARYLAMIDASE	61	lMLTa	L-MALATE asismilation
31	URE	UREASE	62	ELLM	ELLMAN
32	dSOR	D-SORBITOL	64	lLATa	L-LACTATE assimilation

[a]Other well numbers between 1 and 64 not designated in this table are empty.

3.2.9 Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.01 software for Windows (GraphPad Software, La Jolla, CA, USA). Differences in excretion of cloacal swabs and colonisation of internal organs by challenge strain were determined using Fisher's exact test. Normality of the data from ELISA, flow cytometry and RT-qPCR were tested with the D'Agostino & Pearson omnibus test and differences were determined using Kruskall-Wallis and followed by Dunn's multiple comparison test. P values ≤ 0.05 were considered significant.

3.3 RESULTS

3.3.1 Bacteriological examination of cloacal swabs before challenge

Vaccine strain excretion was detected in cloacal swabs at 7 and 14 days after vaccination and it was significantly higher in V1 group vaccinated by oral gavage than in V2 group vaccinated by spray route at 14 days after vaccination. The excretion in V2 group was very low and was not the expected after vaccination. *Salmonella* was not detected in the Control group before the infection with *Salmonella* Typhimurium field strain (Figure 3-1).

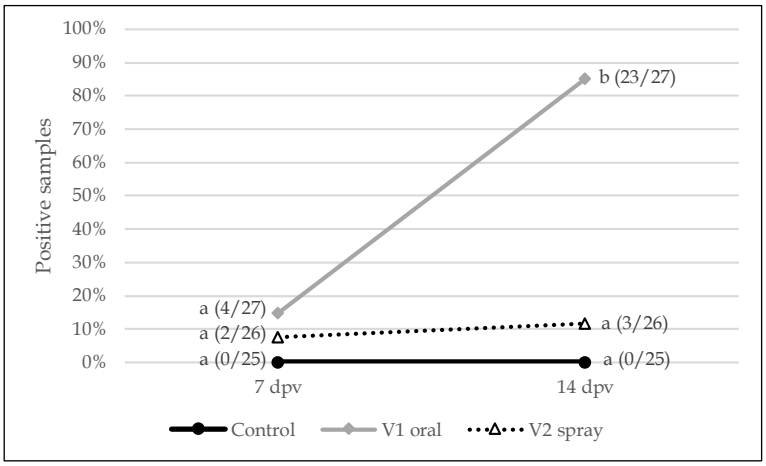

Figure 3-1. Excretion of Salmonella spp. in cloacal swabs after vaccination. dpv: days post-vaccination. Different letters indicate significant differences between the groups (p ≤ 0.05). The number of positive animals with respect to the total is shown in brackets (+/n).

3.3.2 Bacteriological examination of cloacal swabs and internal organs after challenge

Cloacal swabs of all animals were analysed for the presence of *Salmonella* Typhimurium field strain at 3, 5, 7, 10 and 14 days after challenge (Figure 3-2). Excretion of field strain was lower in the group V1 than in the control group in all sampling points except at 5 dpi, and this difference was statistically significant at 7 dpi. Excretion was lower in group

V1 than in group V2 in all sampling points, and this difference was also statistically significant at 7 dpi. There was no significant difference in field strain excretion between the group V2 and the control group (Figure 3-2).

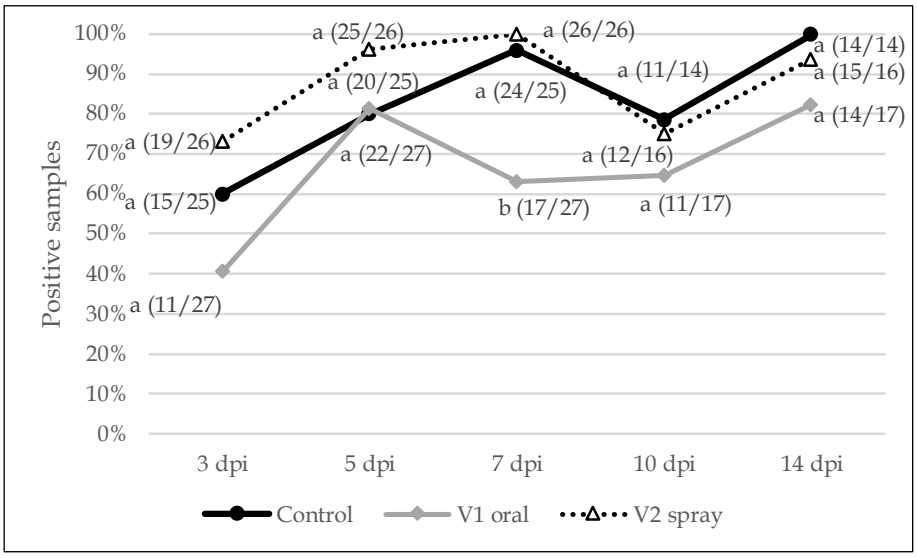

Figure 3-2. Excretion of Salmonella spp. in cloacal swabs after challenge. dpi: days post-infection. Different letters indicate significant differences between the groups ($p \leq 0.05$). The number of positive animals with respect to the total is shown in brackets (+/n).

Field strain of *Salmonella* Typhimurium was detected in internal organs in a representative number of animals at 7 and 14 days after challenge (Figure 3-3). Colonisation of liver was significantly lower in the group V1 compared with group V2 and control group at 7 dpi, and colonisation of spleen was significantly lower in the group V1 compared with group V2 and control group at 7 and 14 dpi. However, there were no significant differences in the colonisation of liver and spleen between the group V2 and the control group. There was no significant difference in the colonization of the caecum between any of the groups of the study.

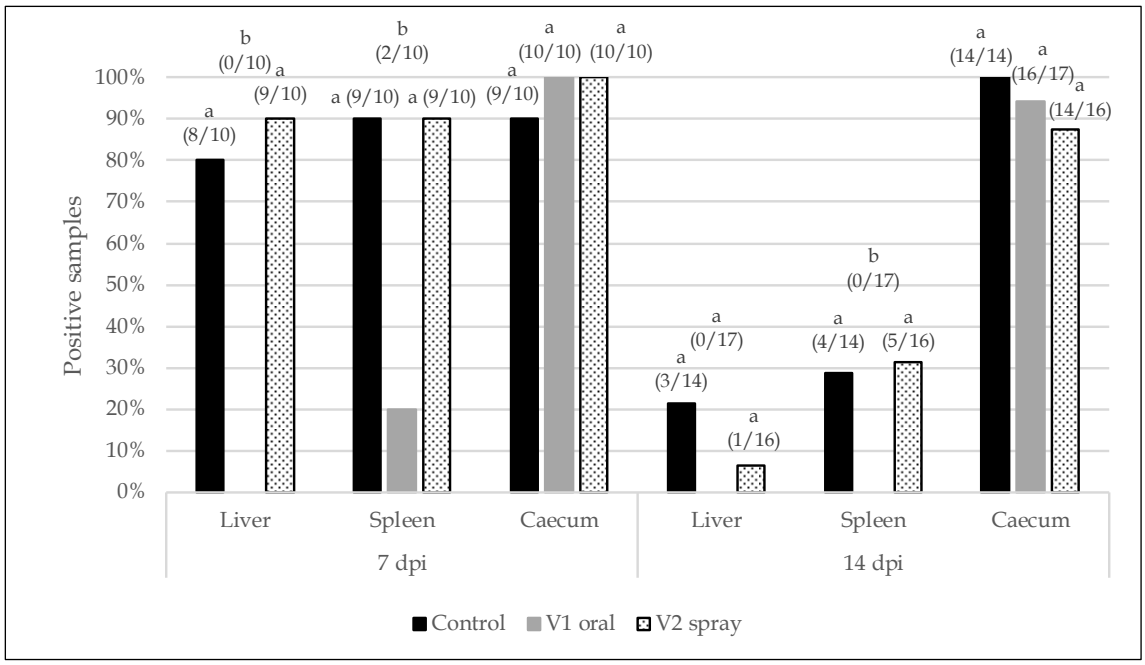

Figure 3-3. Internal organ colonisation of Salmonella spp. after challenge. dpi: days post-infection. Different letters indicate significant differences between the groups ($p \leq 0.05$). The number of positive animals with respect to the total is shown in brackets (+/n).

3.3.3 Antibodies detection in serum and bile

Serum was analysed for the presence of IgY antibodies against *Salmonella* spp. (Figure 3-4). Humoral immune response was delayed until 4 weeks after a first interaction with *Salmonella* vaccine. The challenge with field strain of *Salmonella* induced a humoral response in the vaccinated group V1 detected at 14 days after infection, and the level of antibodies was significantly higher than in the control group. The vaccinated group V2 also developed a mild humoral response but the titre of antibodies was not significantly different from the V1 group or the control group.

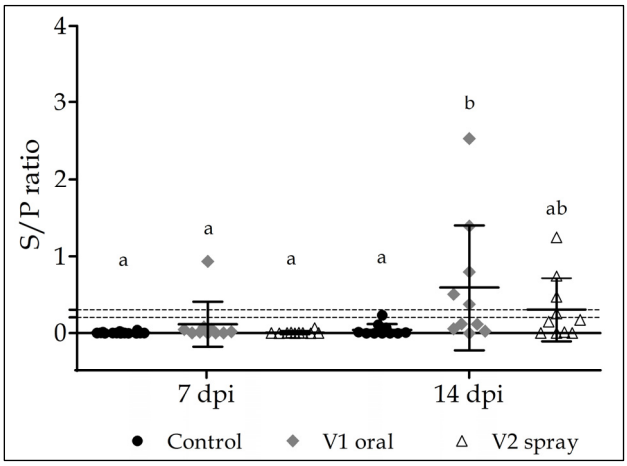

Figure 3-4. Detection of IgY antibodies against Salmonella in serum. Each point represents an animal. Error bars express mean S/P ratio ± SD. Additional discontinuous lines indicate the threshold determined by manufacturer (negative: S/P < 0.2; doubtful: 0.3 > S/P ≥ 0.2; positive: S/P ≥ 0.3). dpi: days post-infection. Different letters indicate significant differences between the groups ($p \leq 0.05$).

Bile was analysed for the presence of secretory IgA antibodies against *Salmonella* spp. (Figure 3-5). Vaccinated groups V1 and V2 developed an earlier response detected at 7 days after challenge, although there were no significant differences between the groups. At 14 days after challenge, the vaccinated groups V1 and V2 and the control group developed a comparable high immune response without significant differences between them.

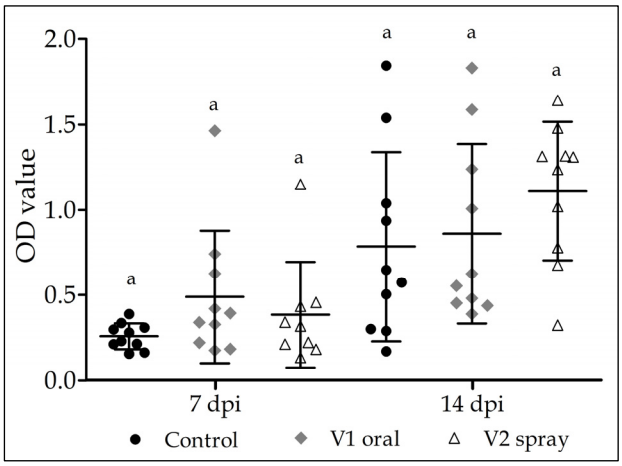

Figure 3-5. Detection of IgA antibodies against Salmonella in bile. Each point represents an animal. Error bars express mean OD value ± SD. dpi: days post-infection. Different letters indicate significant differences between the groups ($p \leq 0.05$).

3.3.4 Cellular composition of ileum

Some cellular components of the ileum were quantified by flow cytometry to determine cellular immune response in vaccinated and control animals after the challenge with *Salmonella* Typhimurium field strains. Figure 3-6 shows the results of the flow cytometry analysis. Macrophages were studied as representatives of the innate immune response. MHC II cells were studied as representatives of the antigen presentation. B cells were studied as representative of the humoral response and T cells subpopulations as representative of the cellular response.

At 7 days after challenge, percentage of MHC II positive cells was significantly higher in control group compared with V1 group. The percentage of CD8+ T cells was significantly lower in V1 group than in V2 group. No significant differences were found between groups in macrophages, B cells, CD3+, CD4+ or double positive CD4+CD8+ cells.

At 14 days after challenge, cellular immune response was evident in more cell subtypes. Macrophages, CD4+ T cells, and double positive CD4+CD8+ T cells percentage were significantly higher in control than in V1 group. CD8+ percentage followed the same

pattern than CD8+ at 7 dpi, and population was significantly lower in V1 group than in V2 group. No significant differences were found in MHC II cells, B cells or CD3+ T cells.

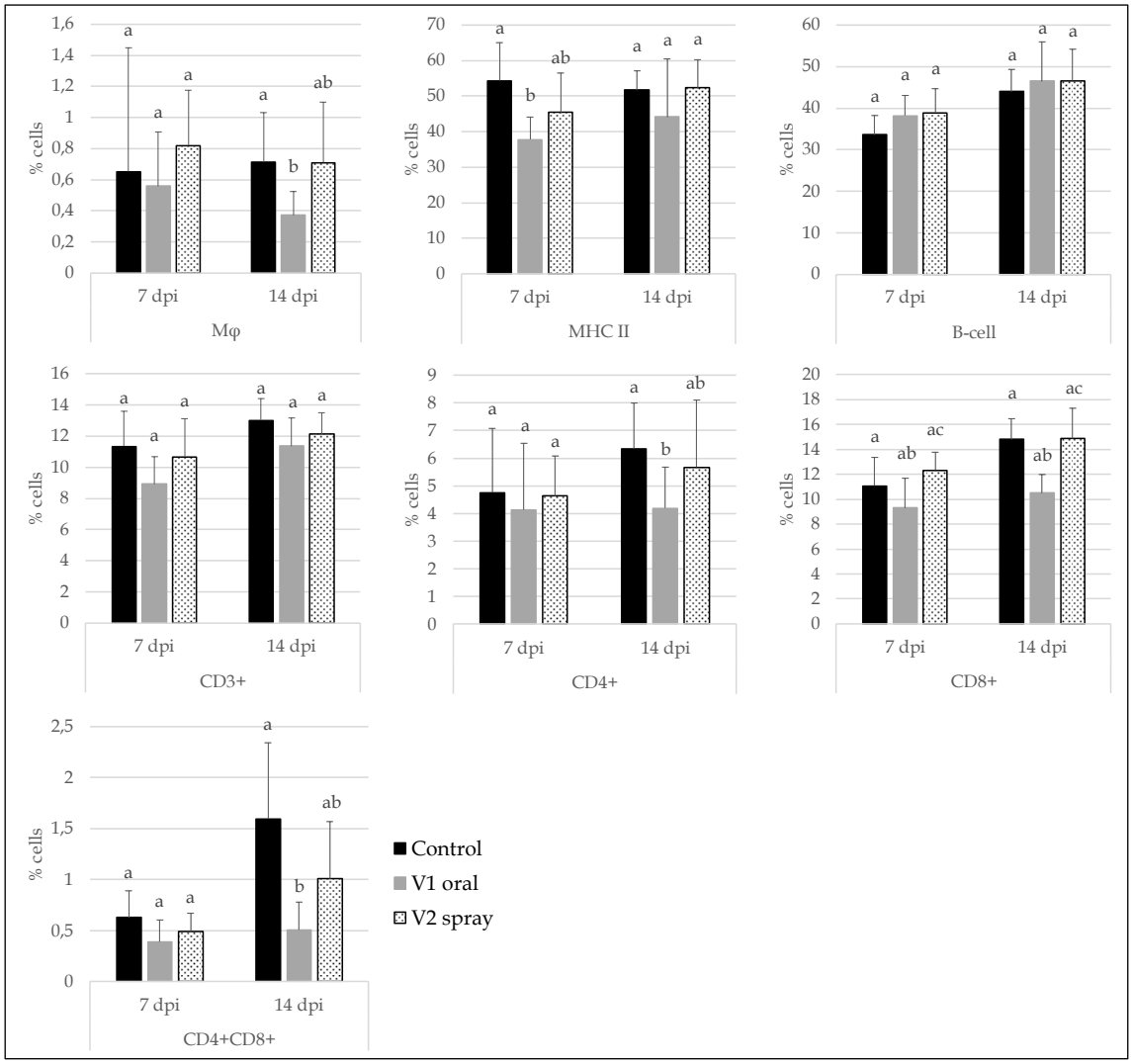

Figure 3-6. Percentage of cell populations in the ileum determined by flow cytometry. Results are expressed as mean cell percentage + SD. dpi: days post-infection. Different letters indicate significant differences between the groups ($p \leq 0.05$).

3.3.5 Gene expression in the ileal mucosa

Changes in the gene expression in the ileal mucosa were compared in vaccinated and control groups after infection with field strains of *Salmonella* Typhimurium and are shown in Figure 3-7. The cytokines IFNγ and TNFα were studied as representatives of proinflammatory response and IL-10 as anti-inflammatory response. TLR4 and TLR5 were studied as receptors of *Salmonella* on the innate immune response and MHC II as a marker of antigen presenting cells.

At 7 days after challenge, IFNγ, IL-10, TLR4, and MHC II were significantly upregulated in V1 group compared with control group and there were no differences between V2

group and the other groups. TNFα and TLR5, however, were upregulated in V2 group compared with control group, and there were no differences between V1 and the other groups.

At 14 days after challenge, there were no significant differences in the expression of IFNγ and TNFα genes. IL-10 and MHC II genes were significantly upregulated in the vaccinated groups V1 and V2 compared with the control group. TLR4 was upregulated in V2 compared with V1. And TLR5 was downregulated in V1 group compared with the control group.

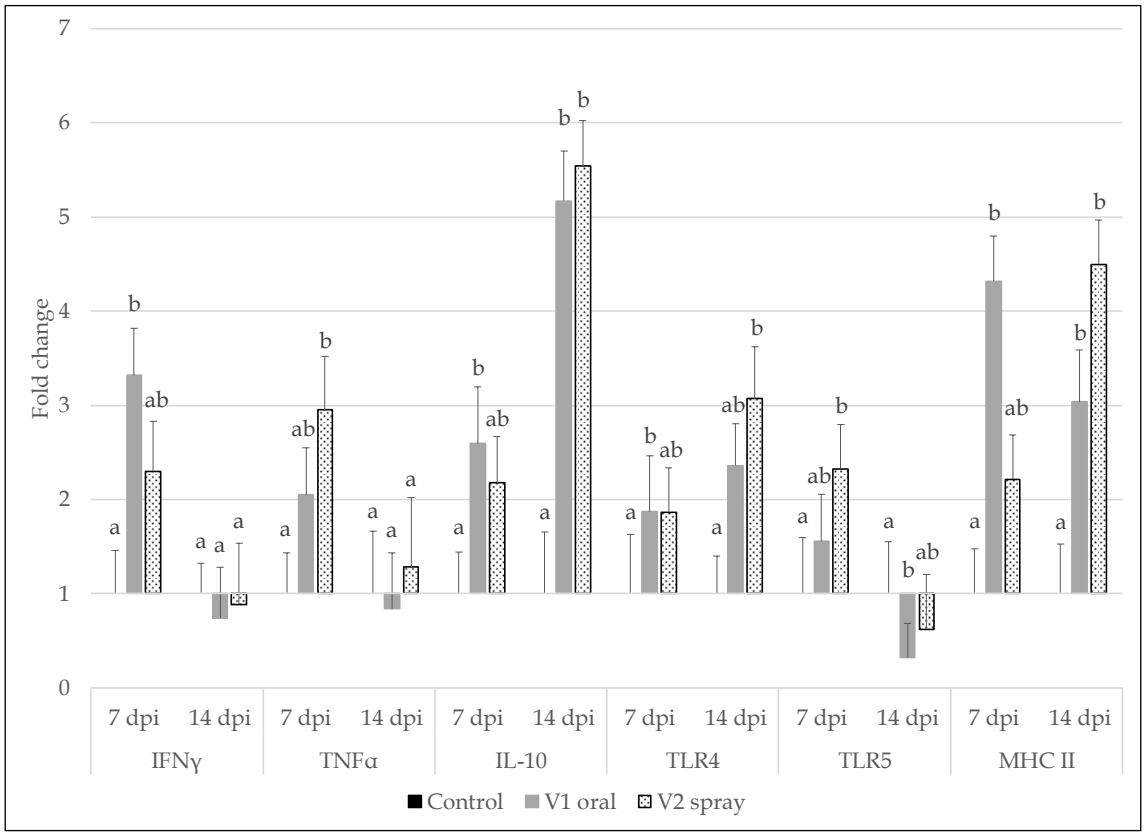

Figure 3-7. Relative fold change in gene expression of cytokines and cellular surface proteins from the ileal mucosa. Data are expressed as mean expression levels for vaccinated group normalised to reference gene and relative to control group + SD. dpi: days post-infection. Different letters indicate significant differences between the groups ($p \leq 0.05$).

3.3.6 Biochemical kinetics profile in challenge strain

The biochemical kinetics profile of the colonies isolated from vaccinated V1 and V2 and control groups were analysed using Vitek 2 Compact Gram-negative identification cards.

The readings of the percentage of transmittance reduction (PCT) were transferred to a spreadsheet. In Table 3-5 there is an example of the arithmetical mean of the absolute differences between the different colonies isolated at 10 dpi. As an example, the distance

between colonies isolated at 10 dpi from an animal from the control group (identification 2432-2) and an animal from the V1 group (identification 561-2) are highlighted in blue (control versus vaccinated) and green (vaccinated versus control).

Table 3-5. Array with the arithmetical mean of the differences between the pairs of colonies isolated from animals at 10 dpi. In the first column appears the identification of group, day of sampling, animal and colony; and the corresponding number in the first row is indicated in brackets.

	(01)	(02)	(03)	(04)	(05)	(06)	(07)	(08)	(09)	(10)	(11)	(12)	(13)	(14)	(15)	(16)	(17)	(18)	(19)	(20)	(21)	(22)	(23)	(24)	(25)	(26)
(01) V1_D10_561_1		-1,25	-1,52	-1,64	-2,04	-1,41	-2,33	-0,86	-1,81	-2,18	-2,83	-3,37	-2,8	-3,25	-3,31	-2,9	-3,21	-2,68	-3,4	-2,53	-3,34	-3,21	-5	-3,2	-3,07	-3,43
(02) V1_D10_561_2	1,25		-0,28	-0,39	-0,8	-0,17	-1,08	0,39	-0,57	-0,94	-1,58	-2,12	-1,55	-2	-2,06	-1,65	-1,96	-1,44	-2,15	-1,28	-2,09	-1,96	-3,75	-1,95	-1,82	-2,18
(03) V1_D10_571_1	1,52	0,28		-0,12	-0,52	0,11	-0,8	0,67	-0,29	-0,66	-1,3	-1,85	-1,27	-1,72	-1,79	-1,38	-1,68	-1,16	-1,87	-1	-1,82	-1,69	-3,48	-1,68	-1,54	-1,91
(04) V1_D10_571_2	1,64	0,39	0,12		-0,4	0,23	-0,69	0,78	-0,17	-0,54	-1,19	-1,73	-1,15	-1,61	-1,67	-1,26	-1,57	-1,04	-1,76	-0,88	-1,7	-1,57	-3,36	-1,56	-1,43	-1,79
(05) V1_D10_597_1	2,04	0,8	0,52	0,4		0,63	-0,28	1,18	0,23	-0,14	-0,79	-1,33	-0,75	-1,2	-1,27	-0,86	-1,17	-0,64	-1,35	-0,48	-1,3	-1,17	-2,96	-1,16	-1,02	-1,39
(06) V1_D10_597_2	1,41	0,17	-0,11	-0,23	-0,63		-0,91	0,55	-0,4	-0,77	-1,42	-1,96	-1,38	-1,83	-1,9	-1,49	-1,8	-1,27	-1,98	-1,11	-1,93	-1,8	-3,59	-1,79	-1,65	-2,02
(07) V1_D10_611_1	2,33	1,08	0,8	0,69	0,28	0,91		1,47	0,51	0,14	-0,5	-1,04	-0,47	-0,92	-0,98	-0,57	-0,88	-0,36	-1,07	-0,2	-1,01	-0,88	-2,67	-0,87	-0,74	-1,1
(08) V1_D10_611_2	0,86	-0,39	-0,67	-0,78	-1,18	-0,55	-1,47		-0,95	-1,32	-1,97	-2,51	-1,94	-2,39	-2,45	-2,04	-2,35	-1,83	-2,54	-1,67	-2,48	-2,35	-4,14	-2,34	-2,21	-2,57
(09) V2_D10_625_1	1,81	0,57	0,29	0,17	-0,23	0,4	-0,51	0,95		-0,37	-1,02	-1,56	-0,98	-1,43	-1,5	-1,09	-1,4	-0,87	-1,58	-0,71	-1,53	-1,4	-3,19	-1,39	-1,25	-1,62
(10) V2_D10_625_2	2,18	0,94	0,66	0,54	0,14	0,77	-0,14	1,32	0,37		-0,65	-1,19	-0,61	-1,06	-1,13	-0,72	-1,03	-0,5	-1,21	-0,34	-1,16	-1,03	-2,82	-1,02	-0,89	-1,25
(11) V2_D10_643_1	2,83	1,58	1,3	1,19	0,79	1,42	0,5	1,97	1,02	0,65		-0,54	0,03	-0,42	-0,48	-0,07	-0,38	0,14	-0,57	0,3	-0,51	-0,38	-2,17	-0,37	-0,24	-0,6
(12) V2_D10_643_2	3,37	2,12	1,85	1,73	1,33	1,96	1,04	2,51	1,56	1,19	0,54		0,57	0,12	0,06	0,47	0,16	0,69	-0,03	0,84	0,03	0,16	-1,63	0,17	0,3	-0,06
(13) V2_D10_649_1	2,8	1,55	1,27	1,15	0,75	1,38	0,47	1,94	0,98	0,61	-0,03	-0,57		-0,45	-0,52	-0,1	-0,41	0,11	-0,6	0,27	-0,54	-0,41	-2,2	-0,41	-0,27	-0,63
(14) V2_D10_649_2	3,25	2	1,72	1,61	1,2	1,83	0,92	2,39	1,43	1,06	0,42	-0,12	0,45		-0,07	0,35	0,04	0,56	-0,15	0,72	-0,09	0,04	-1,75	0,04	0,18	-0,18
(15) V2_D10_665_1	3,31	2,06	1,79	1,67	1,27	1,9	0,98	2,45	1,5	1,13	0,48	-0,06	0,52	0,07		0,41	0,1	0,63	-0,09	0,79	-0,03	0,1	-1,69	0,11	0,24	-0,12
(16) V2_D10_665_2	2,9	1,65	1,38	1,26	0,86	1,49	0,57	2,04	1,09	0,72	0,07	-0,47	0,1	-0,35	-0,41		-0,31	0,22	-0,5	0,37	-0,44	-0,31	-2,1	-0,3	-0,17	-0,53
(17) C_D10_2411_1	3,21	1,96	1,68	1,57	1,17	1,8	0,88	2,35	1,4	1,03	0,38	-0,16	0,41	-0,04	-0,1	0,31		0,52	-0,19	0,68	-0,13	0	-1,79	0,01	0,14	-0,22
(18) C_D10_2411_2	2,68	1,44	1,16	1,04	0,64	1,27	0,36	1,83	0,87	0,5	-0,14	-0,69	-0,11	-0,56	-0,63	-0,22	-0,52		-0,71	0,16	-0,66	-0,53	-2,32	-0,52	-0,38	-0,74
(19) C_D10_2418_1	3,4	2,15	1,87	1,76	1,35	1,98	1,07	2,54	1,58	1,21	0,57	0,03	0,6	0,15	0,09	0,5	0,19	0,71		0,87	0,06	0,19	-1,6	0,2	0,33	-0,03
(20) C_D10_2418_2	2,53	1,28	1	0,88	0,48	1,11	0,2	1,67	0,71	0,34	-0,3	-0,84	-0,27	-0,72	-0,79	-0,37	-0,68	-0,16	-0,87		-0,81	-0,68	-2,47	-0,68	-0,54	-0,9
(21) C_D10_2421_1	3,34	2,09	1,82	1,7	1,3	1,93	1,01	2,48	1,53	1,16	0,51	-0,03	0,54	0,09	0,03	0,44	0,13	0,66	-0,06	0,81		0,13	-1,66	0,14	0,27	-0,09
(22) C_D10_2421_2	3,21	1,96	1,69	1,57	1,17	1,8	0,88	2,35	1,4	1,03	0,38	-0,16	0,41	-0,04	-0,1	0,31	0	0,53	-0,19	0,68	-0,13		-1,79	0,01	0,14	-0,22
(23) C_D10_2427_1	5	3,75	3,48	3,36	2,96	3,59	2,67	4,14	3,19	2,82	2,17	1,63	2,2	1,75	1,69	2,1	1,79	2,32	1,6	2,47	1,66	1,79		1,8	1,93	1,57
(24) C_D10_2427_2	3,2	1,95	1,68	1,56	1,16	1,79	0,87	2,34	1,39	1,02	0,37	-0,17	0,41	-0,04	-0,11	0,3	-0,01	0,52	-0,2	0,68	-0,14	-0,01	-1,8		0,13	-0,23
(25) C_D10_2432_1	3,07	1,82	1,54	1,43	1,02	1,65	0,74	2,21	1,25	0,89	0,24	-0,3	0,27	-0,18	-0,24	0,17	-0,14	0,38	-0,33	0,54	-0,27	-0,14	-1,93	-0,13		-0,36
(26) C_D10_2432_2	3,43	2,18	1,91	1,79	1,39	2,02	1,1	2,57	1,62	1,25	0,6	0,06	0,63	0,18	0,12	0,53	0,22	0,74	0,03	0,9	0,09	0,22	-1,57	0,23	0,36	

Average V1 vs C	-1,92475
Average V1 vs V2	-1,424375
Average V2 vs C	-0,500625
Average C vs V1	1,925
Average V2 vs V1	1,424
Average C vs V2	0,501

As an example, in Figure 3-8 there is a graphical representation of biochemical kinetics profile comparing the two colonies isolated at 10 dpi from the animal from the control group (identification 2432-2, discontinuous lines) and the animal from the V1 group (identification 561-2, continuous lines) highlighted in the Table 3-5. The colony isolated from the control group had faster kinetics than the strain isolated from the group V1 in almost all the examined metabolic pathways.

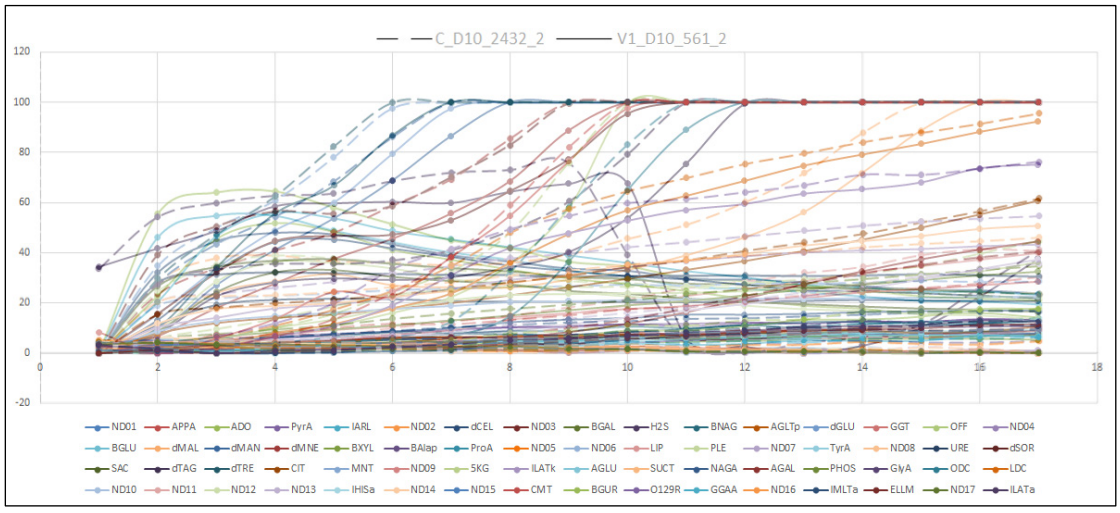

Figure 3-8. Comparative biochemical kinetics profile of metabolic pathways of two colonies isolated at 10 dpi from animal 2342-2 from the control group (discontinuous line) and animal 561-2 from the V1 group (continuous line). The x axis shows the time in hours and the y axis shows the percentage of transmittance extinction.

The dendrogram created with the distances between isolated colonies at 10 dpi is shown in Figure 3-9. The dendrograms created at 3, 5, 7 and 14 dpi are included in the APPENDIX section at the end of the present thesis.

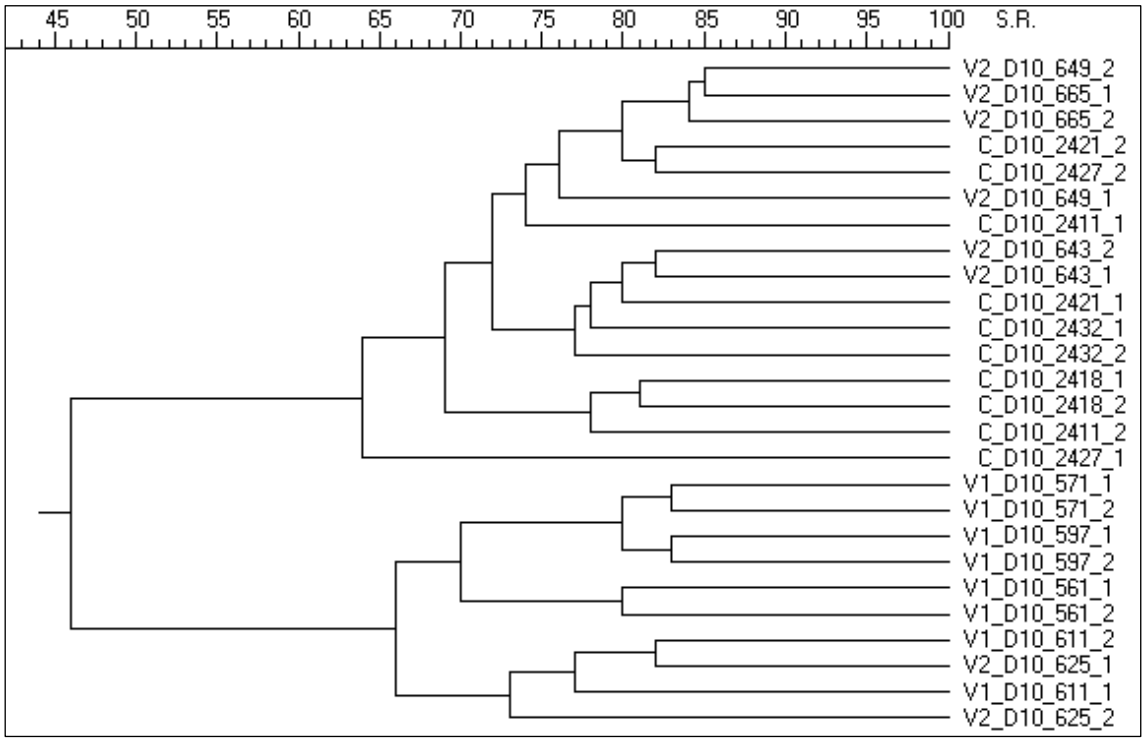

Figure 3-9. Dendrogram obtained with the comparative analysis of biochemical kinetics of different pairs of strains isolated at 10 dpi.

3.4 DISCUSSION

In the present study, we determined the efficacy of a live attenuated vaccine delivered by two different routes in day-old chicks after challenge at 14 days of age, and we studied the humoral and cellular immune response. We also determined the capacity of vaccinated and non-vaccinated chicks to modify the metabolism of the challenge bacteria.

Overall, only V1 group vaccinated by oral gavage presented some protection degree after infection with *Salmonella* field strain. The low results of vaccine excretion in group V2 and the absence of protection indicate that this group did not receive the proper dose of vaccine. The vaccination via spray requires that an adequate amount of the vaccine should be distributed in drops on the chicks and be available long enough for the animals to ingest the appropriate dose, and also that environmental conditions such as temperature or photointensity be high enough (up to 35 °C and 115.5 lumens per square foot after spray are optimal, respectively) (Caldwell et al., 2001a). Environmental conditions could have influenced negatively in the ingestion of the vaccine, since the

animals were transported from the hatching farm to the research farm the same day of vaccination. Due to this transport, animals could have suffered stress or could be at a low uncomfortable temperature, which would modify preening behaviour and therefore reduce the associated ingestion of vaccine (Caldwell et al., 2001a). Additionally, there are stabilizer products available on the market that confer colour and viscosity to the drops and could have improved the vaccine intake by chicks, but were not used in this study (Caldwell et al., 2001b). Despite the parameters to consider when vaccinating via spray, some studies have found that this route of administration is equally effective or can even improve the results of other routes such as drinking water. In a study where the efficacy of a live attenuated *Salmonella* Typhimurium vaccine was compared between field conditions (spray route) and laboratory conditions (oral route), no significant differences were found between both methods considering qualitative or quantitative determination of field strain of *Salmonella* Enteritidis in the caecal samples (Parker et al., 2011). By contrast, another study determined that the administration of an attenuated *Salmonella* Enteritidis strain by coarse spray at day-old to broiler chicks resulted in higher reduction of caecal colonisation and faecal shedding of a wild-type *Salmonella* Enteritidis strain administered at 2 days of age compared with administration of the attenuated strain by drinking water (De Cort et al., 2015). Additionally, when comparing the efficacy of a *Salmonella* Enteritidis vaccine administered via spray and drinking water route against homologous intravenous challenge, the spray vaccination induced stronger systemic immunity (reduction of liver colonisation), although lower protection against caecum colonisation compared with oral vaccination (Varmuzova et al., 2016).

As it has been mentioned, oral vaccination conferred to group V1 partial protection to *Salmonella* Typhimurium and reduced liver and spleen colonisation after challenge with a field strain. However, there was no qualitative reduction of colonization of the caecum, although there was a reduction in the cloacal excretion, which indicates a probable reduction of the bacterial counts in the intestine. The existence of partial protection at early ages has been determined in other vaccine tests, where chickens vaccinated at day-old and at 5 weeks of age with a *Salmonella* Typhimurium live attenuated strain and challenged at 10 weeks of age with a *Salmonella* Enteritidis field strain, had lower bacterial counts in the caecum but were not protected against colonisation of liver and spleen (Parker et al., 2011). Also in another study testing the efficacy of a live attenuated *Salmonella* Enteritidis vaccine administered at day 1 of life, after homologous challenge with a field strain at day 3 of life, vaccination only reduced systemic invasion of the liver

but did not reduce bacterial counts in the caecum compared with non-vaccinated animals (Braukmann et al., 2016). In other study where one week old chicks were inoculated with a *Salmonella* Typhimurium field strain and reinfected at 9 weeks old, the partial protection was also evident: after reinfection, liver and spleen were totally protected against *Salmonella* infection but the infection of the caecum was not reduced (Withanage et al., 2005). From this last study, we can conclude that even with primoinfection with a field strain (considered the gold standard in *Salmonella* protection) the degree of protection is not complete (Methner et al., 2011a). The partial protection observed in our study could be improved by boosting the animals with additional doses of the vaccine. The number and time of booster immunisations should be determined in other studies (Methner, 2018).

However, the efficacy results of STUDY I are not easily comparable with other studies due to the different experimental design, since most studies in young chicks perform the challenge few days after first vaccination (one or two days), and not at 14 days after vaccination as stated by the European Pharmacopoeia. The immune status of animals as well as the protective mechanisms are slightly different at these time points because immune populations of chicks develop the first weeks of life with stronger T cell responses (Beal et al., 2004b, 2005).

The humoral immune response was detected first in serum of group V1 at 28 days after first vaccination. Group V2, however, also developed a mild humoral response compared with non-vaccinated control group which indicates that they could have received an undetermined low dose of vaccine, although not enough to protect them from the inoculated challenge dose. The dynamics of antibody production against *Salmonella* in chickens depends on the age of infection. When chicks are infected at day-old, IgY production is delayed compared with infections at older ages, at 6 weeks of age or more (Beal and Smith, 2007). Initially only IgM are produced at approximately 2 weeks after infection, and class switching to IgY and IgA occurs later, as we can see from our study. In a study with SPF chicks infected with *Salmonella* Hadar at day-old, IgY started to be detected at 4 weeks after inoculation (Desmidt et al., 1998a). These findings explain that adaptive immune response takes time to develop, and in between, protection is conferred by innate immunity or competitive exclusion effect.

Antibody levels in the gut may reflect more accurately the immune response at mucosal level than those antibodies from the serum. From the results observed in serum and bile

ELISA, we can also conclude that the humoral immune response is triggered one week earlier at the intestinal level, because this is the first site for interaction of the pathogen. By contrast, other authors have found that in chickens of 4 lines with different susceptibility to *Salmonella* infected at day-old with *Salmonella* Enteritidis, IgY in serum (detected 2-3 weeks after infection) increased one week before than IgA in intestinal secretions (3-4 weeks after infection) (Berthelot-Hérault et al., 2003). This difference could be explained because in our study, animals received vaccination and second infection with a *Salmonella* Typhimurium field strain, and this booster could stimulate an earlier mucosal immune response, since it is the first place of *Salmonella* invasion and replication (Foley et al., 2013). In addition, in our study IgA antibodies were detected in bile and may be more concentrated than in intestinal secretions (Rose et al., 1981).

The correlation of humoral immune response and resistance to *Salmonella* has not always been proven, even though infection with *Salmonella* in chickens generates high level of antibodies (Beal and Smith, 2007). As seen in the introduction of this thesis, it has been demonstrated that B cells are not essential for clearance of either primary or secondary *Salmonella* Typhimurium infection (Beal et al., 2006). Nevertheless, it is not surprising that in some studies there is an association between high levels of IgY in serum or IgA in the intestine and higher counts of *Salmonella* in the caecum (Berthelot-Hérault et al., 2003). This could be explained by the fact that susceptible chickens to *Salmonella* contain high numbers of bacteria to be presented to the immune system, and consequently higher antibody titres (Beal and Smith, 2007). This situation was found in our study at 14 dpi, where a rise in IgA antibodies together with high *Salmonella* infection in the caecum were found in all the groups, although in group V1 the excretion rate of *Salmonella* was lower, indicating that other mechanisms are responsible for conferring protection.

To obtain information about the mechanisms of protection in vaccinated animals, we investigated the cellular immune response in the ileum by flow cytometry.

The kinetics of the infiltration of immune cells in the intestine was previously determined to start very early after contact with *Salmonella.* Vaccination of day-old chicks with a live attenuated strain of *Salmonella* Enteritidis and challenge 1 day later caused a rapid immune response: macrophages were the fastest and started to infiltrate at 16 h post vaccination (peaked at 2 dpi until 6 dpi), T cells infiltrated at 16 h post vaccination (peaked at 2-4 dpi until 8-10 dpi), and B cells infiltrated at 20 h post

vaccination (peaked at 5 dpi and remained constant); non-vaccinated but challenged animals had the same cellular response but starting from the time of challenge inoculation (Van Immerseel et al., 2002a). This infiltration indicates that there is an early cellular immune response after infection with *Salmonella*, and its contribution to protection will be explained below based in our results.

Macrophages contribute to the early innate immune protection against *Salmonella* infection due to their phagocytic, bactericidal, and antigen-presenting functions (Vazquez-Torres and Fang, 2001; Kaspers and Kaiser, 2013; Braukmann et al., 2015). The influx of macrophages in the intestinal mucosa after day-old vaccination and early challenge seems to contribute to a low number of *Salmonella* organisms in the liver, spleen, and caecum (Van Immerseel et al., 2002a). In other studies, higher invasiveness of a *Salmonella* strain has been linked to higher macrophages influx and higher salmonella phagocytosis, and this should mean higher protection against subsequent infections (Berndt et al., 2007). Contrary to these results, in our study the percentage of macrophages was lower in V1 group compared with control and V2 (significantly lower at 14 dpi). This could be explained by the fact that V1 group had the peak of macrophages before the sampling point at 7 dpi. However, this should be confirmed in future studies analysing cell populations both before challenge and at early stages after challenge (1 or 2 days after inoculation).

Cellular immune response against *Salmonella* is thought to be driven by CD8+ T cells (Berndt and Methner, 2001). However, all T cells subpopulations are involved in protection in some way (Holt et al., 2010). The CD3 molecules mediate recognition of antigens by all T cells (Smith and Göbel, 2013). Additional surface molecules are used to subdivide T cells into CD4+ T helper population, which recognises antigens trough MHC class II molecules; and CD8+ T cytotoxic population, which recognises antigens through MHC class I molecules (Smith and Göbel, 2013). Other T cell population that has been found in chicken and other species as humans or swine is CD4+CD8+ double positive T cells, which seem to have the function of a CD4+ T helper cell (Zuckermann, 1999). The increase of both CD4+ and CD8+ T cells have been largely reported after infection or immunisation of very young chicks with *Salmonella* (Berndt and Methner, 2001; Bai et al., 2014). Other groups have reported the increase of only CD8+ T cells (Berndt et al., 2007; Schokker et al., 2010), some of them correlated with protection against *Salmonella* (Penha Filho et al., 2012). In our study, there were no differences

between groups in the percentage of CD3+ T cells. Interestingly, T cells subpopulations of CD4+, CD8+ and double positives were lower in the V1 group (compared to control and V2 groups) which was surprisingly the group that showed protection against internal organ colonisation and lower *Salmonella* excretion rates after challenge. This situation could be explained similarly to macrophages population. As mentioned before, T cells infiltration peak very early after *Salmonella* infection in intestine and caecum (Van Immerseel et al., 2002b). The dynamics of cellular infiltration in our study could be due to an earlier peak of T cells in protected group that happened before the first time point of euthanise (7 dpi). This should be confirmed with sampling points earlier after challenge inoculation.

B cells have been described as a more stable population after vaccination or infection with *Salmonella*. The influx of B cells in the intestine of young chicks after *Salmonella* infection occurs after T cells (at 2 dpi) and is maintained over time (Van Immerseel et al., 2002b). B cell population is higher in chicks stimulated by *Salmonella* than in non-vaccinated control groups (Berndt and Methner, 2004). In our study, similarly to IgA secretion, B cells increased over time in all the groups and there was no difference between them, indicating a strong humoral stimulation at intestinal level that was not related with protection.

MHC II molecules can be expressed by macrophages, dendritic cells and B cells; and are recognised by CD4+, including helper and regulatory T cells (Kaufman, 2013). In our study, a lower percentage of cells expressing MHC II was detected in the V1 group during all the study, and this difference was statistically significant at 7 dpi. This could represent a lower stimulation of antigen-presenting cells by a lower count of *Salmonella* bacteria in the intestine.

An important point to consider after analysing flow cytometry results is that the technique for isolating the intestinal cells can affect the results of cellular populations (Lillehoj and Chung, 1992). Depending on whether only mechanical or enzyme digestion methods are used and subsequent separation of leukocytes from the other cells, the percentages can vary markedly (Goodyear et al., 2014; Couter and Surana, 2016).

Cytokines are signalling molecules that mediate intercellular communication in response to different stimuli. Cytokines are functionally redundant: different cytokines share similar functions and cells may express more than one receptor for a given

cytokine. To generalize the effect of a particular cytokine is a difficult task (Arango Duque and Descoteaux, 2014).

The IFNγ is a proinflammatory cytokine with many different functions, between them macrophage activation, induction of MHC II expression and increase of antigen processing (Wigley and Kaiser, 2005). This cytokine is upregulated in many studies after *Salmonella* infection (Berndt et al., 2007; Matulova et al., 2013). Also, the up-regulation of IFNγ after first vaccination has been linked to protection against secondary *Salmonella* infection (Withanage et al., 2005). The TNFα is other proinflammatory cytokine that can be produced by macrophages, T cells and NK cells and stimulates acute inflammatory response, endothelial activation and attracts other cells (Wigley and Kaiser, 2005; Arango Duque and Descoteaux, 2014). The expression of TNFα after *Salmonella* infection could represent leucocytes recruitment (Rychlik et al., 2014). Finally, IL-10 is an anti-inflammatory cytokine that can be produced by activated macrophages, B and T cells, and its main function is suppression of macrophages activation, reduction of MHC II expression, inhibition of IFNγ production by Th1 cells, activation of B cells, and induction of antibodies secretion (Rousset et al., 1992; Chadban et al., 1998; Arango Duque and Descoteaux, 2014). In *Salmonella* infection studies, IL-10 has been downregulated in vaccinated animals or even non detected, which could explain a shift in vaccinated animals to inflammatory and protective responses (Withanage et al., 2005; Penha Filho et al., 2012).

As an overview, in the present study the level of proinflammatory cytokines in vaccinated groups was upregulated at 7 dpi (IFNγ in V1 and TNFα in V2) and downregulated at 14 dpi compared with control group. By contrast, anti-inflammatory cytokine IL-10 was upregulated at 14 dpi in the vaccinated groups V1 and V2. This situation reflects a change from the inflammatory status towards the resolution of infection. The cytokine expression at 7 dpi reflects that immune system is trying to resolve the *Salmonella* infection by activation and recruitment of immune cells, although this situation is not reflected in the flow cytometry results, maybe due to a lag in cell and gene response or a low correlation between gene transcript and protein levels (Edfors et al., 2016).

The expression of three surface proteins involved in *Salmonella* immune response has also been determined. TLR4 and TLR5 receptors recognise *Salmonella* in the intestine early after infection (St. Paul et al., 2013). When these receptors bind their respective

ligands, proinflammatory cytokines are expressed (Rychlik et al., 2014). In our study, TLR4 was upregulated in V1 at 7 dpi and in V2 at 14 dpi. TLR5 was only upregulated at 7 dpi in group V2, and downregulated at 14 dpi, possibly due to a decrease in the binding of *Salmonella* to receptor cells. There is not a clear relation between up-regulation of TLR4 and TLR5 receptors and proinflammatory cytokines, which makes difficult to relate these markers to the immune response against *Salmonella.* Finally, the expression of MHC II molecule was upregulated in vaccinated groups in all the study and increased at 14 dpi, contrary to the results in flow cytometry, situation that questions whether the antigen-presentation increased in vaccinated groups or not.

The profiles of kinetics in metabolic pathways were different in colonies isolated from vaccinated animals compared with non-vaccinated controls. The amount of time that an isolated colony requires to transform a metabolic substrate may influence its virulence. If nutritional factors do not vary and environmental conditions are constant, genetic factors can be related with these changes in kinetics of metabolic pathways (de la Torre et al., 2005).

In Figure 3-9 we can observe that all the strains isolated from V1 Group are included in a singular cluster with only the two strains isolated from the animal 625 from V2 group. Some differences were observed between the biochemical kinetics of strains isolated from V2 and C groups but almost all of them are included in the upper cluster. These results indicate that at 10 dpi, higher changes on the fitness of the *Salmonella* Typhimurium strains isolated from animals from V1 group were induced when compared with the *Salmonella* Typhimurium strains isolated from V2 and control groups. Overall, the strains isolated from vaccinated groups were different from the strains isolated from the control group (see the APPENDIX section).

Additionally, it is interesting to remark that the 5 animals chosen from group C were positive during all the sampling points. On the other hand, from the 5 animals chosen from group V1, some of the animals were negative to the excretion of the challenge strains and only six colonies from three animals were isolated on day 7 after challenge, and eight colonies from four animals were isolated during the last two days of sampling. The 5 animals in group V2 were positive during all the study except for day 10, when only 8 colonies were isolated from four animals. We can suggest than vaccination against *Salmonella* Typhimurium induced changes on different biochemical pathways of bacteria that can influence the survival inside the host.

In our experimental conditions, the administration of the vaccine by spray route was not successful because animals did not receive the correct amount of vaccine, as reflected by the reduction in the excretion level of vaccine strain after vaccination, and the lack of protection after challenge. More studies are needed with an effective spray vaccination in order to compare the efficacy conferred by the different routes of administration of the vaccine.

Oral vaccination with a live attenuated *Salmonella* Typhimurium in day-old chicks protected them from internal organ colonisation when challenged at 14 days of age. However, the protection was not correlated by a humoral immune response or an evident cellular immune response. Despite this, the data presented here characterise the immune response to the *Salmonella* infection after first vaccine dose and can be of utility for further studies in vaccine efficacy.

4. STUDY II:

Vaccine effect in the mucosal immune response against *Salmonella* Typhimurium in laying hens with two vaccination protocols

4.1 INTRODUCTION

Salmonellosis is the second most common food-born zoonosis in the European Union. There were 91,662 human cases and 156 deaths of salmonellosis in 2017. Since the implementation of the National Control Programmes (NCP) from 2007 to 2010, the number of human salmonellosis cases has decreased, but in recent years the trend has stabilized. Non-host specific *Salmonella* serovars are the responsible for human food-born infections. The main source *Salmonella* infections are eggs and poultry meat, that is why vaccination of flocks against most common *Salmonella* serovars was one of the measures implemented in the NCP (EFSA and ECDC, 2018). Vaccination can reduce *Salmonella* prevalence in the production stage and therefore decrease the risk of transmission of bacteria to the food-chain (Beal and Smith, 2007).

In order to produce effective vaccines, it is necessary to understand the mechanisms through animals are protected. Commercially available live attenuated and inactivated vaccines have been tested achieving different protection rates, mainly related to the different immune responses elicited (Hahn, 2000; Woodward et al., 2002; Gantois et al., 2006; Atterbury et al., 2009; Filho et al., 2009; Desloges et al., 2010; Groves et al., 2016; Theuß et al., 2018; Huberman et al., 2019). Live vaccines can stimulate both cellular and humoral responses, while inactivated ones fail to induce a strong cellular response even inducing high antibody titres and showing some partial protection (Carvajal et al., 2008; Penha Filho et al., 2012).

Previous studies have shown that humoral and cellular immune responses play different roles in the protection of chickens against *Salmonella* (Van Immerseel et al., 2005). Humoral response seems to be less essential and is limited only at intestinal level, where IgA can have a role in mucosal protection (Desmidt et al., 1998b; Beal et al., 2006; Beal and Smith, 2007). In contrast, recent studies consider that cellular response is pivotal for *Salmonella* clearance (Penha Filho et al., 2012; Wigley, 2014), specifically Th1-mediated response rather than Th2 response (Wigley, 2014).

Although recent studies are focused on the analysis of the cellular immune response, they provide little information about this response at intestinal level in adult laying hens. Since the intestinal barrier is the first defence of chickens against *Salmonella* invasion (Barrow and Methner, 2013), the recruitment of immune cells in this organ can be a first indicator of protection in immunized or non-immunized chickens. Most studies focus

on cell populations in blood (Berndt and Methner, 2001), but few studies have determined the distribution of immune cells in internal organs with associated lymphoid tissue using flow cytometry (Holt et al., 2010). Analysing intestinal fragments where Peyer's patches are present can reflect what is happening in immunological sites that are key for *Salmonella* infection like the Caecal Tonsils (Holt et al., 2010).

In order to understand the protective mechanisms of *Salmonella* vaccine, we investigated the mucosal immune response to field strains of *Salmonella* Typhimurium in vaccinated (with 2 or 3 doses) and non-vaccinated laying hens and compared humoral and cellular response at intestinal level.

4.2 MATERIALS AND METHODS

4.2.1 Animals

Newly hatched day-old Tetra SL LL brown female chicks were obtained from a local hatchery (Granja Gibert, Tarragona, Spain). During the experiment animals were housed in pens with wood shavings. Groups were housed separately. Commercial feed and drinking water were provided ad libitum. This study was approved by the Animal Experimentation Ethics Committee of Clinobs SL and by the Government of the Generalitat de Catalunya (Departament de Medi Ambient, procedure No. 10149).

4.2.2 Study design

The chickens were divided into 4 groups: V1, V2, C1 and C2 to perform 2 experiments. Groups C1 and C2 remained unvaccinated as controls. Group V1 received two vaccine doses at day 1 and at week 7 of age; and group V2 received three vaccine doses: the two initial doses received by Group V1 and an additional one at week 16 of age. Experiment 1 was performed at week 16 of age: groups V1 and C1 were orally infected with 8.65 x 10^8 cfu *Salmonella* Typhimurium (ST) field strains GN-3326 and GN-3760 in 5 ml of inoculum. Experiment 2 was performed at week 35 of age: groups V2 and C2 were orally infected with 6.70 x 10^8 cfu *Salmonella* Typhimurium field strains GN-3326 and GN-3760 in 5 ml of inoculum. The vaccination was administered by drinking water and the challenge was administered by oral gavage using a plastic cannula. The study design is summarized in Table 4-1.

Meconium samples were collected from day-old chicks to confirm *Salmonella*-free status of the experimental groups. Before the experimental infection, serum samples from all

animals were analysed for the presence of *Salmonella* antibodies; and cloacal swabs were sampled from all the animals to detect field strains of *Salmonella* spp. After the infection, cloacal swabs were sampled at 3, 5, 7, 10, 14, and 21 dpi to detect challenge strains. At 7, 14 and 21 dpi approximately 10 animals per group were euthanised by cervical dislocation and samples of blood, bile, liver, spleen, small intestine, caecum, caecal tonsils, ovary and oviduct (only in Experiment 2) were collected. Samples of liver, spleen, caecum, ovary and oviduct were qualitatively examined for detection of challenge strain by culture. Blood and bile were analysed for *Salmonella* antibodies. The small intestine was collected for cells isolation and RNA extraction. Caecal tonsils were collected to perform immunohistofluorescence.

Table 4-1. Study design.

Experiment	Group	Vaccine	Challenge	Euthanasia[a]
1	C1 (n = 34)	No	*S.* Typhimurium (week 16) 8.65 x 10^8 cfu in 5 ml	7 dpi (n=10) 14 dpi (n=10) 21 dpi (n=10)
	V1 (n = 35)	*S.* Typhimurium (day 1 and week 7) 1.83 x 10^8 cfu per dose		7 dpi (n=10) 14 dpi (n=10) 21 dpi (n=10)
2	C2 (n = 27)	No	*S.* Typhimurium (week 35) 6.70 x 10^8 cfu in 5 ml	7 dpi (n=9) 14 dpi (n=9) 21 dpi (n=9)
	V2 (n = 30)	*S.* Typhimurium (day 1, week 7 and week 16) 1.83 x 10^8 cfu per dose		7 dpi (n=10) 14 dpi (n=10) 21 dpi (n=10)

[a]dpi: days post-infection.

4.2.3 Bacterial strains and culture

A live attenuated *Salmonella* Typhimurium spontaneous streptomycin and rifampicin-resistant strain was used as vaccine. Freeze-dried vial was reconstituted in tap water and adjusted to a concentration of 1.83 x 10^8 cfu per dose. Two field strains isolated from adult hens (liver and intestine (GN-3326) and ovary (GN-3760)) in the Laboratory of Animal Health of Barcelona were used as challenge inoculum. The strains used for infection were plated on Columbia agar + 5% sheep blood (bioMérieux, Marcy-l'Etoile, France) at 37 °C. After overnight incubation, the bacteria were suspended in Buffered Peptone Water (BPW, VWR, Leuven, Belgium) and adjusted to the desired titre.

Cloacal swabs and internal organs (liver, spleen, caecum, ovary and oviduct) were qualitatively examined for the detection of *Salmonella* spp. by pre-enrichment in BPW

(37 ± 1 °C for 18 h) followed by cultivation on modified semi-solid Rappaport-Vassiliadis agar (MSRV) (Merck, Darmstadt, Germany) (41.5 ± 1 °C for 48 h) and finally plating on xylose lysine tergitol 4 agar (XLT4) (Merck, Darmstadt, Germany) (37 ± 1 °C for 24 h) where the presence of characteristic colonies resulted in the detection of *Salmonella*.

4.2.4 Enzyme-linked immunosorbent assay (ELISA)

Commercial indirect ELISA kit (*Flocktype* Salmonella Ab QIAGEN, Leipzig, Germany) was used for the detection of antibodies against *Salmonella* Typhimurium in serum and bile. Serum was analysed for the detection of IgY according to the manufacturer's protocol. Bile was analysed for the detection of IgA according to the manufacturer's protocol but changing the dilution of the sample and the secondary antibody solution. Bile samples were diluted 1:20. The secondary antibody solution was prepared with HRP conjugated goat anti-chicken IgA (0.02 µg/ml) (Bethyl, Montgomery, TX, USA) in PBS Tween-20 (0.01%) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 0.5% Fetal Bovine Serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA).

The results of ELISA in serum are expressed as S/P (sample-to-positive) ratio, where S/P:

$$S/P = \frac{(Optical\ Density\ (OD)\ sample - OD\ Negative\ Control)}{(OD\ Positive\ Control - OD\ Negative\ Control)}$$

The threshold determined by the manufacturer is indicated in the graph as additional discontinuous lines (negative: $S/P < 0.2$; doubtful: $0.3 > S/P \geq 0.2$; positive: $S/P \geq 0.3$). The results of ELISA in bile are expressed as OD. OD was measured at 450 nm. Results of each individual animal are represented and error bars express mean ± standard deviation (SD).

4.2.5 Flow cytometry

An intestinal segment of the first 5 cm after Meckel's diverticulum was collected where almost one proximal Peyer's patches is located (Holt et al., 2010). The intestine was flushed first with sterile PBS and then with complete RPMI (CRPMI) (Lonza, MD, USA) supplemented with 5 % FBS (Sigma-Aldrich, St. Louis, MO, USA), 10 U/ml penicillin (Gibco, Life Tecnologies, NY, USA), 10 µg/ml streptomycin (Gibco, Life Tecnologies, NY, USA), and 2 mM L-Glutamine (Gibco, Life Tecnologies, NY, USA). Then intestinal segments were placed in a 50 ml tube with 20 ml of CRPMI on ice.

Intestinal cells isolation protocol was adapted from a previously published article with some modifications (Couter and Surana, 2016). Briefly, fat was removed from intestine and this fragment was cut in a tube with 4 ml of RPMI 1640 containing 1% FBS and 1 mg/ml Type I Collagenase (Sigma-Aldrich, St. Louis, MO, USA). This was incubated for 45 min at 37 °C and 200 rpm in an orbital shaker, vortexing briefly every 15 min. Digested tissue was filtered through a 70 µm cell strainer (Falcon, Corning, NY, USA) and filter was rinsed with 15 ml of RPMI containing 5% FBS. The filtered solution was centrifuged for 10 min at 500 x g at 4 °C. The supernatant was discarded and pellet was resuspended in 15 ml of RPMI containing 5% FBS. The cell suspension was filtered again through a 40 µm cell strainer (Falcon, Corning, NY, USA) and centrifuged for 10 min at 500 x g at 4 °C. The supernatant was discarded and pellet containing isolated cells was resuspended in 20 ml CRPMI containing 0.1 mM non-essential amino acids (Gibco, Life Tecnologies, NY, USA) and 240 U/ml Nystatin (Sigma-Aldrich, St. Louis, MO, USA). Cells were stored over-night at 4 °C with the caps loosely fitted for aeration (approximately 12 hours).

Before staining, viable cell counts were made using the trypan blue exclusion method and 1 x 10^6 cells were aliquoted into each well of a V-bottom 96-well plate. Cells were stained with the antibodies detailed in Table 4-2. All antibody reagents were purchased from Southern Biotech (Birmingham, AL, USA). Cells were stained at 4 °C in the dark for 60 min, then washed twice with PBS, and finally resuspended in PBS containing 5% FBS. Flow cytometry was performed using a MACSQuant Analyzer 10 (Miltenyi biotec, Bergisch Gladbach, Germany). Five parameters, forward scatter (FSC), side scatter (SSC), FITC (filter 525/50 nm), PE (filter 585/40 nm), and Allophycocyanin (APC) (filter 655-730 nm) were collected and evaluated using MACSQuantify software version 2.8 (Miltenyi biotec, Bergisch Gladbach, Germany). Dead cells were removed from the analysis by sequential gating: first by doublet discrimination performed by plotting forward scatter height (FSC-H) vs forward scatter area (FSC-A) and second by viability dye using propidium iodide staining (5 µg/10^6 cells) (Biolegend, CA, USA). Isotype control was used to determine the level of non-specific binding (Goat anti-mouse IgG1 AF647, 0.2 µg/10^6 cells, (Invitrogen, IL, USA)). Results of cell populations are presented as percentage.

Table 4-2. Monoclonal antibodies used for the detection of avian antigens in Flow Cytometry.

Abbreviation	Specificity	Clone	Isotype	Conjugation[a]	Dilution
Mφ	Monocyte/Macrophage	KUL01	Mouse (BALB/c) IgG1κ	FITC	0.5 µg/10^6 cells
MHC II	MHC Class II β-chain	2G11	Mouse (BALB/c) IgG1κ	AF488	0.25 µg/10^6 cells
B cell	Chicken Bu-1a, mainly bursal cells	21-1A4	Mouse (BALB/c) IgG1κ	FITC	1 µg/10^6 cells
CD3+	CD3 T lymphocytes	CT-3	Mouse (BALB/c) IgG1κ	AF647	0.25 µg/10^6 cells
CD4+	CD4 T helper lymphocytes	CT-4	Mouse (BALB/c) IgG1κ	PE	0.05 µg/10^6 cells
CD8+	CD8α T cytotoxic lymphocytes	CT-8	Mouse (BALB/c) IgG1κ	AF647	0.25 µg/10^6 cells

[a]FITC: fluorescein; AF: Alexa Fluor; PE: R-phycoerythrin.

4.2.6 Reverse transcriptase quantitative real-time PCR (RT-qPCR)

The relative gene expression of cytokines and cellular surface immune-relevant proteins at ileal mucosa was determined with RT-qPCR. Ileal mucosal was collected in RNAlater and frozen at ¬80°C. RNA was extracted from 15-20 mg of tissue with RNeasy Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol and treated with RNase-free DNase set (QIAGEN, Hilden, Germany). Quality and quantity of RNAs were determined using BioDrop µLITE spectrophotometer (Biodrop, Cambridge, UK). The mRNA expression rates of Interferon gamma (IFNγ), Tumor necrosis factor alpha (TNFα), Interleukin 10 (IL10), Toll-like receptors 4 and 5 (TLR4 and TLR5) and major histocompatibility complex class II (MHC II) were determined for every individual chicken using the EXPRESS One-Step SYBR GreenER kit (Qiagen, CA, USA) according to the manufacturer's protocol. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as reference gene. Primers for the IFNγ and TNFα (Carvajal et al., 2008) and IL10 (Fasina et al., 2008) had been described previously. Primer sequences for GAPDH, TLR4, TLR5 and MHC II were designed using National Center for Biotechnology Information database (Sayers et al., 2019) and Oligo explorer 1.5 software (http://www.genelink.com). Primer sequences are described in Table 4-3. Amplification and detection of specific products were performed using 7500 Fast Real-Time PCR

system and 7500 software version 2.3 (Applied Biosystems, CA, USA) with the following conditions: cDNA synthesis at 50 °C for 5 min, followed by initial denaturation at 95°C for 5 s, and 40 cycles of denaturation at 95°C for 3 s and annealing/extension at 60 °C for 30 s. Results are expressed as $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001), where ΔΔCT:

$$\Delta\Delta CT = ((CTtarget - CTgapdh)vaccinated - (CTtarget - CTgapdh)control)$$

Statistics were calculated using ΔΔCT values.

Table 4-3. Primer sequences for RT-qPCR.

Target	**Sequence (5′-3′)**		**Product (bp)**
GAPDH	Forward	GGGTGTCAACCATGAGAAATAT	120 bp
	Reverse	CCCTCCACAATGCCAAAGTT	
IFNγ	Forward	CCCGATGAACGACTTGAGAAT	106 bp
	Reverse	AGACTGGCTCCTTTTCCTTTTG	
TNFα	Forward	GCTGTTCTATGACCGCCCAGTT	140 bp
	Reverse	AACAACCAGCTATGCACCCCA	
IL-10	Forward	CATGCTGCTGGGCCTGAA	94 bp
	Reverse	CGTCTCCTTGATCTGCTTGATG	
TLR4	Forward	TCCCTCACACCCATTCCACG	109 bp
	Reverse	ATGGGGAAGGGGCTGAGGA	
TLR5	Forward	TCACACGGCAATAGTAGCAACA	137 bp
	Reverse	TCACACAGTAAGAGAAGCGAT	
MHC II	Forward	CACTACCTGAACGGCACC	156 bp
	Reverse	AATCTCGGCGTTGCTGTTCC	

4.2.7 Immunohistofluorescence (IHF)

The caecal tonsils (n = 3 chickens/group/time-point) were analysed qualitatively for the localisation of *Salmonella* Typhimurium challenge strain and MHC II positive cells. Briefly, selected caecal tonsils were flushed with PBS, embedded in Tissue-tek Optimal Cutting Temperature compound (OCT) (Sakura Finetek, Alphen aan den Rijn, Netherlands), and frozen in dry ice. Cryostat Leica CM 3050S (Leica Microsystems, Nussloch, Germany) sections of 6 µm were mounted onto Superfrost Plus slides (Menzel-Glässer, Braunschweig, Germany) and let air-dry for 10 minutes at room temperature (RT). Sections were fixed with acetone (−20 °C for 3 min) and methanol

(80%, 4 °C for 5 min), washed 3 times in PBS, and blocked in PBS with 5% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA) and 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) at RT for 1 h. Primary *Salmonella* antibody was incubated ON at 4 °C, followed by incubation with secondary and direct labelled MHC II antibody at RT for 1 h. All antibodies used are shown in Table 4-4. Antibodies were diluted in PBS with 2% BSA (Sigma-Aldrich, St. Louis, MO, USA) and 0.01% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA). All washes were performed in PBS at RT (5 times after primary antibody and 3 times after secondary antibody for 5 min). Blocking and antibody incubations were made in a humid chamber. Control staining for *Salmonella* was performed by excluding primary antibody. Slides were mounted in ProLong Diamond Antifade Mountant with 4′,6-diamidino-2-phenylindole (DAPI) (Life Technologies, Eugene, OR, USA).

Images were captured using a confocal microscope Leica TCS-SP5 (Leica Microsystems, Wetzlar, Germany). Images were later analysed using Fiji ImageJ software (Schindelin et al., 2012).

Table 4-4. Antibodies used for immunohistofluorescence.

Antibody	**Specificity**	**Clone**[a]	**Isotype**	**Conjugation**[a]	**Dilution**	**Source**
Salmonella polyclonal antibody	*S.* Enteritidis, *S.* Typhimurium and *S.* Heidelberg	na	Rabbit IgG	Unconjugated	8 µg/ml	Thermo Fisher Scientific, Rockford, IL, USA
Anti-rabbit IgG secondary antibody	IgG heavy and light chains	na	Goat IgG	AF555	2 µg/ml	Thermo Fisher Scientific, Rockford, IL, USA
MHC II	MHC Class II β-chain	2G11	Mouse (BALB/c) IgG1κ	AF488	5 µg/ml	Southern Biotech, Birmingham, AL, USA

[a]na: not applicable.

4.2.8 Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.01 software for Windows (GraphPad Software, La Jolla, CA, USA). Differences in excretion rates of cloacal swabs and colonisation of internal organs between control and vaccinated groups were determined using Fisher's exact test. Normality of the data from ELISA, flow cytometry,

and RT-qPCR was tested with the D'Agostino & Pearson omnibus test and differences between control and vaccinated groups were determined using Mann-Whitney test. P values ≤ 0.05 were considered significant.

4.3 RESULTS

4.3.1 Bacteriological examination of cloacal swabs and internal organs after challenge

All animals were negative for *Salmonella* spp. excretion in cloacal swabs before infection. After the infection with field strains, the excretion rate of *Salmonella* spp. in cloacal swabs was lower in vaccinated than in control groups almost at all sampling points (Figure 4-1). In Experiment 1, excretion was lower in vaccinated than in control groups, and this difference was significant at 5, 7 and 14 dpi. In Experiment 2, excretion was lower in vaccinated than in control groups except at 14 dpi (excretion rate was slightly higher in vaccinated group), and this difference was significant at 7 dpi.

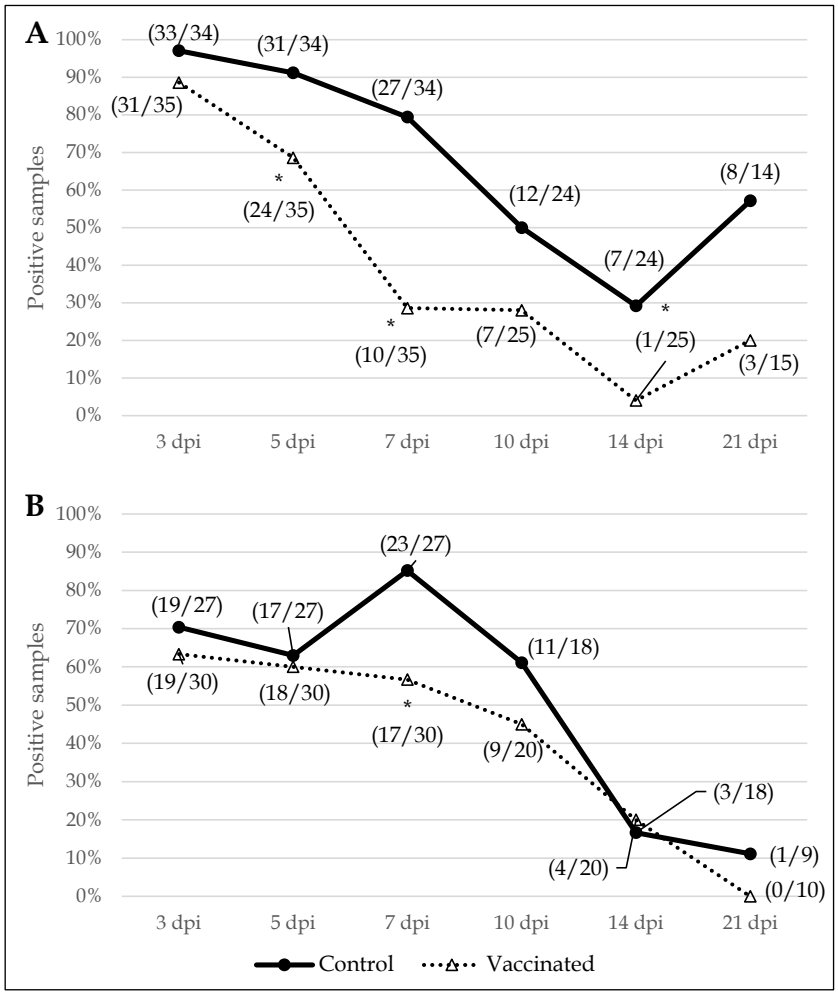

Figure 4-1. Excretion of Salmonella spp. in cloacal swabs after challenge. (A) Experiment 1. (B) Experiment 2. dpi: days post-infection. An asterisk indicates significant differences between control and vaccinated group (p ≤ 0.05). The number of positive animals with respect to the total is shown in brackets (+/n).

Field strain of *Salmonella* Typhimurium was detected in internal organs in a representative number of animals at 7, 14 and 21 days after challenge. In Experiment 1, an early significant reduction in organ colonisation at 7 and 14 dpi in vaccinated group was detected, and later there was a reduction (without statistical significance) in colonisation of caecum at 14 and 21 dpi (Figure 4-2). In Experiment 2, however, there was only a reduction in liver and spleen colonisation at 7 and 14 dpi, which was significant in spleen at 14 dpi. There was a reduction in colonisation of caecum but only at 14 dpi and it was not statistically significant. There was no reduction in the colonization of ovary and oviduct.

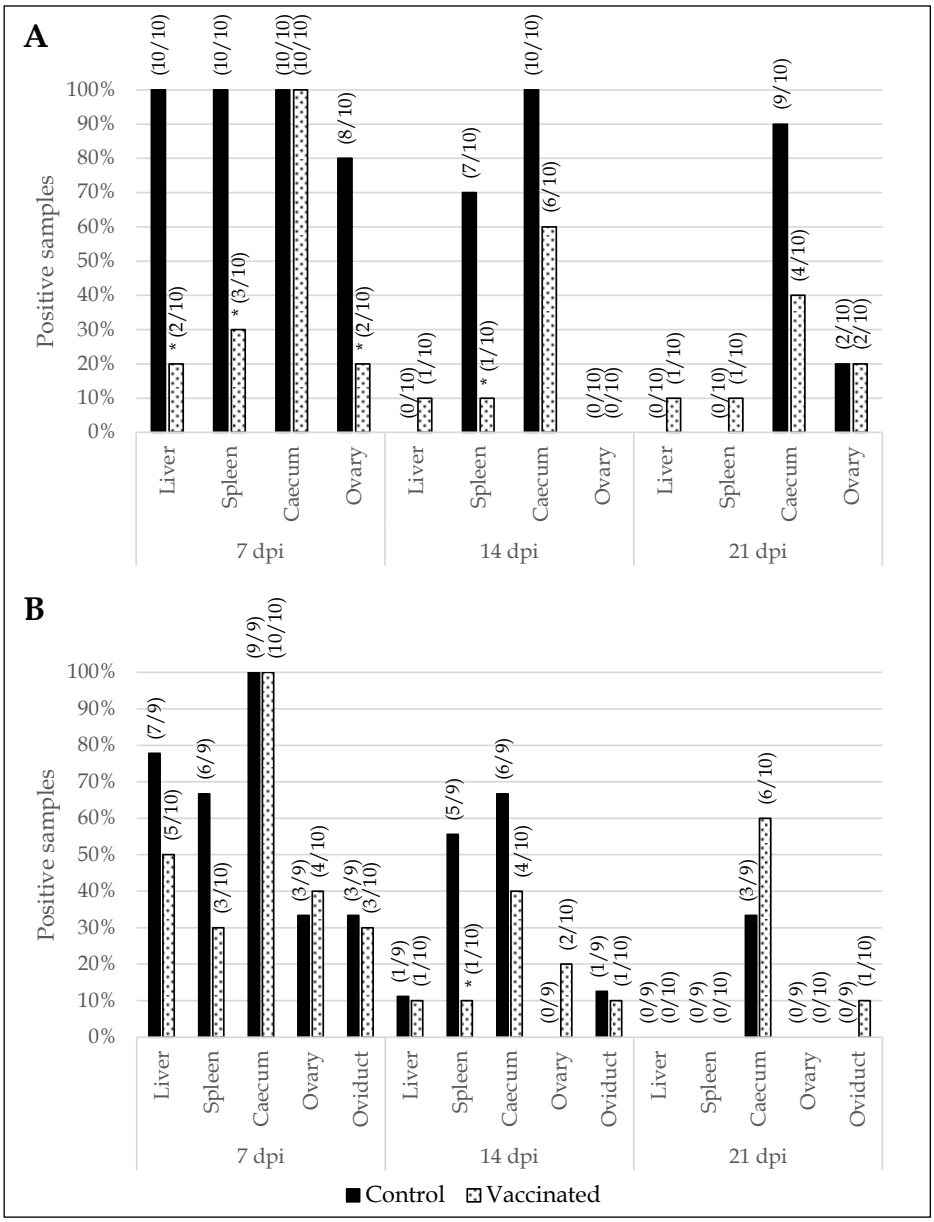

Figure 4-2. Internal organ colonisation of Salmonella spp. after challenge. (A) Experiment 1. (B) Experiment 2. dpi: days post-infection. An asterisk indicates significant differences between control and vaccinated group ($p \leq 0.05$). The number of positive animals with respect to the total is shown in brackets (+/n).

4.3.2 Antibodies detection in serum and bile

Serum was analysed for the presence of IgY antibodies against *Salmonella* Typhimurium.

Serum samples of all animals were analysed before the challenge. Vaccinated animals had higher basal levels of anti-*Salmonella* IgY before the challenge in Experiments 1 and 2 (Figure 4-3) compared to the control group, where all animals were negative.

Infection with field strains of *Salmonella* Typhimurium induced a humoral response in both vaccinated and control groups. In Experiment 1, the level of IgY was significantly higher in vaccinated than controls at 7 dpi; but this response was reversed at 14 and 21 dpi without significant differences between groups. In Experiment 2, the level of IgY was higher in control than in vaccinated group at all sampling points, and this difference was statistically significant at 14 and 21 dpi.

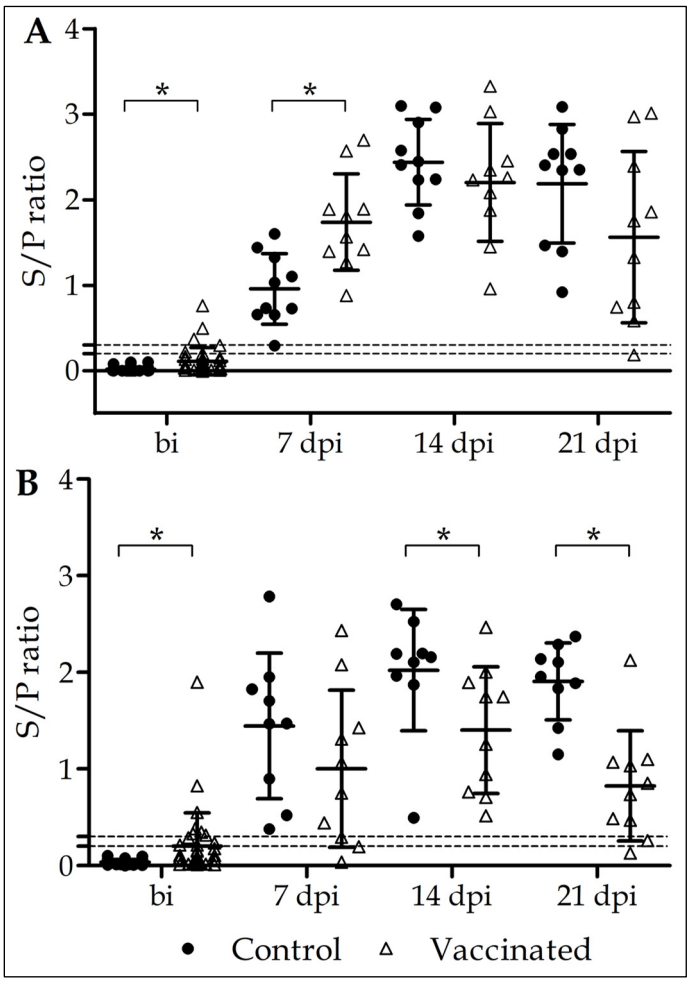

Figure 4-3. Detection of IgY antibodies against Salmonella in serum. (A) Experiment 1. (B) Experiment 2. Each point represents an animal. Error bars express mean S/P ratio ± SD. Additional discontinuous lines indicate the threshold determined by manufacturer (negative: S/P < 0.2; doubtful: 0.3 > S/P ≥ 0.2; positive: S/P ≥ 0.3). bi: before infection; dpi: days post-infection. An asterisk indicates significant differences between control and vaccinated group ($p \leq 0.05$).

Bile was analysed for the presence of secretory IgA antibodies against *Salmonella* Typhimurium. Control group developed a stronger antibody response and levels of secretory IgA were significantly higher than vaccinated group in all sampling points in both Experiments (Figure 4-4).

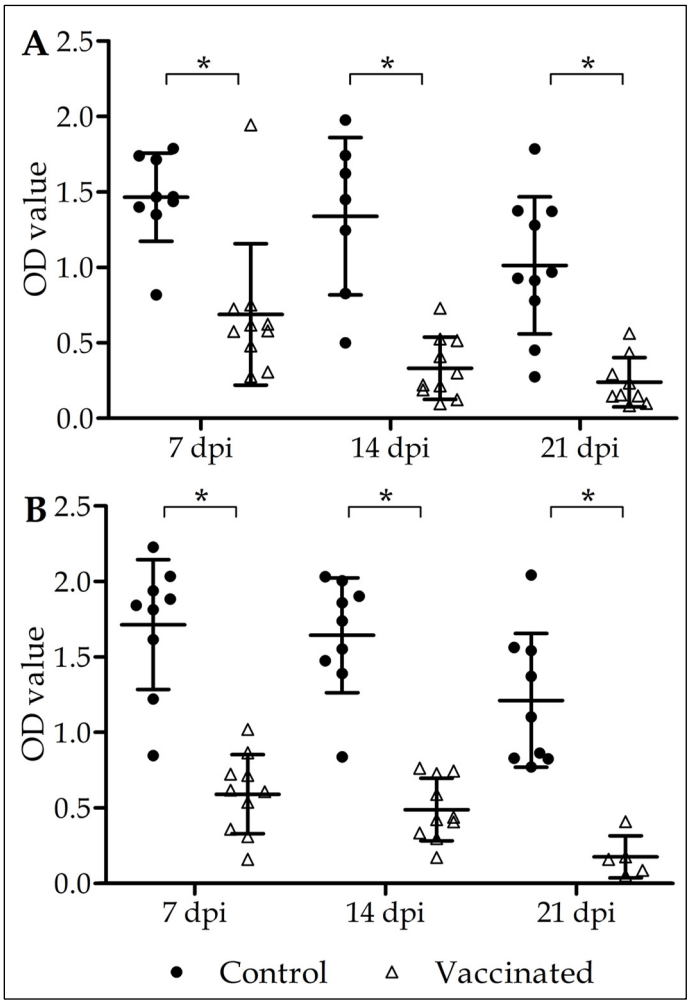

Figure 4-4. Detection of IgA antibodies against Salmonella in bile. (A) Experiment 1. (B) Experiment 2. Each point represents an animal. Error bars express mean OD value ± SD. dpi: days post-infection. An asterisk indicates significant differences between control and vaccinated group ($p \leq 0.05$).

4.3.3 Cellular composition of ileum

Some cellular components of ileum were quantified by flow cytometry to determine different reaction patterns in vaccinated and control animals after challenge with *Salmonella* Typhimurium strains. Macrophages were studied as representatives of the innate immune response. MHC II cells were studied as representatives of the antigen presentation. B cells were studied as representative of the humoral response and T cells subpopulations as representative of the cellular response. Figure 4-5 shows the complete results of the flow cytometry analysis.

The percentage of Macrophages in Experiment 1 was significantly elevated in control group at 7 dpi and in vaccinated group at 14 dpi. In Experiment 2, however, the percentage of macrophages was higher in control group at 7 and 21 dpi.

There were no significant differences between control and vaccinated groups in MHC class II expression and B cell population. But if we consider the progression of cell populations along the time, the expression of MHC class II rises with the progression of the infection in both experiments.

In terms of T cell populations, vaccinated animals had higher CD3+ population in almost all time points of the two experiments except for 7 dpi in Experiment 1, but these differences were significant only at 21 dpi in Experiment 1 and at 7 dpi in Experiment 2.

The distribution of CD4+, CD8+ and CD4+CD8+ T cell subpopulations was different in Experiment 1 and 2 and did not follow a clear pattern. In Experiment 1, CD4+ and CD4+CD8+ were significantly higher in control group at 7 dpi, and CD4+ was significantly higher in vaccinated group at 21 dpi. In Experiment 2, however, only CD8+ and CD4+CD8+ were significantly higher in vaccinated group at 21 dpi.

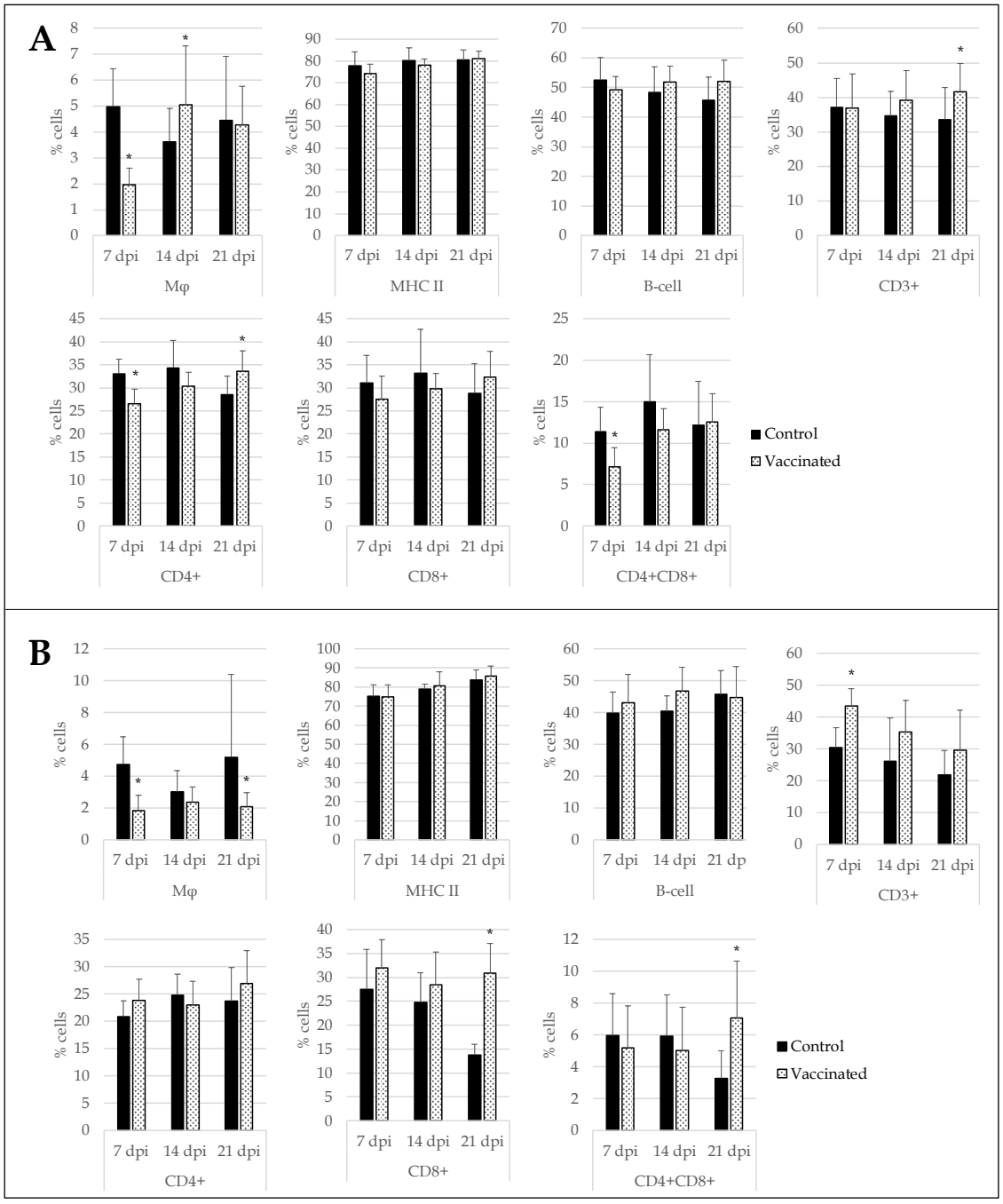

Figure 4-5. Percentage of cell populations in the ileum determined by flow cytometry. (A) Experiment 1. (B) Experiment 2. Results are expressed as mean cell percentage + SD. dpi: days post-infection. An asterisk indicates significant differences between control and vaccinated group ($p \leq 0.05$).

4.3.4 Gene expression in the ileal mucosa

Changes in gene expression in the ileal mucosa were compared in vaccinated and control groups after *Salmonella* Typhimurium infection. In general, major differences in gene expression were found in Experiment 1 than in Experiment 2 (Figure 4-6). The cytokines IFNγ and TNFα were studied as representatives of proinflammatory response and IL-10 as anti-inflammatory response. TLR4 and TLR5 were studied as receptors of *Salmonella* on the innate immune response and MHC II as a marker of antigen presenting cells.

The expression of IFNγ was significantly upregulated after infection in vaccinated groups in all sampling points of Experiments 1 and 2 except at 7 dpi in Experiment 1 (upregulated but not significant).

Generally, TNFα levels upregulated at final stages of infection and were significantly higher in vaccinated than control groups in both experiments at 21 dpi.

Anti-inflammatory IL-10 was significantly downregulated only in Experiment 1 at 14 dpi.

TLR4 marker was only significantly downregulated in Experiment 1 at 14 dpi. TLR5 marker was only significantly downregulated in Experiment 2 at 14 dpi.

The gene expression of MHC II was only significantly downregulated in Experiment 1 at 14 dpi.

During this study, only the gene expression of IFNγ was clearly upregulated, as expected, in vaccinated group. The gene expression of the other cytokines and cell membrane proteins analysed showed strong variation in both experiments, without a clear pattern of up or down-regulation.

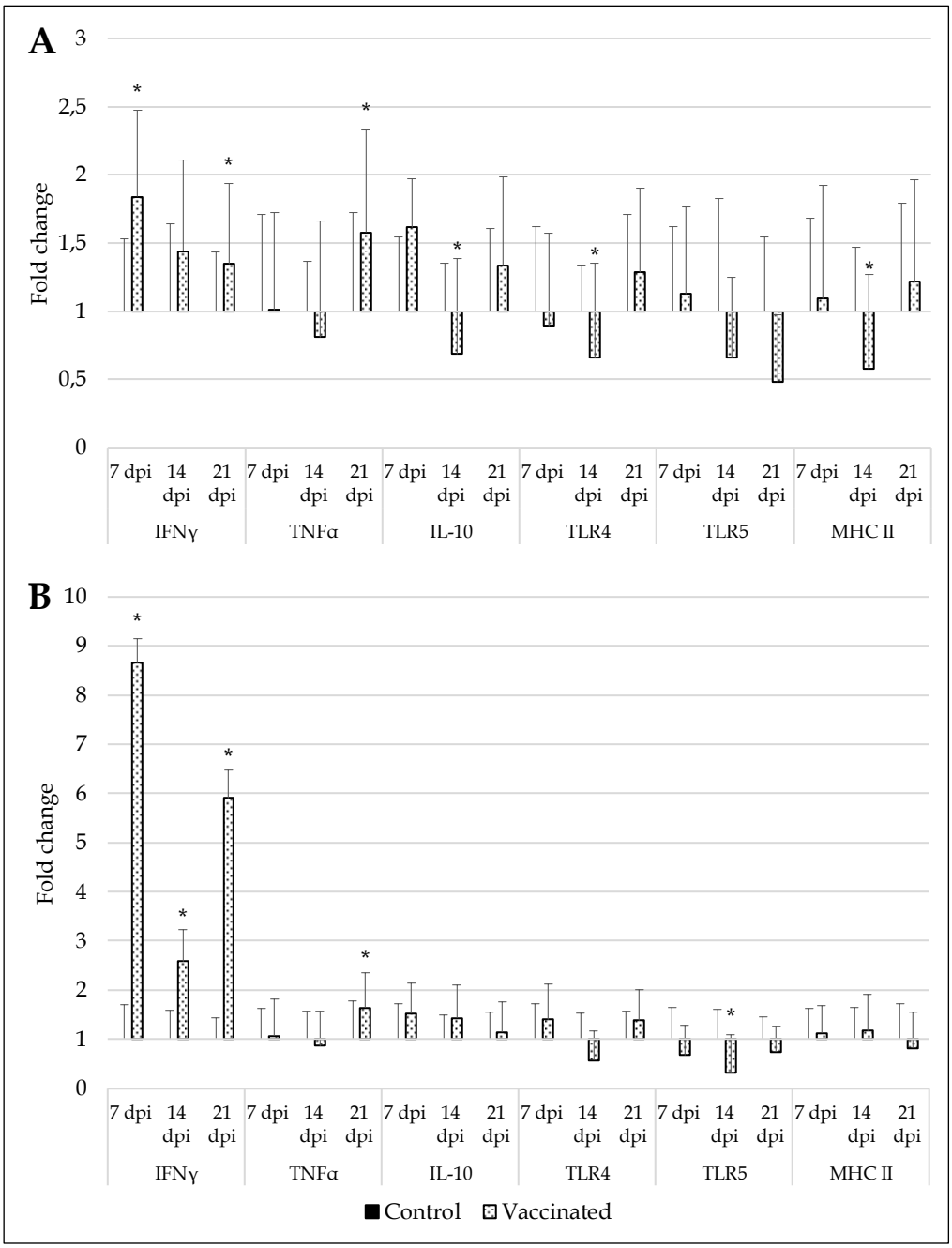

Figure 4-6. Relative fold change in gene expression of cytokines and cellular surface proteins from the ileal mucosa. (A) Experiment 1. (B) Experiment 2. Data are expressed as mean expression levels for vaccinated group normalised to reference gene and relative to control group + SD. dpi: days post-infection. An asterisk indicates significant differences between control and vaccinated group ($p \leq 0.05$).

4.3.5 Immunohistofluorescence (IHF)

Immunohistofluorescence of the caecal tonsils of the chickens revealed that the distribution of the *Salmonella* organisms was related with the progression of the infection and that the MHC II positive cells were distributed mainly in the lamina propria. The localisation of *Salmonella* and MHC II cells was similar in the Experiments 1 and 2 and

in control and vaccinated groups. In Figure 4-7 representative images of the main findings with the confocal microscope images are detailed.

Salmonella bacteria were more numerous in the caecal tonsils at 7 and 14 dpi, when colonisation of internal organs was higher, and were localised attached to the epithelium and in the lumen of the caecal tonsils (Figure 4-7A, 4-7B and 4-7C); and also in the lamina propria (Figure 4-7D). As infection advanced in time, *Salmonella* organisms were located inside lymphoid follicles (Figure 4-7E). At 21 dpi, fewer bacteria were found in the lamina propria, mainly inside MHC II positive cells; and some bacteria were found in the caecal tonsil crypts (Figure 4-7F).

The distribution of MHC II positive cells was comparable in all sampling points of the two experiments. MHC II positive cells were distributed in the lamina propria, which indicates that some of the immune cells that form this tissue are antigen-presenting cells. Antigen-presenting cells are mainly composed of macrophages and dendritic cells.

Colocalization of *Salmonella* Typhimurium and MHC II cells was observed in the lamina propria and inside lymphoid follicles at early stages of the infection (7 and 14 dpi). In Figure 4-8 there is a representative image of an MHC II positive cell that could be a macrophage phagocyting bacterial fragments in the centre of a lymphoid follicle.

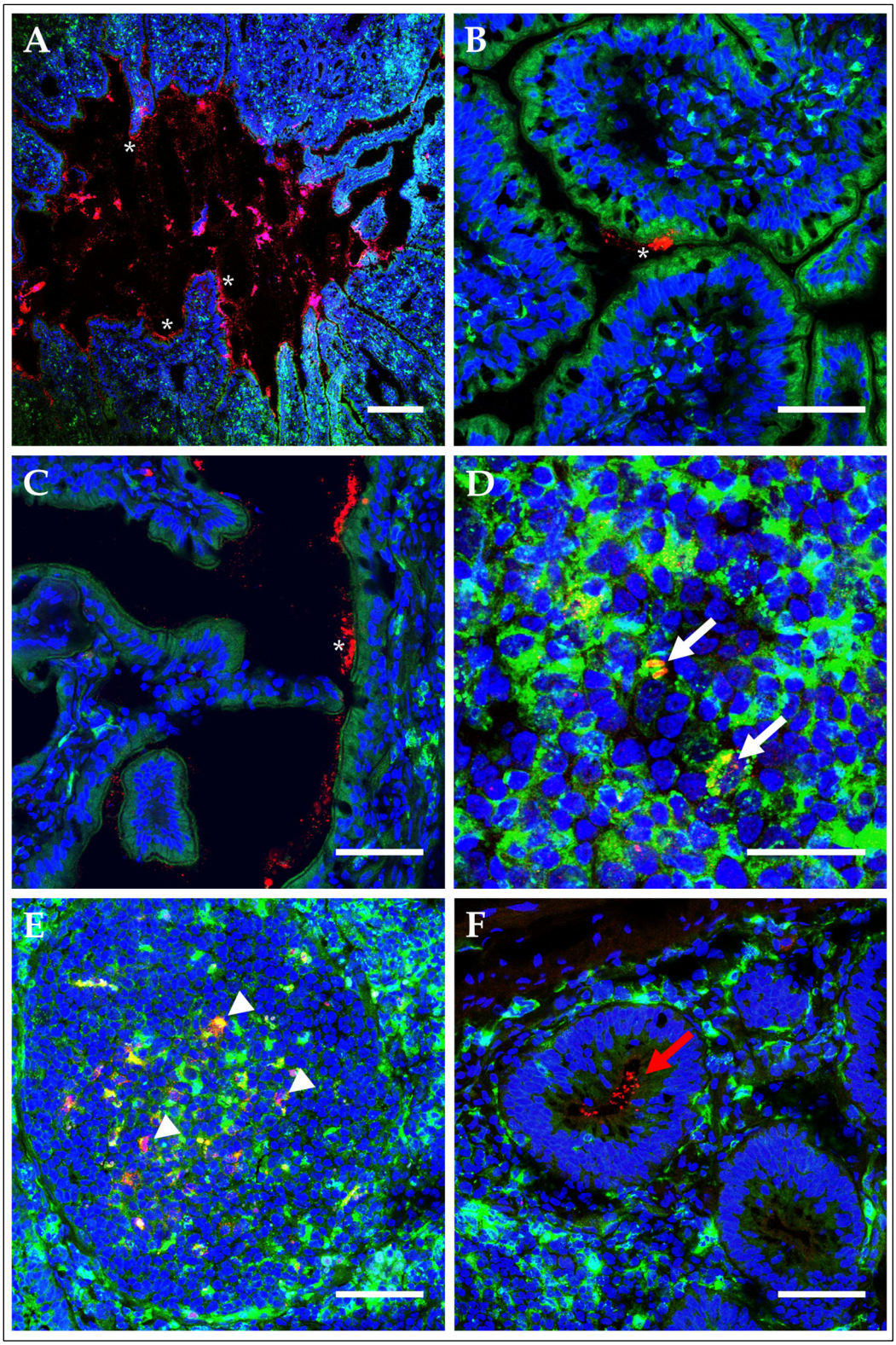

Figure 4-7. Representative confocal images of the caecal tonsils after infection with Salmonella Typhimurium in Experiments 1 and 2. The MHC II cells are stained with green (AF488), Salmonella is stained with red (AF555), and cell nuclei are counterstained with blue (DAPI). (A) Salmonella bacteria attached to the epithelium and in the lumen (white asterisks). Experiment 1, 7 dpi, vaccinated group. Scale bar: 200 µm. (B) Salmonella bacteria attached to the epithelium (white asterisk). Experiment 2, 7 dpi, control group. Scale bar: 50 µm. (C) Salmonella bacteria attached to the epithelium (white asterisk). Experiment 2, 21 dpi, control group. Scale bar: 50 µm. (D). Salmonella inside MHC II cells in the lamina propria (white arrows). Experiment 1, 7 dpi, control group. Scale bar: 25 µm. (E) Salmonella inside MHC II cells in lymphoid follicles (white arrowheads). Experiment 1, 14 dpi, vaccinated group. Scale bar: 50 µm. (F) Salmonella in the caecal tonsil crypts (red arrow). Experiment 2, 21 dpi, vaccinated group. Scale bar: 50 µm.

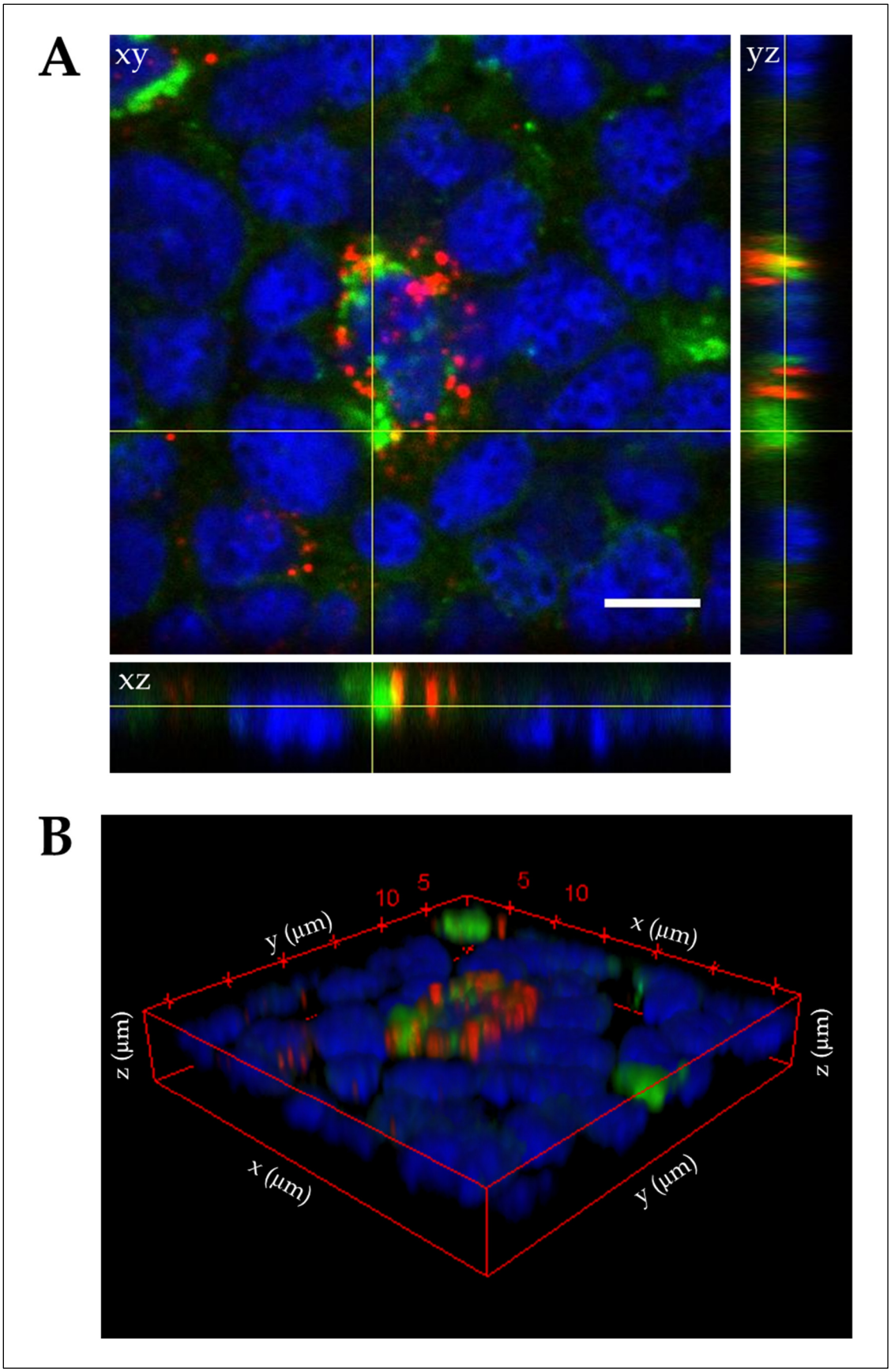

Figure 4-8. Confocal image showing an MHC II positive cell (AF488, green) inside a lymphoid follicle of a vaccinated animal from the challenge at 35 weeks at 7 dpi containing Salmonella bacteria (AF555, red). Nuclei are counterstained with blue (DAPI). Z-stacks were taken at 0.25 µm. (A) Orthogonal views. The x-z and y-z positions are indicated by a yellow line. Scale bar: 5 µm. (B) 3D projection.

4.4 DISCUSSION

The results of this study revealed that the vaccinated animals developed an adaptive immune response at the intestinal level after *Salmonella* Typhimurium infection compared with non-vaccinated controls. In this study we also demonstrated that the number of vaccine doses received can modify this response. Vaccinated animals developed a cellular immune response that reduced *Salmonella* excretion earlier that non-vaccinated ones

Overall, vaccinated groups in Experiment 1 and 2 excreted less *Salmonella* Typhimurium field strain and were protected against internal organ colonisation. After infection, vaccinated animals had lower rate of positive organs mainly in liver and spleen, and in ovary only in Experiment 1 earlier after infection. The reduction of bacterial colonisation of some internal tissues, included the reproductive, indicates a potential use of this vaccine to reduce vertical transmission to eggs (Gast et al., 2019). This fact indicates that vaccination can help to reduce *Salmonella* prevalence in the poultry industry.

It has already been suggested that a strong antibody response does not mean a protective response against *Salmonella* (Barrow et al., 2012). In fact, it was demonstrated by Beal and colleagues that lack of antibodies and B cells did not alter clearance of primary or secondary *Salmonella* Typhimurium infection with a bursectomization method that did not affect other cell types than B cell (Beal et al., 2006). Our findings are in line with the aforementioned literature, because animals vaccinated with live attenuated *Salmonella* Typhimurium vaccine mounted a weaker antibody response than non-vaccinated controls after challenge with a field strain of the same serovar, even though the vaccinated group reduced *Salmonella* excretion. Other researchers that analysed humoral response of vaccinated and control groups in efficacy studies obtained similar results than in Experiment 1: only early after challenge at 3 dpi antibodies were significantly higher in vaccinated than in controls but at late samplings at 14 dpi, the humoral response was stronger in non-vaccinated animals (Methner, 2018). Other groups have found that controls had higher mean antibody values than vaccinated after challenge with field strains of *Salmonella* Enteritidis (Theuß et al., 2018). Similarly, in other studies vaccination with live attenuated *Salmonella* Gallinarum did not induce a strong humoral IgY response after infection with *Salmonella* Enteritidis field strains (Penha Filho et al., 2012). Likewise, other group found that following the re-challenge there was no significant increase in IgY, whereas in age-matched primary-infected birds high levels

of this isotype were measured (Beal et al., 2004a). Despite of the findings on antibody response in our study and in comparable studies in the literature, vaccinated animals were protected, indicating that humoral response is not crucial in protection.

Regarding to secretory IgA production, it has been suggested that persistent antibody responses of IgA correlate with reduced number of *Salmonella* Typhimurium in the gut after secondary infection because it can reduce bacterial adherence and invasion (Beal et al., 2004a; Pasetti et al., 2011). But in our study, the production of secretory IgA was significantly higher in the control group compared with vaccinated group in all the sampling points, and this clear pattern was repeated in Experiment 1 and Experiment 2. This situation could be explained because in the control group there is a higher bacterial load in the intestine that stimulates a stronger humoral response, but in the in vaccinated group there is a reduction of the bacterial load that reduces the production of antibodies. In fact, other studies have confirmed these hypotheses. In a study comparing *Salmonella* Enteritidis infection in different susceptible chicken lines, higher levels of *Salmonella* colonization in the caecum were also accompanied by higher anti-*Salmonella* IgY in serum and IgA in the gut secretions (Berthelot-Hérault et al., 2003). Finally, in another study analysing the immune response against *Salmonella* Enteritidis generated by different vaccine programs, they did not find significant differences in secretory intestinal IgA after challenge between animals vaccinated with a live vaccine and the non-vaccinated control group (but they only analysed until 9 dpi) (Penha Filho et al., 2012). Further experiments are needed to analyse the level of IgA antibodies in bile just before the challenge infection, to determine if these antibodies could be related with adherence or protection. All these results together with those observed in our study suggest that mechanisms other than the humoral response are involved in protection against *Salmonella.*

Immune cell populations in the gut changed during the resolution of the infection, although the bird-to-bird variation was high and the differences between groups were not statistically significant in some cases.

Macrophages are the target cell for *Salmonella* replication early in the infection and interfere in *Salmonella* clearance and in the establishment of systemic infection (Chappell et al., 2009). In the present study, non-vaccinated animals had higher macrophage populations early after challenge in Experiments 1 and 2. This could represent a stronger inflammatory response in non-vaccinated animals that was partially controlled by

immunity to live vaccine in the immunised group. Infiltration of the lamina propria by macrophages has been described in other studies early after infection with virulent and attenuated *Salmonella* Typhimurium strain, but this response was not evaluated after secondary infection (Berndt and Methner, 2004).

There were no differences between groups in B cell or MHC II markers. The results in B cells do not match the results on ELISA in bile, where the control group had significantly higher IgA production. Further studies analysing other more specific immunoglobulin markers in tissue like IgM, IgY or IgA could explain these results (Zheng et al., 1997).

As the infection progresses, T cells and B cells are activated and infiltrate the intestine driving the resolution of infection (Smith et al., 2013). A predominance of cytotoxic CD8+ cells could be expected because this is the representative population of a cell-mediated immune response, although in our study there were no clear differences between vaccinated and control groups in this population (Smith and Göbel, 2013). In our study, CD3+ T cells presence in the intestine was higher in the vaccinated group, statistically significant at 21 dpi in Experiment 1 and 7 dpi in Experiment 2, situation that could indicate an overall higher recruitment of T cells in vaccinated animals. This increase in T cell response indicates that vaccinated animals developed a general adaptive cellular response, but subpopulations of T cells analysis is needed to specify the type of immune response (Kaiser, 2010). The dynamics of CD4+ and CD8+ cells were not clear in our experiments, because in Experiment 1 it seemed that both populations were higher in vaccinated than in controls only at 21 dpi but in Experiment 2 both populations were higher in vaccinated than in controls almost in all sampling days. In some studies that analysed cell populations in the gut after primary infection with *Salmonella*, they found an infiltration of CD8+ and CD4+ T cells compared with non-infected day-old chicks (Berndt and Methner, 2001; Bai et al., 2014). It has also been previously reported a correlation of T cell proliferative responses with *Salmonella* clearance in the spleen in secondary-infected animals (Beal et al., 2004b).

Cytokine response was mainly dominated by IFNγ production. IFNγ is key in Th1 cell-mediated immune responses and in controlling intracellular pathogens such as *Salmonella*. It is produced by T cells and NK cells, but can affect a wide range of cell subsets (Raupach and Kaufmann, 2001; Barrow et al., 2012). This cytokine is required for macrophage activation (Eckmann and Kagnoff, 2001). Up-regulation of IFNγ during all

sampling points of the two studies paralleled *Salmonella* clearance, as we could verify in other studies (Bai et al., 2014).

Up-regulation of proinflammatory TNFα can serve as a modulator against bacterial infections. It is expressed by a wide range of cells (Eckmann and Kagnoff, 2001). In our studies, it was upregulated only at latter stages of the infection, similarly to what happened in other study, where non-vaccinated and challenged animals expressed higher levels due to the increase of *Salmonella* in the intestine tissue (Carvajal et al., 2008).

IL-10 in chickens has a potential anti-inflammatory role and inhibits IFNγ and activation and effector function of T cells, monocytes and macrophages, inclining immune response towards Th2 (Rothwell et al., 2014). The gene expression of this interleukin did not change significantly during our experiments, except a down-regulation in Experiment 1 at 14 dpi in vaccinated animals. Other studies have also found a decrease in IL-10 expression that can represent an active antigen presentation and proinflammatory response against *Salmonella* (Penha Filho et al., 2012). In general, the absence of an IL-10 up-regulation could determine that vaccinated animals developed a protective immune response against an intracellular pathogen, in this case, *Salmonella* (Moore et al., 2001).

Findings in the analysis of caecal tonsils by confocal microscopy showed that MHC II molecule was expressed in a similar way in vaccinated and non-vaccinated groups in the Experiments 1 and 2 (equally to flow cytometry results); and also showed that the amount of *Salmonella* detected by confocal microscopy coincided with the descending trend in *Salmonella* excretion detected by cloacal swabs. Caecal tonsils are the main site for *Salmonella* entry, so it is a target tissue for studying the relation of the pathogen with chicken immune response (Berndt et al., 2007). Also, MHC II cells have an important role on *Salmonella* clearance. MHC II molecules are expressed mainly in antigen presenting cells (macrophages and dendritic cells), but can be also found in B cells; and are recognised by CD4+ T cells (Kaufman, 2013). Therefore, observing the relation of challenge bacteria and MHC II cells could provide information about the immune response and the cell populations involved in protection. In the majority of MHC II cells phagocyting *Salmonella,* low numbers of bacteria are seen, like previous studies in mouse models, and this indicates that the ability of *Salmonella* to multiply in tissues is a result of segregation of bacterial populations and continuous distribution to new phagocytic cells (Mastroeni and Sheppard, 2004).

Surprisingly, protection against *Salmonella* infection after only two vaccine doses was stronger than after three, although 2 or 3 boosters are generally recommended by manufacturers (Desin et al., 2013). Although it should be taken into account that Experiment 1 was carried out 9 weeks after the second vaccine and Experiment 2 was carried out 19 weeks after the third vaccine, so they would not be fully comparable because the time between last vaccination and the challenge infection is crucial for protection (Huberman et al., 2019). It is possible to hypothesise that the different vaccine schedule or the different age in the infection induced a carrier state in some animals. Considering this scenario, *Salmonella* field strains could invade the gut with little activation of innate response and then establish an intracellular infection in macrophages, that could be reactivated lately (Wigley, 2014). This tolerance against infection should be explained by a Th1 to Th2 drift, but it was not the case, because we did not find Th2 markers like antibody proliferation or IL-10 up-regulation (Penha Filho et al., 2012; Kaiser and Stäheli, 2013). An important point to consider is that our experiment was executed under laboratorial conditions with a high dose of challenge inoculum that do not mimic the infection under field conditions. Multiple factors should be considered that affect the outcome of a study: challenge titre and serotype, route of infection, age of infection, and even the time of sampling and observation of immune parameters (Calenge et al., 2010). Further research should be done evaluating protective effect and its immune correlation under field conditions, for example with indirect *Salmonella* exposure model using seeder chickens (Carvajal et al., 2008).

In summary, the results from the present study demonstrated that there was an immune response induced by vaccination with two different vaccine schedules. Vaccinated animals developed a cellular immune response at the gut level, but humoral response was not crucial for *Salmonella* clearance, so a drift to Th1 response can be presumed. The Th1-Th2 polarisation paradigm has been used to understand the adaptive immune response (Kaiser and Stäheli, 2013). The polarisation is regulated by antigen-specific Th cells (Th1 cells drive cellular responses dominated by IFNγ; Th2 cells drive humoral responses dominated by IL-4 and IL-13) (Degen et al., 2005). The study of additional cytokines (as IL-4 and IL-13) in further studies could confirm if presumed Th1 drift is achieved after vaccination or challenge with *Salmonella*. Additionally, more studies evaluating the immune response after modifying crucial parameters like vaccination schedules or challenge model are needed.

5. STUDY III:

Immunogenicity test of combined *Salmonella* Enteritidis and *Salmonella* Typhimurium vaccine at the end of the laying period

5.1 INTRODUCTION

The efficacy of early vaccination during the rearing period in the layer industry needs to be determined at the end of the laying period. *Salmonella* is more likely introduced early in a flock and can persist for long periods, allowing animals to reactivate the excretion (if early infected) or to be infected from the environment during the production period (Davies and Breslin, 2004; Schulz et al., 2011).

A standard laying hen production cycle lasts until 70 to 80 weeks of age. The option of extending this period could reduce environmental impact and financial costs per egg produced, but aspects like lasting of vaccines protection should be studied (Bain et al., 2016; Van De Reep et al., 2018).

With regards to serovars most closely related to cases of human salmonellosis, *Salmonella* Enteritidis and *Salmonella* Typhimurium (including monophasic Typhimurium) accounted for more than 70% of the total confirmed cases of human salmonellosis in the EU in 2018 (EFSA, 2019). In this way, vaccinating with the two most important serovars causing salmonellosis can expand the range of protection and confer cross-protection against other important serovars like monophasic variant of *Salmonella* Typhimurium (Kilroy et al., 2015); or even against *Salmonella* Infantis (Eeckhaut et al., 2018). Also, it has been determined that vaccination with combined *Salmonella* Enteritidis and *Salmonella* Typhimurium strains can increase protection against *Salmonella* Enteritidis challenge, compared with vaccination with only one of the two serovars, demonstrating the existence of synergies between vaccine serovars (Gantois et al., 2006).

Most efficacy studies have been performed in young chicks and have determined protection early after vaccination (Kilroy et al., 2015; Braukmann et al., 2016). Only few studies have tested the long-term efficacy late in the laying period with diverse outcomes. Most of them have determined reductions in organ colonisations and/or in excretion after challenge of laying hens that had been vaccinated during the rearing period (Woodward et al., 2002; Springer et al., 2011; Theuß et al., 2018). However, other studies found that there was no protection against *Salmonella* colonisation in vaccinated laying hens when challenged at around 82 weeks of life, probably because these animals were vaccinated in the field and they may not have been properly immunised (Van De Reep et al., 2018). Studying protection at the end of the laying period is a good indication of whether the vaccine can be effective in the field or not.

In addition to the determination of vaccine efficacy, it is also important to study immune mechanisms that are correlated to protection. Lots of studies have studied humoral and cellular response after *Salmonella* infection (Berndt and Methner, 2004; Bai et al., 2014), but few studies have studied the immune response in vaccinated and non-vaccinated adult animals after challenge with field strains, in order to determine possible mechanisms of protection stimulated by vaccination (Penha Filho et al., 2012).

The main objective of the present study was to evaluate the remaining levels of protection of a bivalent live attenuated vaccine applied during the rearing period at the end of the laying period and the associated mechanisms of immunity. The levels of excretion and colonisation of challenge strains were compared with a non-vaccinated control group, as well as the humoral and cellular immune response at intestinal level.

5.2 MATERIALS AND METHODS

5.2.1 Animals

A total of 58 SPF female chickens were transported to the experimental facilities of AM Animalia Bianya SL (Girona, Spain) at the end of the laying period. During the rearing period, the vaccinated group received three vaccine doses at day-old, at 7 and at 18 weeks of age. The control group came from a different batch of animals and did not receive any *Salmonella* vaccine. During the challenge period, groups were housed in separated buildings in pens with wood shavings. Commercial feed and water were provided ad libitum. This study was approved by the Animal Experimentation Ethics Committee of AM Animalia Bianya (No. 058/17).

5.2.2 Bacterial strains

The bivalent vaccine contained live attenuated *Salmonella* Enteritidis and *Salmonella* Typhimurium strains. The *Salmonella* Enteritidis strain was the commercially available Primun Salmonella E (Laboratorios Calier, Les Franqueses del Vallès, Spain). The *Salmonella* Typhimurium strain was the same used in STUDY I of this Thesis. Freeze-dried vial was diluted in sterile tap water and adjusted to the minimum effective dose of 1×10^8 cfu per ml.

The challenge inoculum was prepared with two *Salmonella* Enteritidis and two *Salmonella* Typhimurium field strains isolated from adult hens. The strains were plated on Columbia Agar + 5% sheep blood (bioMérieux, Marcy-l'Etoile, France) at 37 °C. After

overnight incubation, the bacteria were suspended in buffered peptone water (BPW) (VWR, Leuven, Belgium) and adjusted to the desired titre.

5.2.3 Study design

The animals were divided into 2 groups. The vaccinated group (n = 25) received three vaccine doses at day-old, at 7 and at 18 weeks of age by oral gavage with a plastic cannula. The vaccine was applied at a dose of 1 x 10^8 cfu in 1 ml (1st and 2nd dose) and at 1 x 10^8 cfu in 5 ml the 3rd dose. The control group (n = 33) was kept as non-vaccinated group.

At the end of the laying period, all the animals were inoculated by oral gavage with a challenge inoculum at a dose of 1.95 x 10^8 cfu in 5 ml. In the moment of the challenge infection, the vaccinated group was 63 weeks old and the control group was 46 weeks old. The challenge inoculum contained *Salmonella* Enteritidis strains GN-0825 and GN-1063 and *Salmonella* Typhimurium strains GN-3326 and GN-3760 mixed together. The study design is summarised in Table 5-1.

Before the challenge infection, cloacal swabs and serum samples were collected from all the chickens to confirm that *Salmonella* spp. was not isolated in any of the animals and that control chickens were free from *Salmonella* antibodies. After the infection, cloacal swabs were sampled from each animal at 3, 5, 7, 10, 14 and 21 dpi to detect challenge strains by Multiplex-PCR. At 7, 14 and 21 dpi, 10 chickens per group (or all that were left) were euthanised by intravenous injection of sodium pentobarbital overdose and samples of blood, bile, liver, spleen, caecum, ovary, oviduct and small intestine were collected. Samples of liver, spleen, caecum, ovary and oviduct were qualitatively examined for detection of challenge strains by Multiplex-PCR. Blood and bile were analysed for *Salmonella* antibodies. The small intestine was collected to perform immunohistofluorescence and RNA extraction.

Table 5-1. Study design.

Group	**Vaccine**	**Vaccine doses**	**Challenge**	**Euthanasia[a]**
Control (n = 33)	No	na	*S.* Enteritidis *S.* Typhimurium (week 63)	7 dpi (n = 10) 14 dpi (n = 10) 21 dpi (n = 10)
Vaccinated (n = 25)	*S.* Enteritidis *S.* Typhimurium 1 x 10^8 cfu/animal	1st: Day-old	1.95 x 10^8 cfu/animal	7 dpi (n = 10) 14 dpi (n = 10) 21 dpi (n = 5)
		2nd: week 7		
		3rd: week 18		

[a]dpi: days post-infection. na: not applicable.

5.2.4 Detection of *Salmonella* strains by PCR

Salmonella spp. challenge strains were detected in swabs and internal organs by Multiplex-PCR, in order to differentiate between *S.* Enteritidis and *S.* Typhimurium positive samples. For optimal detection of *Salmonella*, the Multiplex-PCR method comprised a pre-enrichment step and a selective enrichment and subsequent DNA extraction.

First step was non-selective pre-enrichment in BPW (37 ± 1 °C for 18 h). Second step was cultivation in selective enrichment medium Rappaport-Vassiliadis broth (RV) (41.5 ± 1 °C for 48 h). The DNA extraction was performed from 200 µl of the RV broth using the NucleoMag VET kit (Macherey-Nagel, Düren, Germany) and the BioSprint 96 instrument (QIAGEN, Hilden, Germany). The PCR mix was prepared with 3 µl of DNA, 0.5 µl of 2.5 mM each primer of *invA*, 1 µl of 10 mM each primer of *sefA* and *fliB-fliA*, 2 µl of Tween-20 (1:100) (Sigma-Aldrich, St. Louis, MO, USA), and 10 µl of QIAGEN Multiplex PCR Master Mix (QIAGEN, CA, USA). The primers used are detailed in Table 5-2. Thermocycling parameters consisted of an initial denaturation at 95 °C for 5 min; followed by 35 cycles of 95 °C for 1 min, 59.2 °C for 2 min, and 72 °C for 2 min; and a final extension step at 72 °C for 15 min. The PCR products were visualised by electrophoresis on 2% (w/v) agarose gel stained with ethidium bromide.

Table 5-2. Primers used in the Multiplex-PCR assay.

Target gene	**Primer**	**Sequence (5′-3′)**	**Amplicon size (bp)**	**Reference**
invA	Forward	GTGAAATTATCGCCACGTTCGGGCAA	284	(Rahn et al., 1992)
	Reverse	TCATCGCACCGTCAAAGGAACC		
sefA	Forward	TGTGCGAATGCTAATAGTTG	526	(Thomas, 1994)
	Reverse	CTGCTGAACGTAGAAGGTCG		
fliB-fliA	Forward	CTGGCGACGATCTGTCGATG	1000 and 250	(Echeita et al., 2001)
	Reverse	GCGGTATACAGTGAATTCAC		

5.2.5 Enzyme-linked immunosorbent assay (ELISA)

A commercial indirect ELISA kit (*Flocktype* Salmonella Ab) (QIAGEN, Leipzig, Germany) was used for the detection of antibodies against *Salmonella* spp. in serum and bile. Serum was analysed for the detection of IgY according to the manufacturer's protocol. Bile was analysed for the detection of IgA according to the manufacturer's

protocol but changing the dilution of the sample (1:20) and the secondary antibody solution. The secondary antibody solution was prepared with HRP conjugated goat anti-chicken IgA (0.02 µg/ml) (Bethyl, Montgomery, TX, USA) in PBS Tween-20 (0.01%) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 0.5% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA).

The results of serum ELISA are expressed as S/P (sample-to-positive) ratio, where S/P:

$$S/P = \frac{(Optical\ Density\ (OD)\ sample - OD\ Negative\ Control)}{(OD\ Positive\ Control - OD\ Negative\ Control)}$$

The thresholds determined by the manufacturer are indicated in the graph as additional discontinuous lines (negative: S/P < 0.2; doubtful: 0.3 > S/P ≥ 0.2; positive: S/P ≥ 0.3). The results of bile ELISA are expressed as OD. OD was measured at 450 nm. Results of each individual animal are represented and error bars express mean ± standard deviation (SD).

5.2.6 Immunohistofluorescence (IHF)

Ileum was analysed for the quantification of macrophages and CD4+ and CD8+ T cells population. Briefly, ileum was flushed with PBS, and a fragment with macroscopically visible Peyer's patch was embedded in Tissue-tek Optimal Cutting Temperature compound (OCT) (Sakura Finetek, Alphen aan den Rijn, Netherlands), and frozen in dry ice. Cryostat (Leica CM 3050S, Nussloch, Germany) sections of 6 µm were mounted onto Superfrost Plus slides (Menzel-Gläser, Braunschweig, Germany) and let air-dry for 10 minutes at room temperature (RT). Sections were fixed with acetone (−20 °C for 3 min) and methanol (80%, 4 °C for 5 min), washed 3 times in PBS, and blocked in PBS with 5% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA) and 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) at RT for 1 h. All antibodies were incubated at RT for 1 h. Antibodies used are shown in Table 5-3. Antibodies were diluted in PBS with 2% BSA (Sigma-Aldrich, St. Louis, MO, USA) and 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA). After antibody incubation, slides were washed in PBS at RT, 3 times for 5 min. Blocking and antibody incubations were made in a humid chamber. Slides were mounted in ProLong Diamond Antifade light Mountant with DAPI (Life Technologies, Eugene, OR, USA). Images were captured using a confocal microscope Leica TCS-SP5 (Leica Microsystems, Wetzlar, Germany). One section of intestine was stained per bird and five separate images were taken per section.

Table 5-3. Antibodies used for immmunohistofluorescence.

Antibody	Specificity	Clone	Isotype	Conjugation	Dilution	Source
Mφ	Monocyte/ Macrophage	KUL01	Mouse (BALB/c) IgG1κ	FITC	5 µg/ml	Southern Biotech, Birmingham, AL, USA
CD4+	CD4 T helper lymphocytes	CT-4	Mouse (BALB/c) IgG1κ	AF488	2.5 µg/ml	Southern Biotech, Birmingham, AL, USA
CD8+	CD8α T cytotoxic lymphocytes	CT-8	Mouse (BALB/c) IgG1κ	AF647	2.5 µg/ml	Southern Biotech, Birmingham, AL, USA

Macrophages were quantified in the lamina propria and epithelium of ileum. In the case of CD4+ and CD8+ T cells, analysis was performed in lamina propria and epithelium of ileum separately for areas with or without Peyer's patches.

Percentages of each cell population was calculated using ImageJ software (Schindelin et al., 2012). Briefly, the stained areas for each cell population were measured by thresholding and compared with the area occupied by nuclei. In Figure 5-1 there is a diagram of the quantification process.

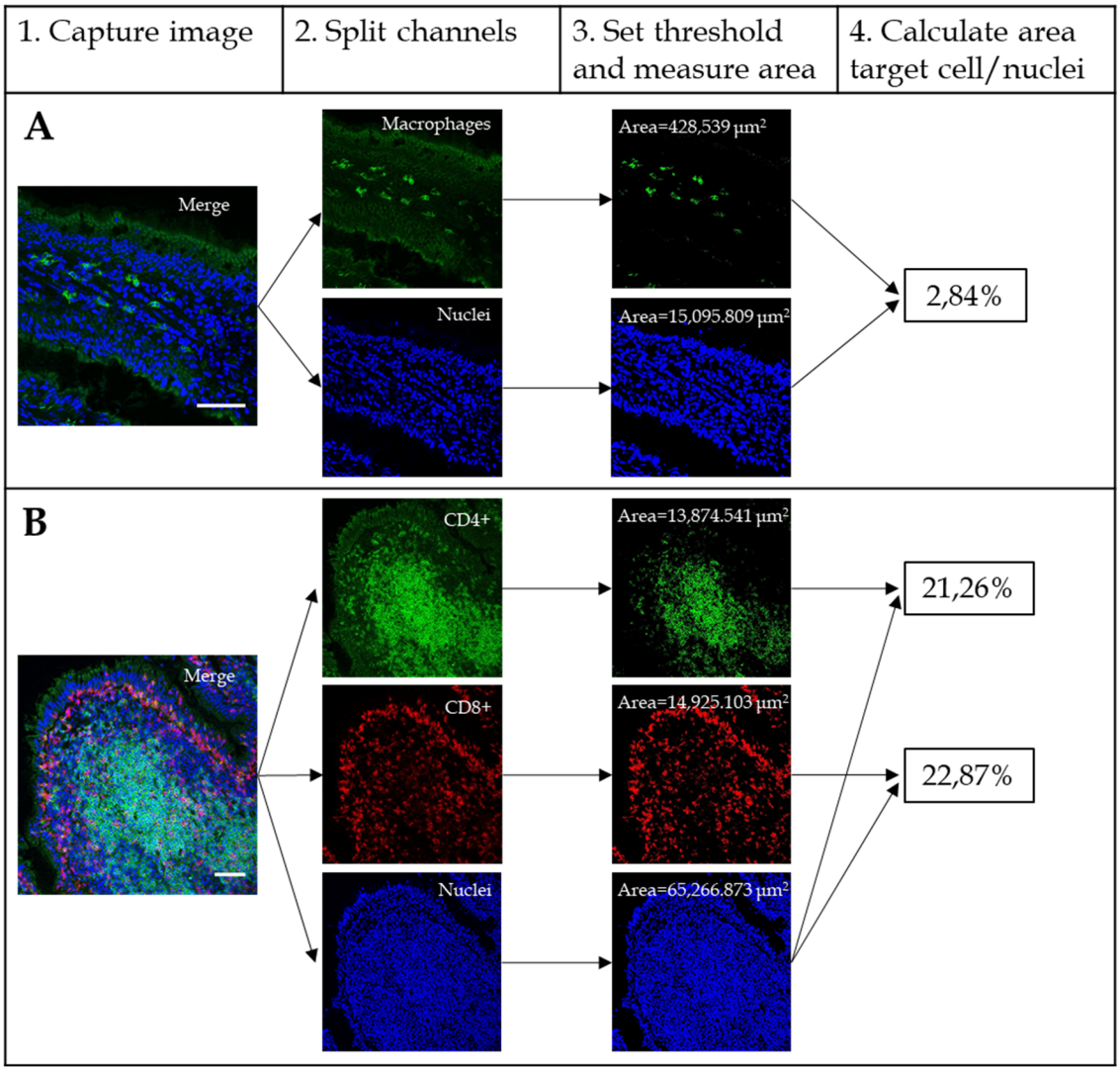

Figure 5-1. Diagram of the quantification steps using ImageJ software. (A) Quantification of macrophages (FITC, green) and nuclei (DAPI, blue). (B) Quantification of CD4+ (AF488, green) and CD8+ (AF647, red) T cells and nuclei (DAPI, blue). Scale bar: 50 µm.

5.2.7 Reverse transcriptase quantitative real-time PCR (RT-qPCR)

The relative gene expression of cytokines and cellular surface proteins at ileal mucosa was determined with RT-qPCR. Ileal mucosal was collected in RNAlater stabilisation solution (Thermo Fisher Scientific, Vilnius, Lithuania) and frozen at −80 °C. RNA was extracted from 15-20 mg of tissue with RNeasy Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol and treated with RNase-free DNase set (QIAGEN, Hilden, Germany). Quality and quantity of RNAs were determined using BioDrop µLITE spectrophotometer (Biodrop, Cambridge, UK). The mRNA expression rates of Interferon gamma (IFNγ), Tumor necrosis factor alpha (TNFα), Toll-like receptors 4 and 5 (TLR4 and TLR5) and major histocompatibility complex class II (MHC II) were determined for every individual chicken using the EXPRESS One-Step SYBR GreenER kit (QIAGEN, CA, USA) according to the manufacturer's protocol.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as reference gene. Primers for the IFNγ and TNFα (Carvajal et al., 2008) had been described previously. Primer sequences for GAPDH, TLR4, TLR5 and MHC II were designed using National Center for Biotechnology Information database (Sayers et al., 2019) and Oligo explorer 1.5 software (http://www.genelink.com). Primer sequences are described in Table 5-4. Amplification and detection of specific products were performed using 7500 Fast Real-Time PCR system and 7500 software version 2.3 (Applied Biosystems, CA, USA) with the following conditions: cDNA synthesis at 50 °C for 5 min, followed by initial denaturation at 95 °C for 5 s, and 40 cycles of denaturation at 95 °C for 3 s and annealing/extension at 60 °C for 30 s. Results are expressed as $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001), where ΔΔCT:

$$\Delta\Delta CT = ((CTtarget - CTgapdh)vaccinated - (CTtarget - CTgapdh)control)$$

Statistics were calculated using ΔΔCT values.

Table 5-4. Primer sequences for RT-qPCR.

Target	**Sequence (5′-3′)**		**Product (bp)**
GAPDH	Forward	GGGTGTCAACCATGAGAAATAT	120 bp
	Reverse	CCCTCCACAATGCCAAAGTT	
IFNγ	Forward	CCCGATGAACGACTTGAGAAT	106 bp
	Reverse	AGACTGGCTCCTTTTCCTTTTG	
TNFα	Forward	GCTGTTCTATGACCGCCCAGTT	140 bp
	Reverse	AACAACCAGCTATGCACCCCA	
TLR4	Forward	TCCCTCACACCCATTCCACG	109 bp
	Reverse	ATGGGGAAGGGGCTGAGGA	
TLR5	Forward	TCACACGGCAATAGTAGCAACA	137 bp
	Reverse	TCACACAGTAAGAGAAGCGAT	
MHC II	Forward	CACTACCTGAACGGCACC	156 bp
	Reverse	AATCTCGGCGTTGCTGTTCC	

5.2.8 Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.01 software for Windows (GraphPad Software, La Jolla, CA, USA). Differences in excretion of cloacal swabs and colonisation of internal organs by challenge strain were determined using Fisher's exact

test. Normality of the data from ELISA, RT-qPCR, and IHF was tested with the D'Agostino & Pearson omnibus test and differences were determined using Mann-Whitney test. P values ≤ 0.05 were considered significant.

5.3 RESULTS

5.3.1 Detection of Salmonella in cloacal swabs and internal organs after challenge

Before the challenge, all animals were negative for *Salmonella* spp. excretion in cloacal swabs.

After the challenge with field strains, the excretion of *Salmonella* Enteritidis and *Salmonella* Typhimurium was lower in the vaccinated than in the control group (Figure 5-2). The excretion of *Salmonella* Enteritidis was very low in vaccinated animals during all the study, significantly different from control group at 5 dpi. The excretion of *Salmonella* Typhimurium, however, decreased fast during the first week of the study, and was significantly lower than control group at 5 and 7 dpi.

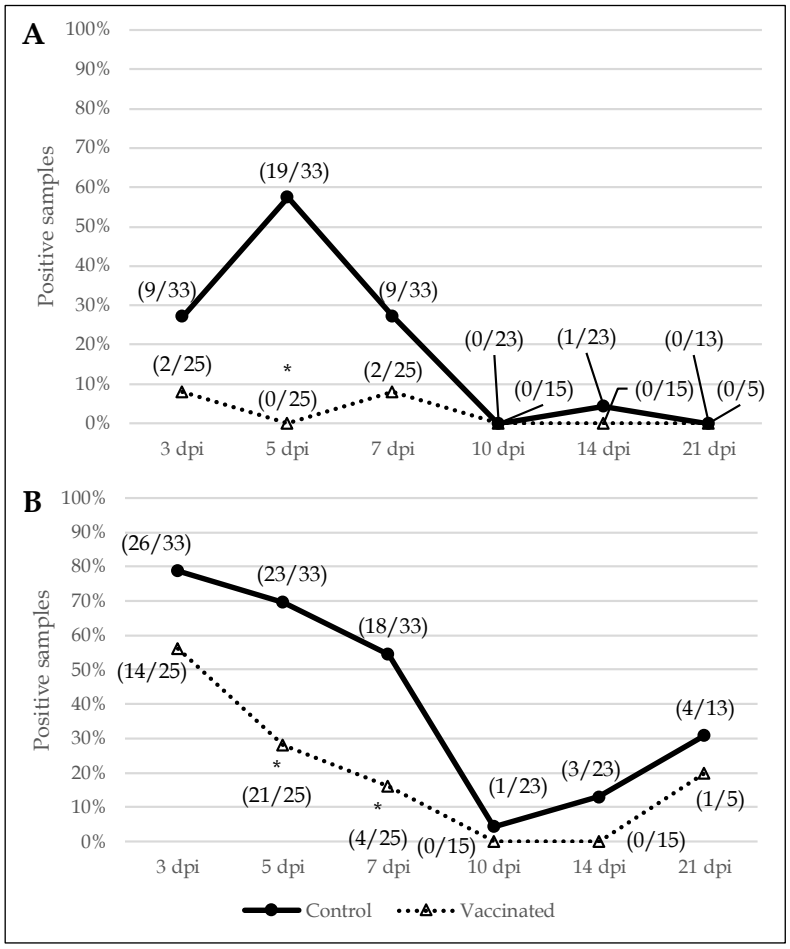

Figure 5-2. Excretion of Salmonella spp. in cloacal swabs after challenge. (A) Salmonella Enteritidis. (B) Salmonella Typhimurium. dpi: days post-infection. An asterisk indicates significant differences between control and vaccinated group (p ≤ 0.05). The number of positive animals with respect to the total is shown in brackets (+/n).

The results of internal organ colonisation after challenge are shown in Figure 5-3. *Salmonella* Enteritidis colonisation of internal organs was lower in the vaccinated than in the control group during all the study, although only some of the organs were highly colonised in control group (liver and caecum had the highest rates at 7 dpi with 50% and 100%, respectively). Hence, only the colonisation of liver at 7 dpi was significantly lower in the vaccinated than in the control group.

Salmonella Typhimurium colonisation was also lower in the vaccinated than in the control group, but like serovar Enteritidis, only caecum, ovary and oviduct were highly colonised in control group. Vaccinated group had significantly lower colonisation of caecum at 14 and 21 dpi and of ovary at 14 dpi.

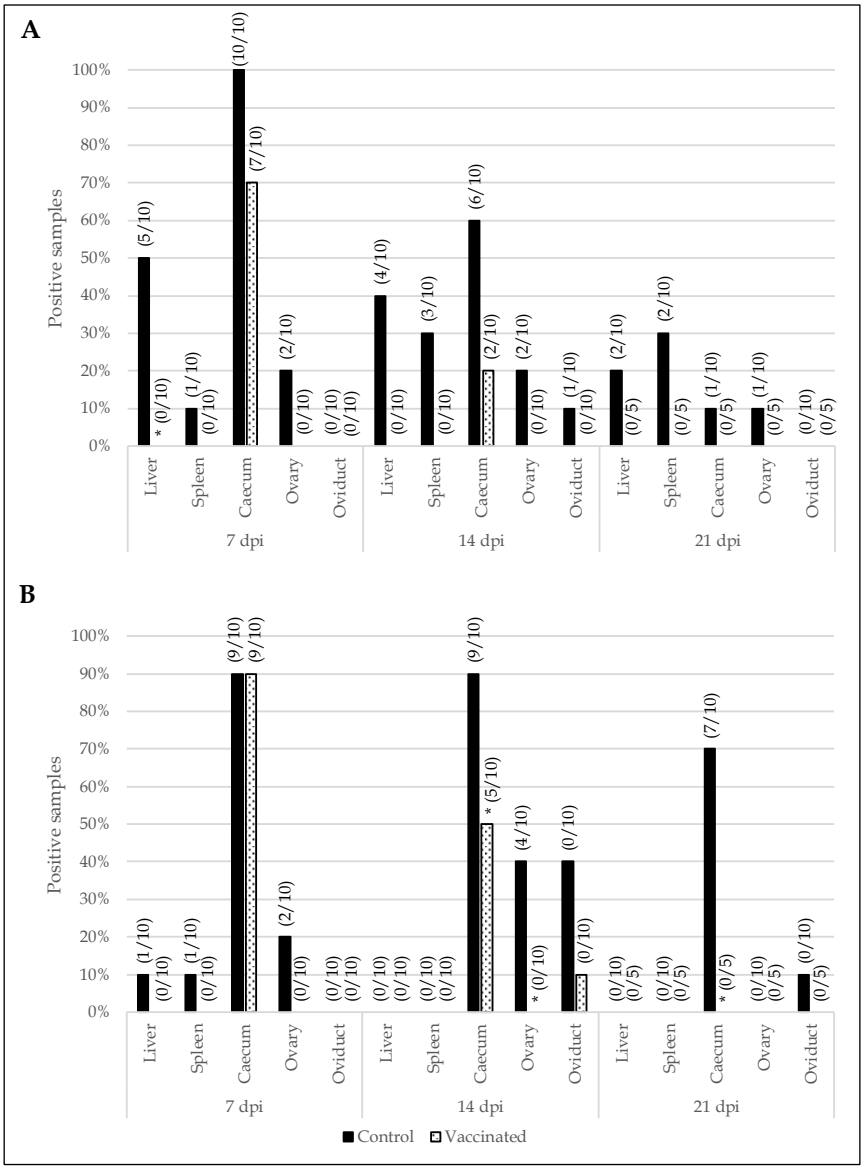

Figure 5-3. Internal organ colonisation of Salmonella spp. after challenge. (A) Salmonella Enteritidis. (B) Salmonella Typhimurium. dpi: days post-infection. An asterisk indicates significant differences between control and vaccinated group ($p \leq 0.05$). The number of positive animals with respect to the total is shown in brackets (+/n).

5.3.2 Antibodies detection in serum and bile

Results of antibody detection against *Salmonella* in serum are shown in Figure 5-4 and antibodies in bile in Figure 5-5. Before the challenge, serum samples from all the animals were analysed for the presence of antibodies against *Salmonella.* All the control animals were negative, while vaccinated animals had a significantly higher titre of antibodies related to the vaccination during the rearing period.

After the challenge, there were no significant differences between vaccinated and control group in serum IgY antibodies or in bile IgA antibodies.

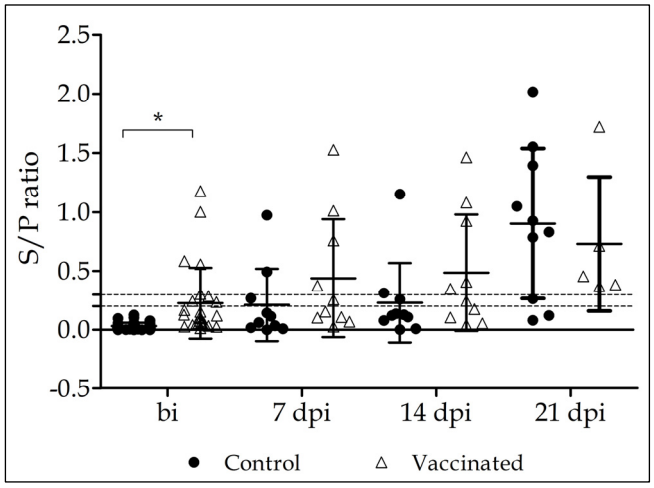

Figure 5-4. Detection of IgY antibodies against Salmonella in serum. Each point represents an animal. Error bars express mean S/P ratio ± SD. Additional discontinuous lines indicate the threshold determined by manufacturer (negative: S/P < 0.2; doubtful: 0.3 > S/P ≥ 0.2; positive: S/P ≥ 0.3). bi: before infection; dpi: days post-infection. An asterisk indicates significant differences between control and vaccinated group (p ≤ 0.05).

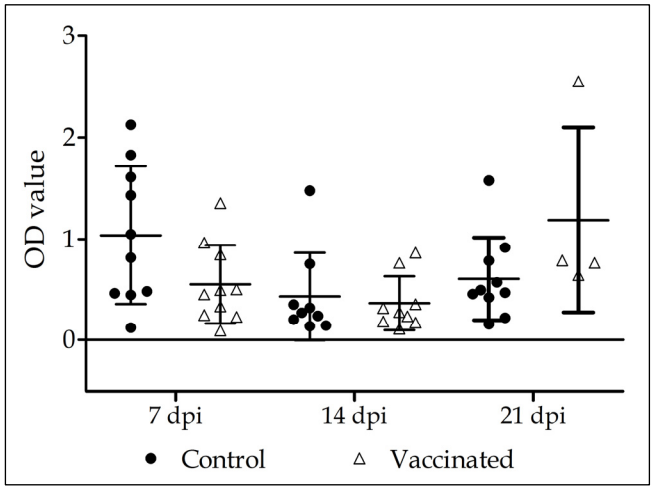

Figure 5-5. Detection of IgA antibodies against Salmonella in bile. Each point represents an animal. Error bars express mean OD value ± SD. dpi: days post-infection. An asterisk indicates significant differences between control and vaccinated group (p ≤ 0.05).

5.3.3 Cellular composition of ileum

To characterise the influx of immune cells in the ileum after challenge, the area occupied by macrophages and CD4+ and CD8+ T cells was quantified. The percentage of macrophages was significantly higher in vaccinated animals at 7 and 14 dpi, and decreased to the same level as the control group at 21 dpi (Figure 5-6).

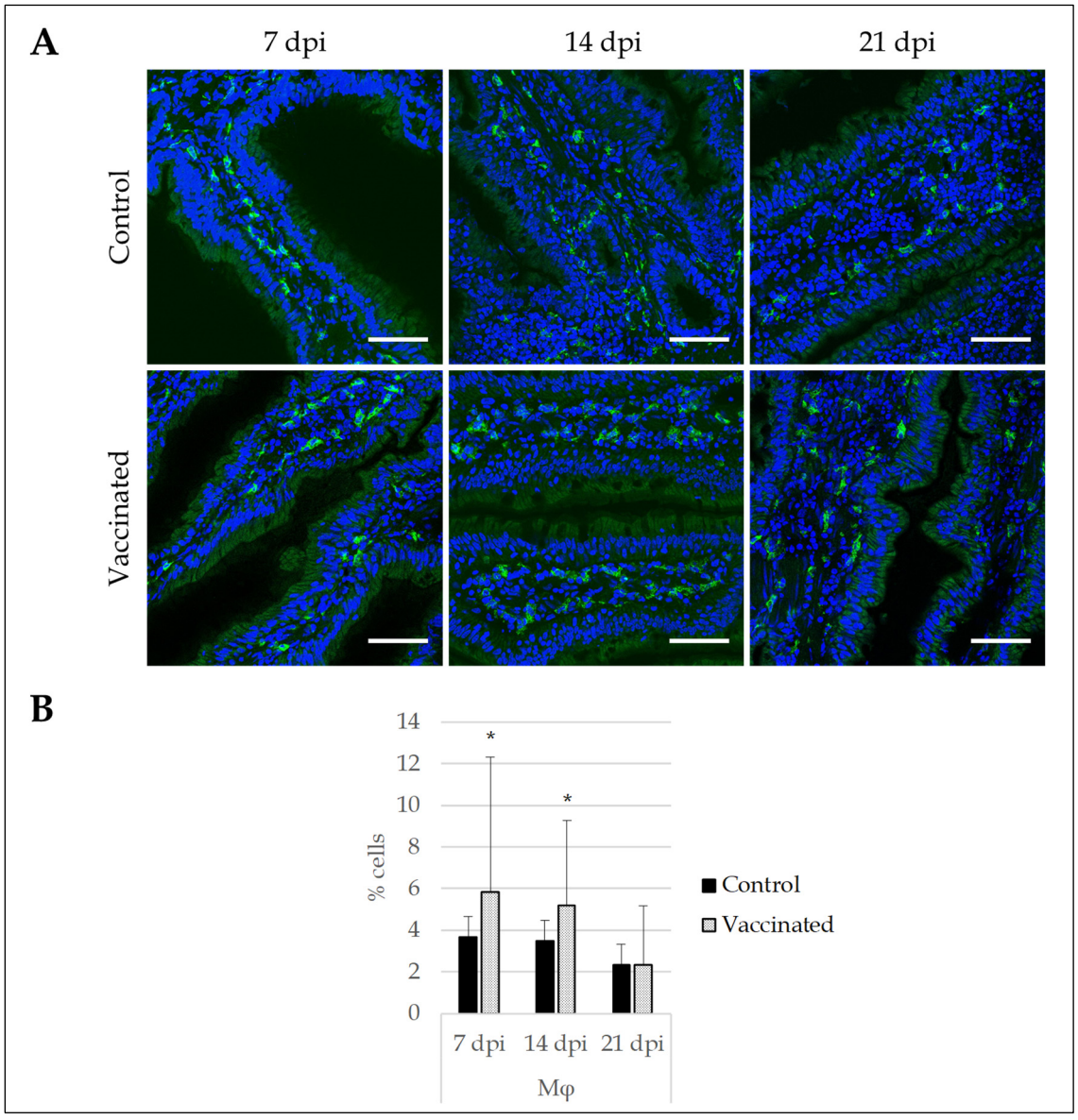

Figure 5-6. Quantification of macrophages in the intestine. (A) Representative images of the stained intestine for macrophages (FITC, green) and nuclei (DAPI, blue). Images were taken at 63x magnification. Scale bar: 50 µm. (B) Results of quantification using ImageJ software. The values represent the mean + SD of 5 images per section and one section per bird. dpi: days post-infection. An asterisk indicates significant differences between control and vaccinated group ($p \leq 0.05$).

The percentage of CD4+ and CD8+ T cells was determined in areas with and without the presence of Peyer's patches. The results of areas without Peyer's patches are shown in Figure 5-7. The population of CD4+ T cells was significantly higher at 14 and 21 dpi

in vaccinated animals compared to control animals. The population of CD8+ T cells was significantly higher in vaccinated animals only at 14 dpi, although an increasing trend can be observed in vaccinated and control groups during the study, corresponding to an active cellular immune response in both groups.

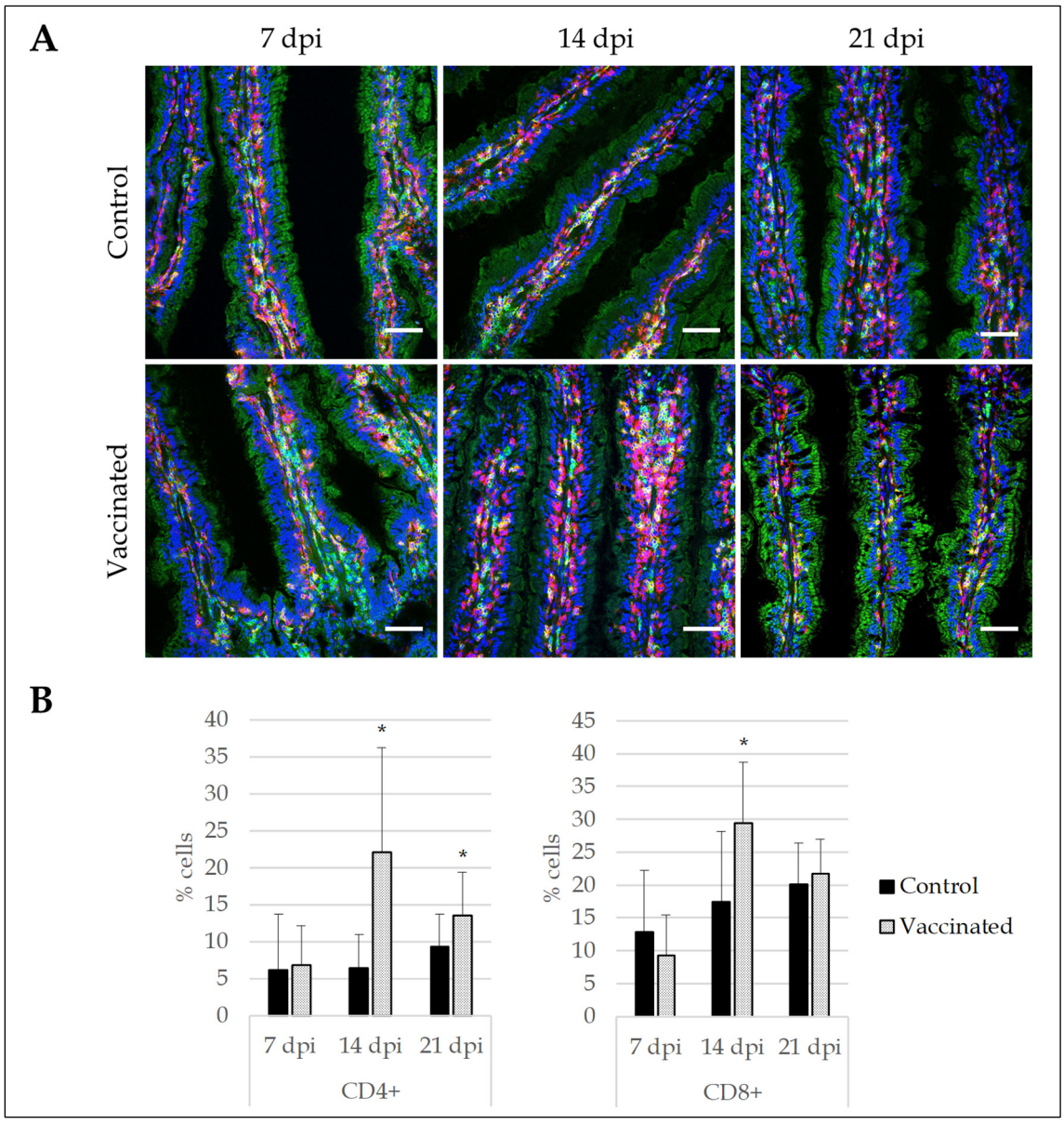

Figure 5-7. Quantification of CD4+ and CD8+ T cells in the villi. (A) Representative images of the stained intestine for CD4+ (AF488, green) and CD8+ (AF647, red) T cells, and nuclei (DAPI, blue). Images were taken at 40x magnification. Scale bar: 50 µm. (B) Results of quantification using ImageJ software. The values represent the mean + SD of 5 images per section and one section per bird. dpi: days post-infection. An asterisk indicates significant differences between control and vaccinated group ($p \leq 0.05$).

The results of areas with Peyer's patches are shown in Figure 5-8. The population of CD4+ T cells was significantly higher in vaccinated animals during all the study. However, there was no difference between groups in CD8+ T cells population at 7 and 14 dpi, although at 21 dpi these cells were significantly higher in control group.

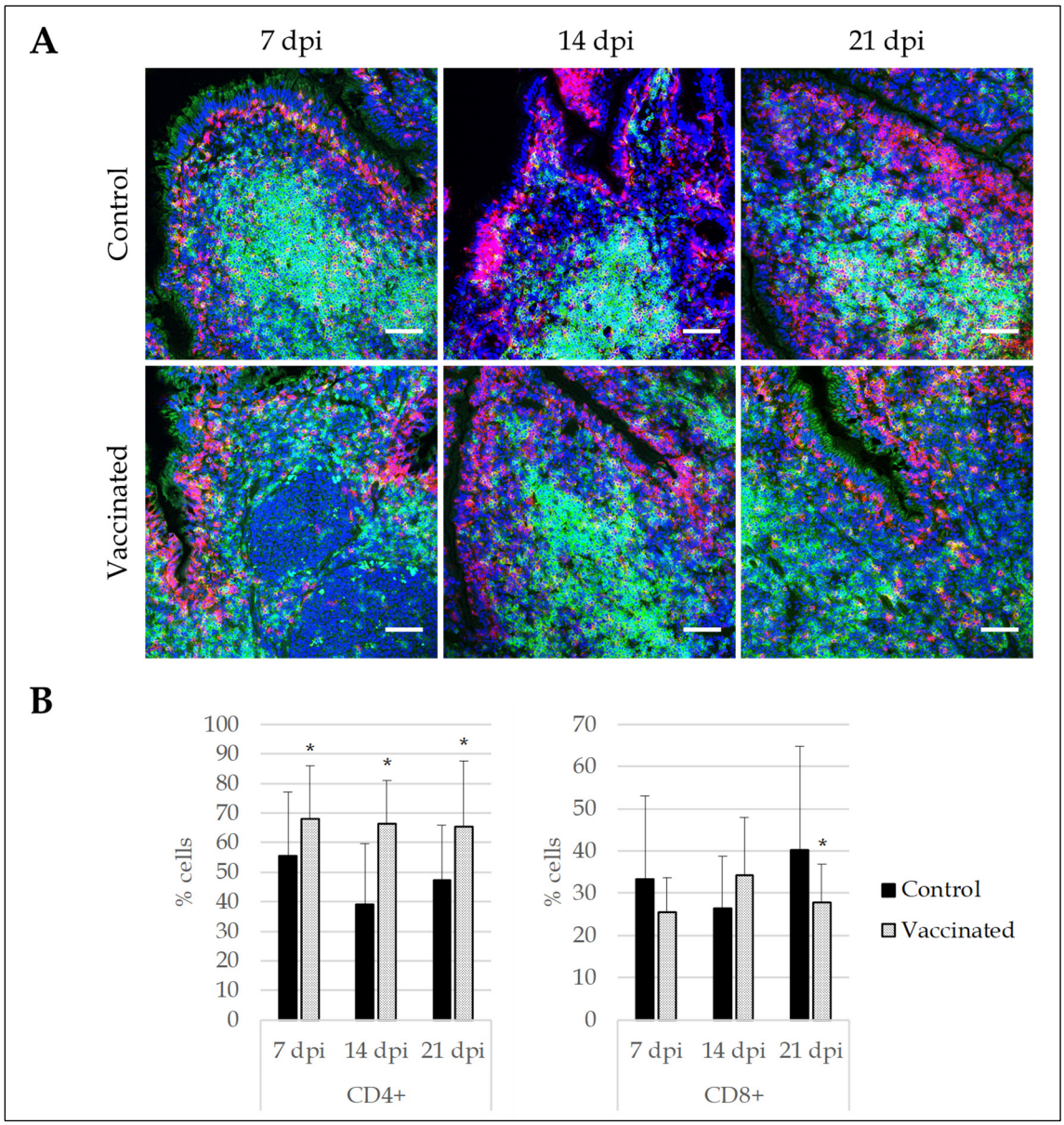

Figure 5-8. Quantification of CD4+ and CD8+ T cells in the Peyer's patches. (A) Representative images of the stained intestine for CD4+ (AF488, green) and CD8+ (AF647, red) T cells, and nuclei (DAPI, blue). Images were taken at 40x magnification. Scale bar: 50 µm. (B) Results of quantification using ImageJ software. The values represent the mean + SD of 5 images per section and per bird. dpi: days post-infection. An asterisk indicates significant differences between control and vaccinated group ($p \leq 0.05$).

5.3.4 Gene expression in the ileal mucosa

Changes in gene expression in the ileal mucosa were compared in the vaccinated group and in the control group after challenge infection. Results are shown in Figure 5-9. The cytokine IFNγ was studied as representative of proinflammatory response and IL-10 as anti-inflammatory response. TLR4 and TLR5 were studied as receptors of *Salmonella* on the innate immune response and MHC II as a marker of antigen presenting cells.

There was high variation in the results between sampling days and a clear pattern was not defined. But in general, there was a significant down-regulation of cytokine TNFα

and surface proteins TLR4, TLR5 and MHC II at 7 dpi; and a following up-regulation of cytokines IFNγ and TNFα and surface proteins TLR4 and MHC II at 14 dpi.

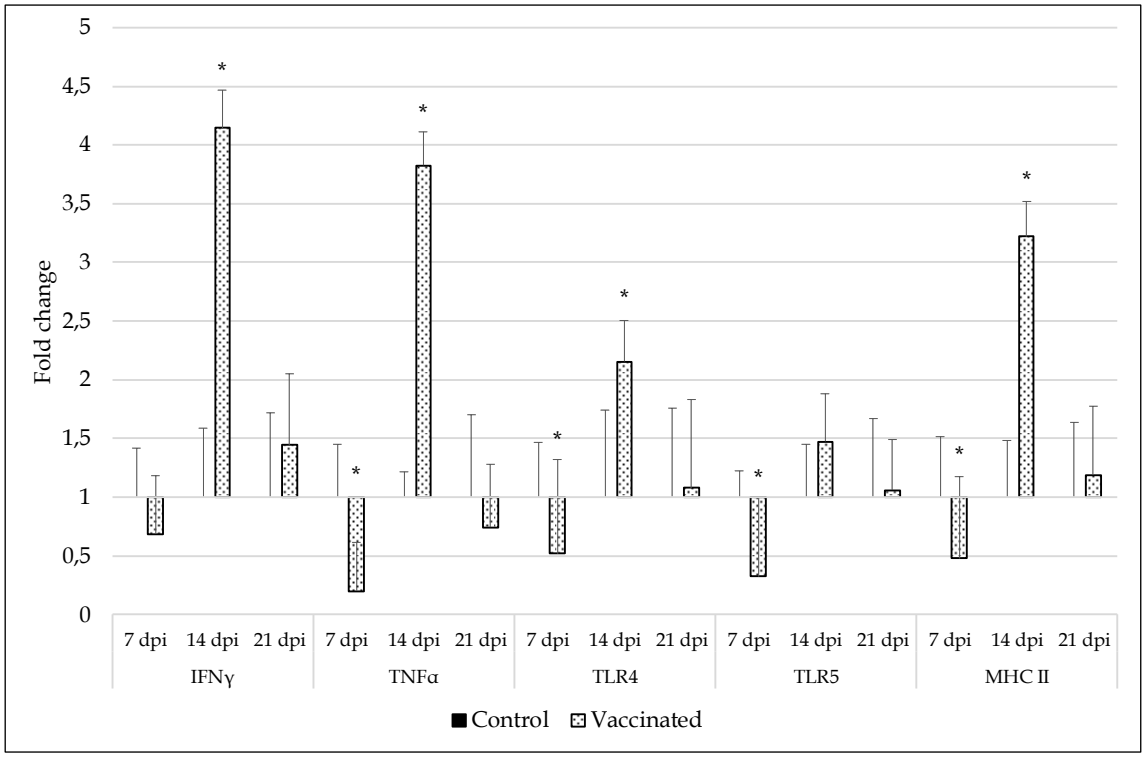

Figure 5-9. Relative fold change in gene expression of cytokines and cellular surface proteins from the ileal mucosa. Data are expressed as mean expression levels for vaccinated group normalised to reference gene and relative to control group + SD. dpi: days post-infection. An asterisk indicates significant differences between control and vaccinated group ($p \leq 0.05$).

5.4 DISCUSSION

The main objective of the present study was to evaluate the levels of protection of a bivalent live attenuated vaccine applied during the rearing period at the end of the laying period and the associated humoral and cellular immune response. Our results showed that vaccinated animals were still protected at week 63 of life, because after the challenge infection, the excretion rates and colonisation of internal organs were lower than in non-vaccinated control animals.

After challenge, vaccinated group had lower excretion rate and lower organ colonisation of both *Salmonella* Enteritidis and *Salmonella* Typhimurium strains. These results demonstrate that the duration of immunity after three vaccine doses at day-old, week 7 and week 18 of age reaches at least until week 63 of life, although it could be longer. In a similar study that followed the European Pharmacopoeia guidelines for determining immunogenicity at the end of the laying period, it was demonstrated also partial protection of a live attenuated vaccine. The laying hens were vaccinated with a live

attenuated *Salmonella* Enteritidis vaccine at 1, 8 and 12 weeks of age and challenged orally with a homologous virulent strain at 77 weeks of age. If compared with our study, they also found a significant reduction of challenge strain excretion at 5 and 7 dpi in vaccinated animals, but internal organ colonisation cannot be compared because they summed the positive and negative organs corresponding to 7 and 14 dpi altogether (Theuß et al., 2018). Other authors have also determined that vaccination during rearing period can last until an advanced age. In one study it was determined that two vaccination protocols consisting in three doses of a live attenuated vaccine or two doses of this live attenuated vaccine plus one booster with a killed vaccine (both *Salmonella* Enteritidis) conferred protection against either *Salmonella* Enteritidis or *Salmonella* Typhimurium at the end of laying period (70 weeks) (Springer et al., 2011). In another study, although the vaccination protocols consisted in two or three doses of a *Salmonella* Enteritidis killed vaccine, after challenge at 59 weeks of age, vaccinated animals had lower rates of cloacal swabs, organs and eggs positive to *Salmonella* Enteritidis field strain (Woodward et al., 2002).

The possibility that a vaccine applied during rearing reaches levels of protection until the end of laying indicates that it is a very useful tool in controlling the prevalence of *Salmonella* in the poultry industry.

Nevertheless, in the present study some of the organs of the control group did not reach high colonization rates, making it difficult to find significant differences comparing with the vaccinated group. Other studies have also revealed that challenge of 20-week old layers by oral gavage with a high dose (5×10^8 cfu) of four different strains (one *Salmonella* Enteritidis, one *Salmonella* Typhimurium and two monophasic *Salmonella* Typhimurium) resulted in low rate of infected organs, mainly in ovaries (Arnold et al., 2014). The infection rate has been tried to solve through an intravenous challenge, which achieves high rates of internal organ colonisation, especially in ovaries and eggs, although it does not mimic the infection in field conditions (Woodward et al., 2002).

Most live attenuated vaccines against *Salmonella* licensed in the EU recommend 3 doses of vaccine (Gantois et al., 2006; Eeckhaut et al., 2018; Theuß et al., 2018; Huberman et al., 2019). It has been seen than the first dose is crucial for protecting animals early after hatching, because very young animals are more susceptible to *Salmonella* infection due to an immune system not fully developed (Bailey et al., 1994; Van Immerseel et al., 2005). The time of second vaccination is not considered important for protection, ranging

between week 3 and 7 of life, without affecting the efficacy of the vaccine against challenge (Methner, 2018). Generally, it has been proven in a study comparing several vaccination protocols that both a higher number of vaccine doses and the proximity between the last dose and the challenge improved the protection of the animals (Huberman et al., 2019). However, a vaccine dose before the onset of lay has been considered crucial to confer stronger immunity until end of the laying period in this last study, and also to prevent a possible decrease in egg laying if a salmonella infection occurs (Gantois et al., 2006; Huberman et al., 2019). The last vaccine dose should be administered almost 3 weeks before the onset of lay to avoid any chance of the vaccine strain to reach the food chain, although the vaccine strain in adult chickens is isolated from cloacal swabs a very short period of time (Huberman et al., 2019).

In the present study, both the vaccine and the challenge inoculum were formulated with a combination of *Salmonella* Enteritidis and *Salmonella* Typhimurium. Therefore, we cannot determine if protection against each strain was conferred by one or both vaccines. But other studies have compared protection levels of vaccines either monovalent with *Salmonella* Enteritidis or *Salmonella* Typhimurium or bivalent with the two serovars; and have determined that there is a potential synergy between serovars, because group vaccinated with bivalent vaccine had lower *Salmonella*-infected eggs compared with groups vaccinated with only one serovar (Gantois et al., 2006).

The humoral response in serum or bile in vaccinated animals did not correlate with the reduction of excretion and colonisation of the challenge strain. In fact, there were no significant differences between groups after challenge, although before challenge the antibodies titre in vaccinated animals was significantly higher than in controls, fact that reflect the baseline immunity levels conferred by vaccination. As can be seen in other studies in adult hens, the level of antibodies against *Salmonella* in serum of vaccinated animals can be already high before challenge (due to immunisation) and do not increase after infection, compared with control groups that develop a significantly stronger humoral response after primary infection (Withanage et al., 2005). In another efficacy study of a live attenuated *Salmonella* Enteritidis vaccine in laying hens, the level of antibodies were low before challenge, but after challenge it was significantly higher in the non-vaccinated group (Theuß et al., 2018). Other authors have detected an absence of correlation between high antibody titres in laying hens vaccinated with a combination of live and inactivated *Salmonella* vaccine and the reduction of the challenge strain in

internal organs or intestine (Springer et al., 2011; Penha Filho et al., 2012). In all the mentioned studies, humoral response was not correlated with protection, either because vaccinated and protected animals had lower levels of antibodies compared with controls, or because higher titres of antibodies (mainly in animals vaccinated with killed vaccines) were not accompanied with protection. These observations confirm that humoral response is not essential for protection against *Salmonella.*

In the present study, the levels of secretory IgA detected in the intestine were not different between vaccinated or control group, as it has been previously seen in other studies (Penha Filho et al., 2012). The possibility of a protective role for IgA immunoglobulins in the intestinal mucosa was proposed previously with the hypothesis that IgA could reduce bacterial adherence to the epithelium (Beal et al., 2004a). Conversely, high IgA levels in intestine have been correlated with also high intestinal colonisation by *Salmonella* (Berthelot-Hérault et al., 2003). High numbers of *Salmonella* in the intestine to be presented to the immune system can induce high antibody titres without a protective function (Beal and Smith, 2007).

In the absence of a protective humoral response, a cellular immune response in the gut should be expected. In general, there was an increase of macrophages and CD4+ and CD8+ T cells in the lamina propria of the villi and an increase of CD4+ T cells in the Peyer's patches in vaccinated animals compared to control group.

Macrophages have different functions in the early innate immune response against *Salmonella.* These cells engulf bacteria, present antigens to B and T cells, and produce proinflammatory cytokines that trigger other cells of the immune response (Arango Duque and Descoteaux, 2014). The population of macrophages in the intestine has been determined to increase in young chicks after *Salmonella* infection (Van Immerseel et al., 2002b; Braukmann et al., 2016). In adults there is little information about the function of macrophages at the intestinal level after a *Salmonella* infection, however, the increase of macrophages in ovaries and oviducts of laying hens after a secondary infection with *Salmonella* Enteritidis has been correlated with an also elevated number of T cells and a reduction in *Salmonella* Enteritidis recovery from the reproductive tract, indicating that these cells play a role in early inflammation and protection (Withanage et al., 2003).

The population of T cells increases after *Salmonella* infection (Berndt and Methner, 2001). In general, CD8+ T cells are expected to lead a Th1 cellular protective response against

secondary infection of *Salmonella,* as seen in previous studies where this population increased both in the intestine and caecal tonsils after primary infection in adult laying hens (Bai et al., 2014). In the same way, the increase of CD8+ T cells in the caecal tonsils has been correlated with a decrease in the *Salmonella* counts in the caecum (Penha Filho et al., 2012). As expected, the CD4+ helper T cell and CD8+ cytotoxic T cell population increased in the villi of the intestine in our study early after infection. However, only CD4+ T cell population seem to increase significantly compared to non-vaccinated group in the Peyer's patches, which is the same result found in a primoinfection of adult hens in the Peyer's patches and caecal tonsils (Holt et al., 2010). CD4+ T cells are also the main T cell population present in the lamina propria of the Peyer's patches (Bucy et al., 1988; Smith et al., 2013). The increase of CD4+ T cells in Peyer's patches could involve a stimulation of the antigen presentation through the MHC class II expressing cells and increase in bacterial clearance (Wosen et al., 2018). The difference detected in the cell populations compared with other studies could be related with the age of the chickens, but also could be related with the limitation of the technique, because the we quantified the cells using immunohistofluorescence and confocal microscope (equivalent to Penha Filho et al., 2012, quantified by immunohistochemistry) but the other mentioned studies quantified the cells by flow cytometry, which could be more accurate and less time consuming than immunohistofluorescence technique (Diederichsen et al., 1998).

Proinflammatory cytokines like IFNγ and TNFα were expected to be upregulated in the vaccinated group, due to its role triggering the immune response after *Salmonella* infection and recruiting cells involved in protection (Wigley and Kaiser, 2005). However, the expression of these genes did not follow a clear pattern. Only at 14 dpi there was an up-regulation of IFNγ and TNFα, but this up-regulation was expected to occur at the same time of S*almonella* clearance like in other studies (Bai et al., 2014), so the results are not conclusive. In another efficacy study, the IFNγ was also found not to increase after *Salmonella* infection and clearance, indicating that other mechanisms are involved or that detection was not accurate (Penha Filho et al., 2012).

The immune response against *Salmonella* involve the recognition of bacteria through TLR4 and TLR5 expression in epithelial cells and presentation of antigen through MHC II pathway (Rychlik et al., 2014; Wosen et al., 2018). These genes were downregulated at 7 dpi and upregulated at 14 dpi (except TLR5) but showed no changes at 21 dpi. These

markers were expected to peak early after infection, because are involved in the innate immunity response.

As a conclusion, early vaccination with a bivalent vaccine during rearing lead to protection against internal organ colonisation and reduced the rates of *Salmonella* Enteritidis and *Salmonella* Typhimurium excretion in cloacal swabs after challenge at the end of the laying period. This protection was not correlated with a humoral immune response, neither at systemic nor intestinal level. The expected cellular response, however, was detected at intestinal level with the presence of higher macrophages and CD4+ and CD8+ T cells populations but was not related with expression of proinflammatory cytokines or cell markers. Other studies are needed to expand the knowledge in the mechanisms involved in protection against *Salmonella* in adult chickens.

6. GENERAL DISCUSSION

Salmonellosis was the second most reported zoonosis in humans in the EU in 2018. Particularly, *Salmonella* caused one third of all foodborne outbreaks (30.7%), and from the *Salmonella* outbreaks almost half were caused by eggs and egg products (45.6%). If we focus on the predominant serovars, most cases of human salmonellosis were caused by only three serovars: *Salmonella* Enteritidis (49.9%), *Salmonella* Typhimurium (13%), and monophasic *Salmonella* Typhimurium (8.1%) (EFSA, 2019).

These numbers exemplify the importance of reducing the *Salmonella* prevalence specially in laying hens, where *Salmonella* can colonise the intestine without production of disease and enter the food chain through contaminated eggs and meat (Barrow and Methner, 2013). But, despite all the measures implemented to reduce the prevalence of this pathogen in the poultry and pig industry through the National Control Programmes, the number of cases of salmonellosis in humans has stabilised over the last five years, after a long period of a declining trend (EFSA, 2019).

It is essential to remark that *Salmonella* prevention and control cannot be achieved by a single measure alone, and a combination of biosecurity and additional measures is needed (World Organisation for Animal Health (OIE), 2019a, 2019b). Vaccination is considered an additional measure for the control of *Salmonella* in poultry, by increasing the resistance of the bird to the organism through stimulation of the immune system (European Commission, 2006).

Considering the significance of vaccines in the control of salmonellosis, the three studies included in this thesis are part of a project for developing a vaccine against the *Salmonella* serovars of major public health relevance. The main objectives of this thesis were to determine the efficacy of the vaccine against field strain infection, and to better understand the associated immune response in three different points of the poultry production: young animals after 1st vaccination (STUDY I), before the onset of lay after 2nd and 3rd vaccination (STUDY II), and finally at the end of the laying period (STUDY III). The development of an effective vaccine relies on understanding the host response to field strain infection, and how vaccination can modulate it. Most of the published studies about *Salmonella* vaccines are based on the systemic effect of adapted serovars in mice (*Salmonella* Typhimurium), and are not fully comparable with other species (like chickens) because these vaccines are not so effective in protecting against intestinal colonisation of non-adapted serovars (Barrow and Methner, 2013). Due to the different response against adapted and non-adapted serovars, the strong protective capacity

against reinfection achieved with *Salmonella* Gallinarum or Pullorum vaccines, is not accomplished for non-adapted serovars related with food poisoning like *Salmonella* Enteritidis or *Salmonella* Typhimurium (Barrow and Methner, 2013).

The criteria followed during the studies of this thesis to determine if a *Salmonella* Typhimurium live attenuated strain could be a good vaccine candidate were protection against intestinal and systemic infection, stable attenuation markers, ability to differentiate from field strains, and long-lasting protection. These are the keys defined by the publications related to the development of vaccines against *Salmonella* (Barrow and Methner, 2013).

In the first study, we determined that a first vaccine dose administered orally early after hatching was able to protect partially against field strain infections and reduced colonisation of liver and spleen. The comparative analysis between the oral and spray administration of the vaccine resulted in higher efficacy of oral route, probably because the spray administration failed. Previous publications have determined that coarse spray vaccination can increase vaccine efficacy compared with oral vaccination, thus it should be studied in future experiments (De Cort et al., 2015; Varmuzova et al., 2016). Although first vaccination did not confer a complete protection (Parker et al., 2011; Braukmann et al., 2016), this situation could be improved by the application of vaccine boosters (Methner, 2018).

In the second study, we determined that after a second and third vaccine dose chickens were partially protected against infection with field strains of *Salmonella* Typhimurium and had lower excretion and lower bacterial colonisation of liver and spleen compared to the control group. It seems that in Experiment 1 after only two vaccine doses animals had higher protection against excretion and internal organ colonisation, compared with challenge after three vaccine doses in Experiment 2. In the Experiment 1 shorter time happened between the last vaccine dose and the challenge, which could cause the animals to experience a stronger immune response and higher protection (Huberman et al., 2019).

In the third study, we determined the efficacy the vaccination with a combined *Salmonella* Enteritidis and *Salmonella* Typhimurium during rearing at the end of the laying period. Vaccinated hens were challenged with a combined inoculum of the *Salmonella* serovars included in the vaccine and showed a reduction in the excretion and

colonisation of internal organs by the challenge strains compared with a non-vaccinated control. By using a combined challenge inoculum, we were able to determine at the same time the protection against two different *Salmonella* serovars using a limited number of animals. This was possible thanks to the ability to discriminate between different serovars by PCR.

The infection with *Salmonella* induces the production of high levels of antibodies in chickens, which can be used for monitoring the possible contact of the flock with the pathogen but are not correlated with the protection degree. In the STUDY I, humoral response was detected first in bile at 3 weeks of age (1 week after challenge) and later in serum at 4 weeks of age (2 weeks after challenge). In STUDY II humoral response at intestinal level was significantly higher in non-vaccinated groups and correlated with higher levels of *Salmonella* in the intestine and internal organs. In STUDY III, no differences between vaccinated and control groups were detected, but animals were considerably older, and this factor could reduce the immune response. The level of antibodies (detected by ELISA) and B cells (Flow Cytometry) were related in the STUDY I (both levels increased without differences between groups) but not in STUDY II (the increase of antibodies in the control group were not observed in B cells with Flow Cytometry).

Salmonella stimulates the infiltration of immune cells in the intestine that are considered key in the protection against infection. In the STUDY I, clearance of the bacteria was not correlated with the cellular immune response, but the peak of involved cells like macrophages or T cell subpopulations could have occurred before the first sampling point. In the STUDY II, however, an increase in CD3+ T cell recruitment in the intestine could be related with protection. In STUDY III, an influx of macrophages and CD4+ and CD8+ T cells were detected in the intestine and Peyer's patches, showing an adaptive cellular immune response.

The gene expression of cytokines was determined to compare with other methods. In STUDY I, only a diffuse proinflammatory response at 7 dpi mediated with IFNγ and the resolution of inflammation by IL-10 at 14 dpi was observed. Key cytokine IFNγ dominated also response in STUDY II, being the only one cytokine upregulated in vaccinated group and indicating a possible drift to Th1 response protective. No relevant changes in gene expression were detected in STUDY III, indicating that association between cellular response and associated cytokines is not easy to determine.

An interesting point to consider when evaluating efficacy tests is the type of challenge performed. There are different parameters that can decisively affect the effectiveness of a vaccine like the titre of the inoculum or the route of administration (animals can be in contact with field strain by direct inoculation or a seeder challenge can be performed, see below) (Immerseel et al., 2004; Bohez et al., 2007).

The titre of the challenge inoculum influences the outcome of the efficacy studies. High challenge doses (10^7 cfu of *Salmonella* Enteritidis per dose) can overcome any immunity that birds may have had as a result of vaccination (Parker et al., 2011). In our studies, only high doses of *Salmonella* challenge were used (ranging from 10^7 cfu in young animals to 10^8 cfu in older ones), and maybe the partial protection observed in our results could improve with a different challenge approach.

With respect to the type of challenge, an alternative to oral inoculation could be the seeder challenge. This type of challenge mimics the natural route of infection in the farms and is more accurate to determine the real capacity of vaccines to protect animals (Springer et al., 2011). Seeder challenge consist on infecting a group of "seeder" chickens with a defined dose of *Salmonella* and subsequently expose vaccinated and control animals to these "seeder" birds by placing all together in the same pen (Springer et al., 2011). At last, the excretion and colonisation of challenge strain can be compared between vaccinated and control groups. Examples of seeder challenge in the literature indicate that it is a valuable tool to evaluate vaccine efficacy under field-like conditions. In a study that compared seeder challenge model and oral challenge with high or low dose, it was determined that only after seeder challenge it was possible to find lower significant number of colonised organs and level of shedding in vaccinated compared to control animals (Clifton-Hadley et al., 2002). In a similar way, other authors have used the seeder challenge successfully to test the efficacy of different vaccines, indicating that it is a validated option to infect animals (De Cort et al., 2015; Kilroy et al., 2015). Unfortunately, the seeder method could not be accepted by the regulatory authorities because this challenge method cannot assure the number of bacteria that will receive the "in-contact" animals, and it is not included in the European Pharmacopoeia guidelines.

When determining the efficacy of a vaccine, different parameters are analysed. One of them is the colonisation or infection of internal organs. The European Pharmacopoeia guidelines (04/2013:2520 and 04/2013:2521 apply for *Salmonella* Enteritidis and Typhimurium live attenuated vaccines, respectively) ask for the determination of the

presence of the challenge strain in the organs, that is, qualitative determination of positive or negative organs. But a quantitative method to detect *Salmonella* in organs has been applied in multiple efficacy studies of licensed vaccines, in addition to the qualitative one (Clifton-Hadley et al., 2002; Carvajal et al., 2008; Atterbury et al., 2009; Filho et al., 2009; Springer et al., 2011; Kilroy et al., 2015; Braukmann et al., 2016; Eeckhaut et al., 2018; Methner, 2018). With this method, organs are weighted, homogenised and diluted 10-fold and directly plated to determine number of cfu per gram of tissue. The organs that are negative after direct plating are, however, enriched and a qualitative detection is also performed. Having both values of quantitative and qualitative evaluation of the *Salmonella* presence in the organs is interesting because allows us to have a more global vision of the performance of a vaccine. Furthermore, the quantitative method is a useful parameter because higher counts of bacteria in the intestine or caecum increase the probability of horizontal infection of eggs after oviposition (Schoeni et al., 1995). Additionally, the quantitative method is more respectful of animal welfare, because significant differences can be determined between groups with a smaller number of animals, as pointed by other authors (Theuß et al., 2018). In addition to the above, caution is needed to compare the effectiveness of different vaccines if different *Salmonella* detection methods have been used.

A fact that awakes my curiosity is that the presence of *Salmonella* in eggs is not considered in most efficacy studies (neither in the studies presented in this thesis). Why not analyse the main food vehicle source of *Salmonella*? (eggs and egg products were the origin of almost 40% of total strong-evidence *Salmonella* foodborne outbreaks in 2018) (EFSA, 2019). In fact, the European Pharmacopoeia does not explicitly request it, although it does ask for ovaries and oviducts, but we have already seen that vertical transmission is not the only source of *Salmonella*, and it is not clear if it is the main one or not (Gantois et al., 2009). One possible explanation is that most studies testing vaccines are carried out in young animals before the onset of laying (Methner et al., 1997; Van Immerseel et al., 2005). Another possibility is that it is difficult to find significant differences between vaccinated and non-vaccinated animals in egg contamination by *Salmonella* because the rate of infection of eggs (and reproductive tissues) is very low, especially after oral challenge (Arnold et al., 2014). Intravenous challenge may increase this ratio, but it moves away from natural route of infection (Woodward et al., 2002). In fact, the reports of EFSA confirm that the prevalence of *Salmonella* in 2018 in table eggs was effectively very low (0.37%), compared with other food matrices like broiler or

turkey meat where positive samples can represent up to 7% (EFSA, 2019). The reason of eggs causing most foodborne outbreaks having a very low *Salmonella* prevalence is, as mentioned before in this thesis, the consumption habits of using undercooked or raw eggs to prepare different food specialities or the high storage temperature for this product (EFSA, 2019). A possible solution to improve the available information about the tandem vaccines/eggs would be to carry out field studies with a high number of animals, in order to determine the real efficacy of a *Salmonella* vaccine plan in conjunction with other biosafety measures to reduce prevalence in table eggs (Sharma et al., 2018).

Finally, the most important part should be to consider the real contribution of vaccines to protect animals. In which extension the vaccines contributed to the reduction of *Salmonella* prevalence and the reduction of human cases of salmonellosis in the EU since the implementation of the NCP? It is known that to reduce *Salmonella* prevalence a holistic approach is needed where biosafety and hygiene in farms are the main tools and vaccination is a complementary tool, because without the two first, the second makes no sense in the control of the bacteria. As an example, in a study of the effects of vaccination and other control measures against *Salmonella* in commercial farms, it was determined that the farms with poorer cleaning, disinfection, and rodent and insect plans had also the highest *Salmonella* prevalence (Davies and Breslin, 2003).

In general, the use of the *Salmonella* Typhimurium live attenuated vaccine with the schedule indicated in this thesis can be a tool for reduction of horizontal transmission (reduction of *Salmonella* excretion in cloacal swabs) and reduction of vertical transmission (reduction of bacterial colonisation of internal organs, included the reproductive).

To sum up, the studies performed in this thesis can contribute to the knowledge of the avian immune response against *Salmonella* in order to formulate better vaccines for the future of poultry farming.

7. CONCLUSIONS

1. The oral vaccination of day-old chicks with a live attenuated strain of *Salmonella* Typhimurium reduces the colonisation of internal organs when challenged at 14 days of age but booster vaccinations are needed to increase resistance against *Salmonella* infections.

2. Two or three vaccine doses can confer protection to animals against a challenge with field strains and reduce excretion and colonisation of internal organs. This protection is clearly not correlated with a production of antibodies at blood or the intestinal level, but CD3+ cells are significantly increased in vaccinated animals. Variations on CD4+ and CD8+ subpopulations of T lymphocytes are related with the animal age or the timing between vaccination and challenge.

3. The vaccination with a bivalent vaccine during rearing period reduces excretion and internal organ colonisation of *Salmonella* Enteritidis and *Salmonella* Typhimurium challenge at the end of the laying period. This protection is not correlated with a humoral immune response, neither at systemic nor intestinal level. An expected cellular response is detected at intestinal level with the presence of higher macrophages and CD4+ and CD8+ T cells populations but is not related with expression of proinflammatory cytokines or other cell markers.

4. Protection is not correlated with the antibody response in serum or bile, but it is correlated with the recruitment of cells of the innate and adaptive immune system, mainly macrophages and subpopulations of T cells.

5. Different parameters like the number of vaccine doses, but also the time between last immunisation and challenge influence the efficacy of a vaccine against *Salmonella*.

8. APPENDIX

STUDY I. Additional dendrograms created with the distances between biochemical kinetics in isolated colonies at 3, 5, 7 and 14 dpi.

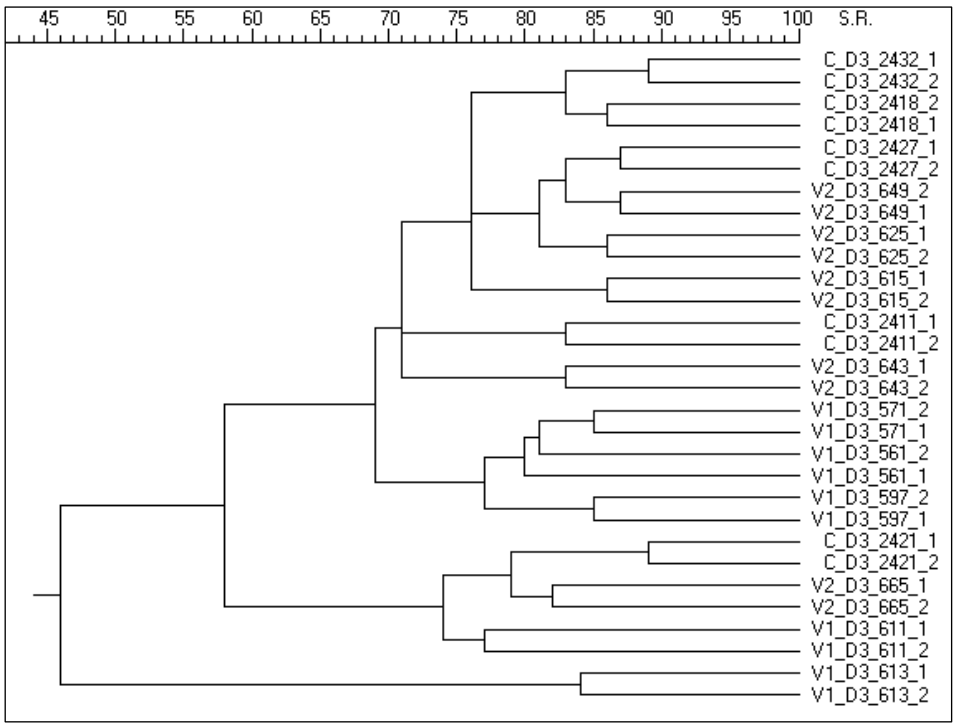

Figure 8-1. Dendrogram obtained with the comparative analysis of biochemical kinetics of different pairs of strains isolated at 3 dpi.

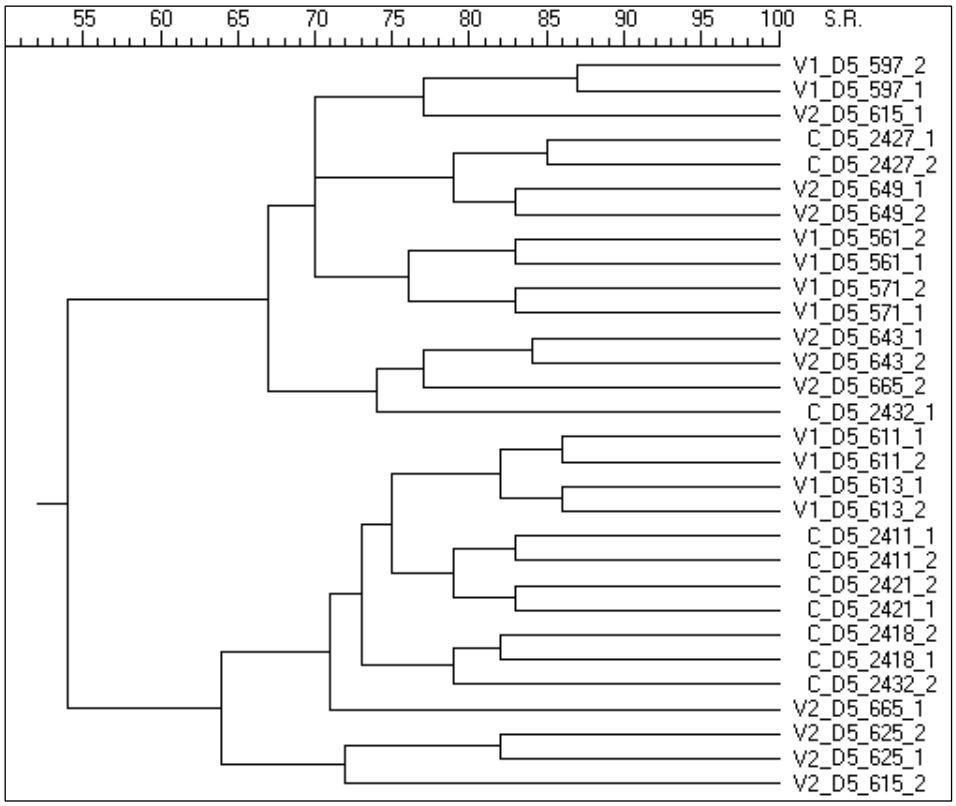

Figure 8-2. Dendrogram obtained with the comparative analysis of biochemical kinetics of different pairs of strains isolated at 5 dpi.

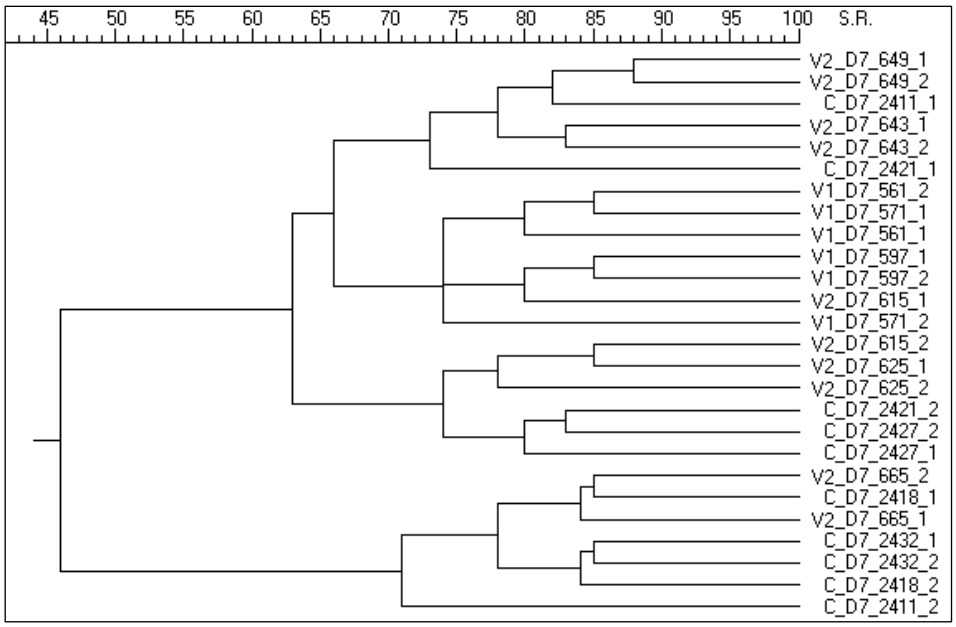

Figure 8-3. Dendrogram obtained with the comparative analysis of biochemical kinetics of different pairs of strains isolated at 7 dpi.

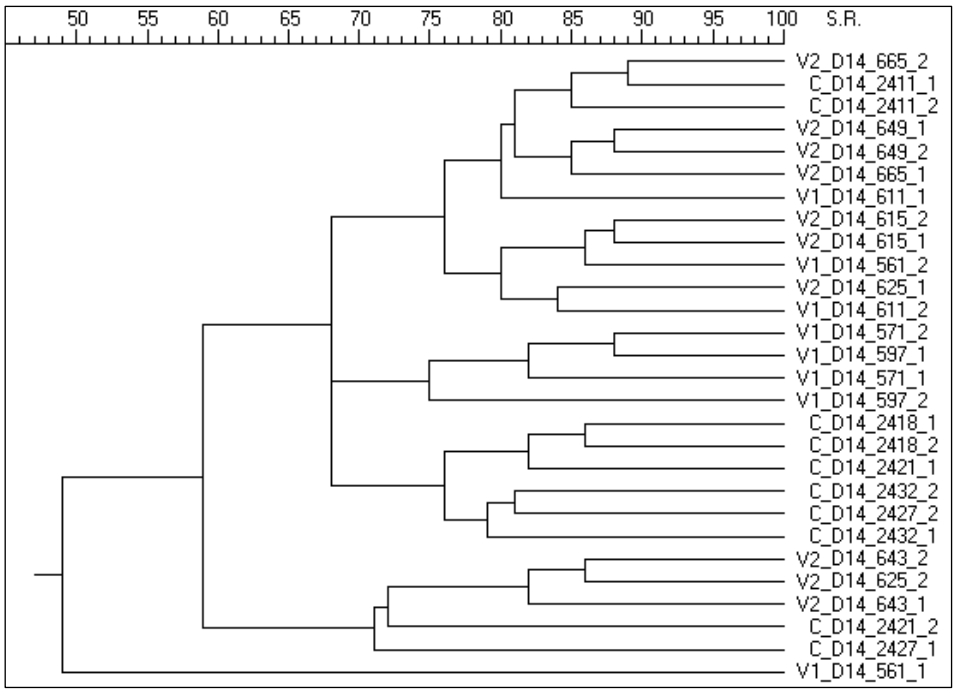

Figure 8-4. Dendrogram obtained with the comparative analysis of biochemical kinetics of different pairs of strains isolated at 14 dpi.

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