Optimization of the control of Salmonella infections in pigs

Lotte De Ridder
The only place where success comes before work is a dictionary

(Vidal Sassoon)
Optimization of the control of *Salmonella* infections in pigs

Lotte De Ridder

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<th>Full Form</th>
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<tbody>
<tr>
<td>ADV</td>
<td>Aujeszky’s Disease Virus</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen Presenting Cells</td>
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<tr>
<td>ATR</td>
<td>Acid Tolerance Response</td>
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<td>BIOHAZ</td>
<td>BIOlogical HAZards</td>
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<tr>
<td>BGA</td>
<td>Brilliant Green Agar</td>
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<td>BPEX</td>
<td>British Pig EXecutive</td>
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<tr>
<td>BPW</td>
<td>Buffered Pepton Water</td>
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<tr>
<td>CFU</td>
<td>Colony Forming Units</td>
</tr>
<tr>
<td>CODA</td>
<td>Centrum voor Onderzoek in de Diergeneeskunde en Agrochemie</td>
</tr>
<tr>
<td>CERVA</td>
<td>Centre d'Etude de Reserche Vétérinaire et Agrochimique</td>
</tr>
<tr>
<td>DGZ-</td>
<td>Dierengezondheidszorg</td>
</tr>
<tr>
<td>ARSIA</td>
<td>Association Régionale de Santé et d'Identification Animales</td>
</tr>
<tr>
<td>DIASALM</td>
<td>DIAgnostic semi-solid SALMonella agar</td>
</tr>
<tr>
<td>DIVA</td>
<td>Differentiation of Infected and Vaccinated Animals</td>
</tr>
<tr>
<td>DNA</td>
<td>DeoxyriboNucleic Acid</td>
</tr>
<tr>
<td>DT</td>
<td>Definitive phage Type</td>
</tr>
<tr>
<td>ECDC</td>
<td>European Centre for Disease Prevention and Control</td>
</tr>
<tr>
<td>EFSA</td>
<td>European Food Safety Authority</td>
</tr>
<tr>
<td>ELFA</td>
<td>Enzyme-Linked Fluorescent Assay</td>
</tr>
<tr>
<td>(AC) ELISA</td>
<td>(Antigen-Capture) Enzyme-Linked Immuno-Sorbent Assay</td>
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<tr>
<td>EU</td>
<td>European Union</td>
</tr>
<tr>
<td>FA</td>
<td>Fatty Acids</td>
</tr>
<tr>
<td>FAO</td>
<td>Food and Agriculture Organization</td>
</tr>
<tr>
<td>FASFC</td>
<td>Federal Agency for the Safety of the Food Chain</td>
</tr>
<tr>
<td>FCC</td>
<td>Federal Communications Commission</td>
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<tr>
<td>FIA</td>
<td>Freund’s Incomplete Adjuvant</td>
</tr>
<tr>
<td>FLF</td>
<td>Fermented Liquid Feeding</td>
</tr>
<tr>
<td>GRAS</td>
<td>Generally Recognized As Safe</td>
</tr>
<tr>
<td>HACCP</td>
<td>Hazard Analysis and Critical Control Points</td>
</tr>
<tr>
<td>IFC</td>
<td>Information of the Food Chain</td>
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<tr>
<td>ISO</td>
<td>International Standardisation Organisation</td>
</tr>
<tr>
<td>LAMP</td>
<td>Loop-mediated isothermal AMPlification</td>
</tr>
<tr>
<td>LPS</td>
<td>LipoPolySaccharide</td>
</tr>
</tbody>
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MALDI-TOF MS: Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry
MKTTn: Muller Kauffmann TetraThionate novobiocin broth
MLST: MultiLocus Sequence Typing
MLVA: Multi-Locus Variable-number of tandem-repeats Analysis
MS: Member States
MSRV: Modified Semi-solid Rappaport Vassiliadis agar
NRSS: National Reference Centre for Salmonella and Shigella
OD: Optical Density
OMP: Outer Membrane Porine
OR: Odds Ratio
PAMP: Pathogen-Associated Molecular Pattern
PCR: Polymerase Chain Reaction
PFGE: Pulsed-Field Gel Electrophoresis
RFLP: Restriction Fragment Length Polymorphism
R0/Ra: Basic/Adjusted Reproduction ratio
RV: Rappaport Vassiliadis broth
RVS: Rappaport Vassiliadis broth with Soya
S/P ratio: Sample-to-Positive ratio
SAP: Salmonella Action Plan
SSAP: herd-Specific Salmonella Action Plan
TSI: Triple Sugar Iron agar
VIDAS: Vitek Immuno Diagnostic Assay System
WGS: Whole Genome Sequencing
WHO: World Health Organization
WIV-ISP: Wetenschappelijk Instituut voor Volksgezondheid
ISP: Institut Scientifique de Santé Publique
XLD: Xylose Lysine Desoxycholate agar
XLT-4: Xylose Lysine Tergitol-4
YOPI: Young, Old, Pregnant and Immunocompromised
ZAP: Zoonoses Action Plan Salmonella monitoring scheme
ZNCP: Zoonoses National Control Programme for Salmonella in pigs
Chapter 1 – General Introduction
1. **Salmonella taxonomy and characteristics**

*Salmonella* is a zoonotic, gram-negative bacterium with facultative-anaerobic capacities, and which possesses peritrichous flagellae for its motility. The bacterium belongs to the family of Enterobacteriaceae, as is *Escherichia coli*, where it diverged from 100-150 million years ago (Dougan *et al.*, 2011). *Salmonellae* are ubiquitous pathogens, due to i) their many reservoir hosts (warm- and cold-blooded animals) and transmission vectors (feed, farm material, rodents, pets, birds), ii) the stress-induced faecal shedding of carriers, and iii) their environmental persistence, *e.g.* multiplication is possible between 7-45°C and 4.0-9.5 pH; survival outside a host can last for months; and freezing does not eliminate the organism. The genus ‘*Salmonella*’ consists of two species, ‘*Salmonella enterica*’ and ‘*Salmonella bongori*’, of which the first is further divided into six subspecies (Fig. 1).

![Classification of the Genus Salmonella in species and subspecies](Guibordenche *et al.*, 2010).

These six subspecies of *S. enterica* and *S. bongori* comprise together 2610 characterized serotypes or serovars (Grimont and Weill, 2007), based on different, highly immunogenic surface antigens: the somatic ‘O’, flagellar ‘H’ and capsular ‘Vi’ antigens. The antigenic formulae of these *Salmonella* serovars are listed according to the White-Kauffmann-Le Minor scheme (Grimont and Weill, 2007). Some serovars are adapted to a single host species (*e.g.* *S. Typhi* in humans and *S. Choleraesuis* in swine), but the majority of the serovars are capable of causing disease in a broad range of hosts (*e.g.* *S. Typhimurium* and *S. Derby*). Most serovars with zoonotic potential belong to *S. enterica* subspecies *enterica* (Guibourdenche *et al.*, 2010). *Salmonella enterica* subspecies *enterica* serovar Typhimurium for instance, or shortly ‘*Salmonella Typhimurium*’ in common use, has been the most and second most prevalent serotype in pigs and humans respectively, in the EU during the past years (Hendriksen *et al.*, 2011; EFSA and ECDC, 2014). This serovar Typhimurium has therefore been investigated the most in recent studies on control strategies in pigs (Letellier *et al.*, 2000; Anderson *et al.*, 2004; Nagy *et al.*, 2008; Martín-Peláez *et al.*, 2010; Hur *et al.*, 2011; Leyman *et al.*, 2011; Arguello *et al.*, 2013), as is the case for the transmission experiments and the field trial in this thesis.
Based on their susceptibility to antimicrobials and to a defined set of bacteriophages, *Salmonella* strains within the same serotype can be further divided. With the latter method, different phage types (DT) are determined within the same serotype. In the experiments conducted within the scope of this thesis, two different strains of a *Salmonella* Typhimurium were used: *Salmonella* Typhimurium phage type DT 9 (a commercial and experimental one) for immunization, and a *Salmonella* Typhimurium strain 112910a, phage type 120, for infection. More details can be found in Chapter 3.

2. *Salmonella* infection in humans

2.1 Epidemiology

In 2012, 91034 and 3101 confirmed cases of human salmonellosis were reported in the EU and in Belgium, respectively (NRSS, 2011; EFSA and ECDC, 2014). A total of 61 deaths due to non-typhoidal salmonellosis were reported in the EU, resulting in an estimated case-fatality rate of 0.14 %.

Although a significant decrease in the number of human salmonellosis cases has been observed in recent years (mainly related to the successful *Salmonella* control programmes in poultry), it was still the second most commonly reported zoonotic disease in 2012, after campylobacteriosis. In terms of reported food-borne outbreaks in the EU, *Salmonella* even accounted for the largest number in 2012 (28.6 % of all outbreaks), followed by bacterial toxins (14.5 %), viruses (14.1 %) and *Campylobacter* (9.3 %) (Fig. 2; EFSA and ECDC, 2014). Globally, approximately 93.8 million cases of *Salmonella*-induced gastroenteritis occur each year, resulting in 155000 fatalities (Majowicz et al., 2010).

![Figure 2: Distribution of food-borne outbreaks per causative agent in the EU in 2012 (EFSA and ECDC, 2014).](image-url)
Obviously, the aforementioned reported cases are an underestimation of the real salmonellosis occurrence in humans, since not all infected people develop symptoms, not all ill persons are sampled, and not all positive sample results are reported to public health authorities.

In the past years, *Salmonella Enteritidis* and *Salmonella Typhimurium* were the most frequently reported serovars in confirmed human cases, not only in Belgium (20.9% and 53.7% respectively in 2012 (NRSS, 2012)) (Fig. 3), but also in the EU (41.3 % and 22.1 % respectively in 2012 (EFSA and ECDC, 2014)), and worldwide (43.5 % and 17.1 % respectively in 2001-2007 (Hendriksen et al., 2011)). As a result of a harmonised reporting and also several large outbreaks, monophasic *Salmonella Typhimurium* 1,4,[5],12:i:- was the third most commonly reported serovar in the EU (7.2 %) (EFSA and ECDC, 2014).

Although human infection can occur through direct and indirect animal contact or environmental contamination (Hoelzer et al., 2011), most human salmonellosis cases are caused by the consumption of raw or undercooked contaminated food (i.e. meat or vegetables already contaminated before preparation, or cross contaminated during preparation in the kitchen). In the most recent Biological Hazards (BIOHAZ) report from EFSA, it was estimated that approximately 56.8% of the human salmonellosis cases in the EU could be attributable to pigs, while the contribution associated with poultry (laying hens (eggs), broilers and turkeys) was 30.2% (EFSA, 2012b). In the preceding years (2006-2009) in Belgium, this percentage of human salmonellosis due to pork consumption was even estimated to be 73.7% by Pires et al. (2011). However, when observing the risk of the different sources related to the tonnage of consumed food, the EFSA study stated that egg consumption was still the
main risk for human salmonellosis in the EU, closely followed by pig meat. Considering the
transmission of Salmonella serovars separately, human salmonellosis due to Salmonella Enteritidis is
generally associated with the consumption of contaminated eggs and poultry meat, while Salmonella
Typhimurium cases are mostly linked to the consumption of contaminated pig meat.

2.2 Symptoms

Salmonella-infections often occur asymptptomatically in humans. In case a non-typhoidal
salmonellosis takes place, this generally manifests as diarrhoea (and consequently dehydration),
abdominal cramps, vomiting and fever. These symptoms usually begin 12 - 24h after oral uptake, and
resolve after four to seven days without treatment. In a low percentage of diseased individuals however,
a systemic infection develops, possibly resulting in arthritis, pneumonia, organ abscesses, kidney
failure and even death. Certain human subpopulations are more susceptible to infection and to
developing these symptoms: the so-called ‘YOPI’ group (young, old, pregnant and immunocompromised).

2.3 Therapy

Rehydration and electrolyte supplementation may be indicated in severe diarrhetic cases.
Antimicrobial therapy of uncomplicated non-typhoidal Salmonella gastroenteritis however, is not
routinely used, as this does not shorten the symptomatic period and may actually prolong the duration
of carriage. Moreover, emerging antimicrobial resistance over the past 20 years has limited therapeutic
alternatives (Crump et al., 2011). In 2012 for instance, 62.5% of the Salmonella Typhimurium isolates
from humans in Belgium showed to be multi-drug resistant (i.e. to ampicillin, streptomycin,
sulfonamides and tetracyclines) (NRSS, 2012). Therefore, antibiotics should be reserved for patients with
(a high risk for) severe disease.

3. Salmonella infection in swine

3.1 Epidemiology

The latest baseline survey in pig holdings that was carried out in the EU (EFSA, 2009), based on
pooled faecal samples, reported an EU prevalence of 28.7% (95% CI [26.3-31.0]) Salmonella-positive
breeding holdings and 33.3% [30.9-35.7] Salmonella-positive production holdings. For Belgium, the
prevalence in this survey of Salmonella-positive breeding herds was 18.8% [7.3-45.1], and the
production holdings’ prevalence was 36.4% [30.5-43.1] (EFSA, 2009). The EU-prevalence of slaughter
pigs with Salmonella-positive lymph nodes was 10.3% in 2007, whereas 8.3% of the carcasses were
Salmonella-positive over all EU Member States (MS) (EFSA, 2008a). The observed Salmonella
prevalence in Belgium amounted to 13.9% and 18.8% in the lymph nodes and on the carcasses of slaughtered pigs, respectively (EFSA, 2008a).

*Salmonella* Typhimurium was by far the most commonly reported serovar in the EU over the period 2004-2011 in pigs and pork, and *Salmonella* Derby the second. As in humans, monophasic *Salmonella* Typhimurium 1,4,[5],12:i:- was the third most frequently reported serovar in 2011 in pigs and pork (Fig. 4; EFSA and ECDC, 2013a). In Belgian pig isolates, the most commonly reported serovar was also *Salmonella* Typhimurium (30.8%), followed by its monophasic variant (22.3%) and *Salmonella* Derby (13.5%) (Fig. 5; CODA, 2012).

![Figure 4: The main Salmonella serovars from pigs and pig meat in the EU in 2004-2011 (EFSA and ECDC, 2013a).](image)
As a slaughter pig with Salmonella-infected lymph nodes is twice as likely to yield a Salmonella-contaminated carcass (EFSA, 2008b), it is critical to understand the dynamics of Salmonella infections in swine herds supplying the slaughterhouses. However, the number of potential infection sources for pig populations are numerous, varying from Salmonella-contaminated feed (Molla et al., 2010) or farm materials (Twomey et al., 2010), over Salmonella-infected rodents and pets (Barber et al., 2002; Meerburg et al., 2007), to residual environmental pen contamination (Beloeil et al., 2007; Lurette et al., 2011). Moreover, an evidence-based link with outbreaks is generally lacking, since the host and vector range for pig-related Salmonella bacteria are wide, and the variety of environmental and management situations seemingly endless (Carlson et al., 2012).

Salmonella transmission between pigs mainly occurs via the faecal-oral route (Griffith et al., 2006; Boyen et al., 2008a), although oral-pharyngeal secretions may also allow nose-to-nose transmission of Salmonella (Oliveira et al., 2007), or even aerosol transmission over short distances (Fedorka-Cray et al., 1995; Proux et al., 2001). Transmission studies exposing pigs to Salmonella-contaminated environments have shown that low numbers of the bacteria \(10^2 - 10^3\) CFU can be sufficient for infection (Boughton et al., 2007a) and that Salmonella can be excreted within only two hours post-infection (Boughton et al., 2007b; Rostagno et al., 2011). More details concerning risk factors for Salmonella transmission can be found below paragraph ‘5. Salmonella control on-farm’.

**Figure 5:** The main Salmonella serovars from pig origin in Belgium in 2012 (CODA, 2012).
3.2 Pathogenesis, lesions and symptoms

After oral uptake, the antimicrobial peptide β-defensin 1, present in the pig’s salivae and excreted by the tongue mucosa, is the first host barrier *Salmonella* is confronted with (Shi *et al.*, 1999). Antimicrobial peptides are capable of both killing bacteria by membrane disruption and chemotactically attract immature T-cells and monocytes, providing a link between innate and adaptive immunity (Veldhuizen *et al.*, 2007). As secondary lympho-epithelial tissue, the tonsils in the oropharynx also participate in both innate and adaptive immune responses, through the production of antimicrobial peptides, lymphocytes, cytokines and chemotactic molecules (Horter *et al.*, 2003). However, some microorganisms can circumvent the tonsillar immune defences and even utilize the tonsils as a colonization site. *Salmonella Typhimurium* for instance, can reside extracellularly on the porcine tonsillar epithelium and in the tonsillar crypts (Fedorka-Cray *et al.*, 1995; Van Parys *et al.*, 2010).

Once ingested, *Salmonella* bacteria encounter the acidic environment of the stomach, with a pH of 2.5 - 4.0 in adult pigs, or up to 7.0 in weaned piglets. This gastric acidity is considered a major barrier for most orally ingested microbial pathogens to colonize the gastrointestinal tract (Smith, 2003). *Salmonellae* however, have been shown to possess adaptive acid stress response systems that facilitate survival and even enhance colonization in acidic environments (Audia *et al.*, 2001). One such system is the acid tolerance response (ATR) with the production of many acid shock proteins (Baik *et al.*, 1996; Viala *et al.*, 2011). Obviously, when the gastric pH is increased, e.g. through the buffering presence of (fatty) feed, more bacteria will survive and reach the gut (Mikkelsen *et al.*, 2004).

When *Salmonellae* arrive in the intestines, again they are faced with several host defence mechanisms: the intestinal peristalsis, antibacterial bile salts, antimicrobial peptides on the intestinal epithelium and competitive exclusion by the normal gut microbiota (Viala *et al.*, 2011). Yet, *Salmonella* bacteria are able to attach to the intestinal mucosa by using adhesins on their envelope, especially type 1 fimbriae (Althouse *et al.*, 2003). Thereafter, *Salmonellae* enter the epithelial cells (enterocytes and microfold cells, dendritic cells and goblet cells) of the intestinal mucosa, and reach the subepithelial lamina propria through vacuole transport. This adhesion and invasion occurs predominantly at the Peyer’s Patches (Schauer *et al.*, 2004), which harbour large populations of lymphoid (T- and B-cells) and non-lymphoid (macrophages, dendritic cells) immune cells. After endocytosis, the *Salmonella* bacteria can survive and even multiply inside these cells, and consequently enter the blood and lymph stream and spread to gastrointestinal lymph nodes (Tam *et al.*, 2008).

After this most efficient infection process, *Salmonella* pathogens can persist in the tonsils, the intestines (ileum, caecum and colon) and the gut-associated lymphoid tissue (Wood *et al.*, 1989; Boyen *et al.*, 2008a). These ‘carrier’ pigs often do not show any clinical symptoms, but may shed *Salmonella* intermittently (van Winsen *et al.*, 2001). Recrudescence and re-excretion of the pathogen may occur in
these pigs due to stress, caused by for instance the commingling of pigs, feed withdrawal (Verbrugghe et al., 2011), and transport towards the slaughterhouse (Rostagno et al., 2005). This re-excretion may eventually result in carcass contamination.

The abovementioned passage of Salmonella bacteria through the epithelium induces the production of inflammatory enzymes, and chemotactic recruitment of neutrophils and macrophages. The presence of these immune cells in the gut enables the pig to overcome a Salmonella infection on one hand (Foster et al., 2003), but this intestinal inflammation also induces mucosal and endothelial necrosis on the other hand. This may result in watery yellow diarrhoea, indicative for Salmonella infections (Carlson et al., 2012). Moreover, the resorption of endotoxins into the blood stream leads to fever and consequently to lethargy and a decreased feed intake. Usually only few pigs die, due to endotoxemia (acute) or dehydration (more slowly), and some may waste chronically, the so-called ‘runts’ (Carlson et al., 2012).

The described symptoms are mainly applicable to infections with broad host range serotypes like Salmonella Typhimurium, and differ from the more severe symptoms seen after infections with the host-adapted ones like Salmonella Choleraesuis (Boyen et al., 2008a; Carlson et al., 2012). The latter serotype however, is predominantly found in Asian countries, while it is only rare in the United States (Foley et al., 2008) and eradicated in most western European herds (EFSA and ECDC, 2012a); therefore, these symptoms are not deepened in this thesis.

4. Salmonella detection

As various other diseases in pigs (e.g. colibacillosis, coccidiosis, dysentery) may resemble the clinical signs of salmonellosis, these symptoms do not provide a clear-cut diagnosis. Moreover, Salmonella infections in pigs mostly occur subclinically. Confirmation of infection through bacteriological or serological examination is thus necessary.

4.1 Bacteriology

Many different matrices can be used for the detection of Salmonella species: faeces or intestinal content, oral fluid, tissues such as lymph nodes or intestines, environmental or carcass swabs, and feed. Regardless of the sample source, the isolation of Salmonella generally includes: (i) non-selective pre-enrichment; (ii) selective enrichment; (iii) inoculation of plating media; (iv) biochemical confirmation of suspect colonies; and (v) phenotypic and genotypic characterization. Different detection methods were established by the International and European Standardisation Organisations (EN/ISO methods) for different matrices to allow an inter-laboratory comparison of the outcome. As the experiments in
Chapter 1

General Introduction

This thesis mostly investigated faeces and tissues, ISO 6579:2002/Amd1:2007 Annex D is the main ISO method referred to here (Fig. 6). All following techniques are summarized in Table 1 and 2.

A direct culture by inoculating samples directly on non-selective plating media (e.g. blood agar) is considered useful only in clinical-disease situations, whereas the numbers of Salmonella bacteria in the samples from carrier animals or the environment are too low to detect with this method.

Enrichment steps have the disadvantage to limit the assay speed, but in most cases they are necessary in surveillance to increase the number of Salmonella bacteria (and others) for optimal recovery, and to dilute the effect of possible inhibitors. In a first pre-enrichment step (Fig. 6), in which the revival of injured cells is enhanced, buffered peptone water (BPW) is the broth of choice (ISO, 2007). In this BPW, the sample is diluted 1:10 and homogenized in a stomacher if necessary (e.g. in case of tissues). For the subsequent selective enrichment, different fluid and semi-solid media are currently available. The main broths used are ‘Rappaport-Vassiliadis’ with Soya (RVS) and ‘Muller-Kauffmann tetra-thionate’ supplemented with novobiocine (MKTTn), and the main semi-solid agars are ‘diagnostic semi-solid Salmonella’ (DIASALM) and ‘modified semi-solid Rappaport-Vassiliadis’ (MSRV). MSRV is used in ISO 6579:2002/Amd1:2007 Annex D (Fig. 6), as it provides added novobiocin, more nutrients, a higher buffering capacity, and less magnesium chloride in comparison with RV. Although semi-solid media are considered to be the most suitable selective enrichment media, several authors showed that different media led to different patterns of strain dominance, so that a combination of these media increases the relative sensitivity (Gorski, 2012; De Busser et al., 2013).

The next, selective isolation step is usually performed on plates of xylose-lysine-deoxycholate agar (XLD), xylose-lysine-tergitol-4 agar (XLT-4), brilliant green agar (BGA) and RAPID'Salmonella Medium (RAPID) agar. For ISO 6579:2002/Amd1:2007 Annex D, an XLD plate and a second medium are inoculated with a loopful from the growth zone edge on the MSRV plate.

A biochemical confirmation is performed thereafter, when colonies with a Salmonella-typical morphology are present. One such colony is then inoculated in triple sugar iron agar (TSI), lysine-decarboxylase broth and sorbitol agar according to ISO 6579:2002/Amd1:2007 Annex D (Fig. 6).
### Figure 6: Scheme of the bacteriological isolation of *Salmonella* spp., performed in CODA-CERVA.

(Based on ISO 6579:2002/Amd1:2007 Annex D)
Although culture methods are recommended for *Salmonella* detection, since they provide a 100% specificity (ISO, 2007), it is well recognized that their sensitivity depends on the matrix of the sample (faeces or tissue), on the type of sample (single or pooled) and on the used combination of culture media (Wilkins *et al.*, 2010). A culture sensitivity of 74 and 90% was estimated by the systematic review of Wilkins *et al.* (2010) for faecal and lymph node samples, respectively. In addition, culture methods are labour-intensive and above all time-consuming, which is not desirable in cases when urgent diagnosis is necessary or when many samples have to be tested. Therefore, developing more convenient and rapid, but reliable alternatives for the culture methods has been the goal of many research so far.

Real-time polymerase chain reaction (Real-time PCR) for instance, is the most common approach in rapid testing (Hoorfar, 2011). The PCR amplifies a targeted region of the *Salmonella* genome by a three-step process: (i) denaturation of double-stranded DNA into single strands; (ii) annealing of specific primers to the single-stranded DNA; and (iii) enzymatic extension of the primers to produce an exact copy of the original double-stranded DNA sequence. However, PCR has a low diagnostic sensitivity when testing samples with a low number of *Salmonella* bacteria (Nga *et al.*, 2010; Jensen *et al.*, 2013). Therefore, a preceding enrichment in a culture broth is nevertheless recommended (Jasson *et al.*, 2011).

A more rapid, simpler and cheaper molecular detection method however, is the loop-mediated isothermal amplification (LAMP) assay. It relies on the autocycling amplification of six distinct regions of a target gene in a water-bath, after which the amplified products can be detected by turbidity (visually or by a turbidimeter) due to the formation of insoluble magnesium pyrophosphate (Techathuvanan *et al.*, 2010). Another rapid method is the VIDAS® UP *Salmonella* assay (Vitek Immuno Diagnostic Assay System), an enzyme-linked fluorescent assay (ELFA), for the detection of *Salmonella* in food products, environmental samples, and primary production samples within two days (Bird *et al.*, 2013). Similarly, the antigen-capture enzyme-linked immunosorbent assay (ELISA) of Brooks *et al.* (2014) showed to detect a broad range of *Salmonella* serovars in field samples, this with a diagnostic sensitivity and specificity comparable to culture, but with a higher cost-efficiency. A very different detection approach is performed with matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). Starting from a single colony or even a selective enrichment broth, this technique generates profile spectra of small (ribosomal) proteins, revealing a peak pattern (Sparbier *et al.*, 2012). Matching the pattern against a reference database enables the discrimination of different bacterial species.
When the identification of a *Salmonella* isolate is required, a phenotypic or genotypic characterization can be carried out. Genotypic methods are progressively replacing the phenotypic assays, but the latter are still routinely used in many reference laboratories (EFSA, 2013b). Often a combination of phenotypic and genotypic typing methods is used.

For a phenotypic examination, options like serotyping, phage-typing and an assessment of the antimicrobial resistance patterns are available. Serotyping is generally performed according to the Kauffman-White-Le Minor scheme (Grimont and Weill, 2007; Guibordencche et al., 2010). This system classifies the *Salmonella* isolate as a certain serotype or serovar, based on the slide agglutination of somatic (O), flagellar (H) and capsular (Vi) antigens present on its cell surface with *Salmonella*-specific rabbit-antibodies. Although serotyping by agglutination is widely used for epidemiological purposes for over 80 years, as serovars are discriminatory and suitable for global communication, it is
time-consuming, expensive, and requires carefully trained personnel as well. A more automated way of serotyping is possible when using Check&Trace Salmonella (or previously known as ‘Premi®Test’) (Check-Points), a molecular serotyping method. It combines a multiplex PCR with the simultaneous detection of the PCR products on a diagnostic microarray (Wattiau et al., 2008). Starting from a pure colony after culture, the test produces unique microarray hybridization profiles within 9 - 10h, that enable identification at both the genus and serovar level of the most commonly encountered Salmonella spp.

Salmonella strains can also be examined by phage typing, which assesses the strains’ susceptibility to a defined set of bacteriophages (Tolba et al., 2010; López-Cuevas et al., 2011). However, only important serovars such as Salmonella Enteritidis and Typhimurium are investigated this way, and only a limited number of bacteriophages are available, which demand in addition a continuous quality control. Also, a careful inter-laboratory coordination is vital to ensure the assay’s reproducibility (Baggesen et al., 2010).

As antimicrobial resistance is an emerging problem worldwide, another phenotypic technique is increasingly important: the determination of antimicrobial resistance profiles among isolated Salmonella strains (Miller et al., 2011; Bonardi et al., 2013; Kerouanton et al., 2013).

Genotypic examination can be performed by several techniques as well. Pulsed-field gel electrophoresis (PFGE) is considered the ‘gold standard’ method for the subtyping of foodborne pathogens. With this technique, long strands of DNA are separated through an agarose gel matrix and are visualized as bands. The actual DNA content of each band is thus unknown, but the isolates can be identified when matching with the used reference strains. PFGE was historically one of the earliest DNA subtyping systems for fingerprinting Salmonella isolates and has become a frequently used procedure, since it has been shown to be reliable and highly discriminating (Goering, 2004; Ribot et al., 2006), with the exception of certain strains, like Salmonella Typhimurium DT 104. Its usefulness is limited however, by the labour-intensive, time-consuming protocol and the expensive equipment required. Inter-laboratory comparisons of i.a. these PFGE fingerprints are notably improved by PulseNet, The International Molecular Subtyping Network for Foodborne Disease Surveillance (http://www.pulsenetinternational.org/networks/europe).

Multi-locus variable-number of tandem-repeats analysis (MLVA) has recently become a widely used method as well for the typing of Salmonella Typhimurium (Hopkins et al., 2007; Larsson et al., 2009; Kruy et al., 2011), and it showed an even higher discriminatory power in comparison with PFGE (Dewaele et al., 2012). This method, based on the amplification and fragment size analysis of five repeat loci, is much more labour-friendly, but requires a specific nomenclature to match MLVA profiles between laboratories (Larsson et al., 2009).
Restriction fragment length polymorphism (RFLP) is another molecular subtyping tool in epidemiologic investigations (EFSA, 2013b). In this technique, a target DNA sequence known to differ between strains, is cleaved with restriction endonucleases to generate fragments of varying length. Before this cleavage, the target DNA sequence can be amplified at high annealing temperatures in a PCR-RFLP, to maximise stringency. The isolates are then typed by comparing their RFLP pattern after gel electrophoresis. RFLP-analysis provides a moderate discriminatory capability, and is moderately comparable between laboratories (EFSA, 2013b).

Unlike PFGE, MLVA and RFLP, the method of multi-locus sequence typing (MLST) is based on DNA sequencing (Maiden, 2006). MLST determines the DNA sequence variations in multiple housekeeping genes depending on the degree of discrimination desired, and characterizes strains by their unique allelic profiles or sequence types (STs). This sequence-based typing method is considered moderately to highly discriminatory for general examinations, but too low for outbreak investigations. Its reproducibility between laboratories is however, regarded as high. Achtman et al. (2012) investigated isolates of Salmonella enterica from diverse hosts from all continents and showed that this MLST technique recognized multiple evolutionary groups that were confounded by traditional serotype designations. Therefore, they concluded that serotyping can be misleading concerning the disease potential of Salmonella sp., and they advised the replacement of serotyping by MLST or its equivalents for routine diagnostic purposes.

Progress is also ongoing in the whole genomic sequencing (WGS) technique, which results in the fully sequenced genome of a pathogen. Therefore, WGS achieves the maximum capability for discriminating isolates. The international harmonisation of the huge amount of information will be a major challenge in future however, which makes WGS not readily applicable as a routine subtyping method (EFSA, 2013b).

Although these genotyping technologies become increasingly accepted, an important limitation must be taken into account. Namely, they only indicate that bacteria with those gene sequences are present, and not whether the gene is actually expressed or whether the bacterium is viable. The latter issue might be overcome by a method with immobilized bacteriophages capturing metabolically-active host bacterial cells, which can be detected afterwards using real-time PCR (Tolba et al., 2010).
### Chapter 1

#### General Introduction

Table 2: Main bacteriological typing techniques for *Salmonella* analysis.

<table>
<thead>
<tr>
<th>Technique *</th>
<th>Characteristics</th>
<th>Advantage(s)</th>
<th>Disadvantage(s)</th>
</tr>
</thead>
</table>
| **Serotyping** (agglutination) | • Standard classification cfr. Kauffman-White-Le Minor scheme  
• Agglutination of anti-*Salmonella* rabbit-antibodies with antigens on a slide | • Discriminatory serotypes  
• Widely used  
• High inter-laboratory comparisons  
• Relatively rapid (1-2 days) | • Only possible after isolation  
• Rather expensive  
• Trained personnel  
• Little discriminatory for outbreak analysis |
| **Serotyping** (molecular) | • Standard classification cfr. Kauffman-White-Le Minor scheme  
• Multiplex PCR + microarray (Check&Trace®) | • Discriminatory serotypes  
• Widely used  
• High inter-laboratory comparisons  
• Relatively rapid (1-2 days)  
• No trained personnel | • Best after isolation (Low Se from MSRV; better Se from BGA)  
• Very expensive  
• Little discriminatory for outbreak analysis |
| **Phage-typing** | • Determination of growth inhibition round bacteriophage drops | • Widely used  
• Rapid (1 day) | • Only possible after isolation  
• Limited number + careful quality control of bacteriophages  
• Not available for all serovars  
• Trained personnel  
• Difficult inter-laboratory comparisons (exc. for Definite phage Types (DT)) |
| **Antibiogramme** | • Determination of growth inhibition round antimicrobial tablets | • Worldwide increasing importance  
• Rapid (1 day) | • Only possible after isolation  
• Not very discriminatory |
| **PFGE** | • Gold standard genotyping  
• Separating DNA molecules in a gel matrix by an electric field that periodically changes direction | • Widely used  
• Highly discriminatory, but depending on serotype  
• Moderate-high inter-laboratory comparisons (e.g. high with PulseNet protocol) | • Only possible after isolation  
• Labour-intensive  
• Time-consuming  
• Rather expensive |
| **MLVA** | • Amplification + fragment length analysis (electrophoresis) of repeat loci | • Widely used  
• Highly discriminatory  
• Labour-friendly  
• Moderate-high inter-laboratory comparisons (e.g. high with PulseNet protocol and in case of *Salmonella* Typhimurium) | • Only possible after isolation  
• Specific assay is needed per species/serovar |
| **(PCR-)RFLP** | • (Amplification +) fragmenting DNA by restriction enzymes + gel electrophoresis | • Moderately-highly discriminatory  
• Relatively inexpensive | • Only possible after isolation  
• Low-moderate inter-laboratory comparisons  
• Time-consuming |
| **MLST** | • Amplification + sequencing DNA in a set of genetic loci (housekeeping genes), producing allelic profiles or sequence types (ST) | • Moderately discriminatory  
• High inter-laboratory comparisons  
• Correlation between ST and serotypes | • Only possible after isolation  
• Little discriminatory for outbreak analysis  
• Expensive  
• Time-consuming |
| **WGS** | • DNA extraction + sequencing of entire genome  
• Usually performed on isolated outbreak strains | • Rapid  
• Highly discriminatory  
• High inter-laboratory comparisons | • Variable results between DNA preparation protocols  
• Complex analysis due to enormous dataset |

*PFGE = Pulsed-Field Gel Electrophoresis; MLVA = Multi-Locus Variable-number of tandem-repeats Analysis; (PCR-)RFLP = (Polymerase Chain Reaction-)Restriction Fragment Length Polymorphism; MLST = Multi-Locus Sequence Typing; WGS = Whole Genome Sequencing; Se = Sensitivity
4.2 Serology

The detection of Salmonella-specific antibodies in pigs can be performed on two matrices: serum derived from blood samples and meat juice from muscle tissues. Monitoring of Salmonella-specific antibodies in blood samples of fattening pigs is carried out in several countries such as Belgium and the Netherlands, whereas Denmark and Germany use meat juice (EFSA and ECDC, 2012a). As meat juice comprises serum, lymphatic moist and intracellular fluid, it is a natural diluted form of blood serum, and needs therefore a ten-times lower dilution in the preparation step of serological testing. Collecting muscle samples at the slaughterhouse is less labour-intensive than taking blood samples at the herd, but large variations occur in the obtained meat juice volume per gram muscle tissue. This results in an indefinable dilution of the present antibodies, and thus in a bias of the serological outcome. When comparing serum and meat juice analysis, the serum results were consistently higher than those from meat juice, and the overall correlation coefficient between both was moderate to low (Vico et al., 2011). Wilhelm et al. (2007) recommended to recalculate the optical density percentage (OD%) obtained from meat juice, according to a specific regression equation, to adjust the results to those obtained from sera. In addition, the meat juice from different muscles provides different levels of antibodies. Antibody levels in the meat juice from the diaphragm pillar for instance, resembled the levels in serum better than the levels in meat juice from neck or belly muscle (Nobmann et al., 2011). These findings suggest that the choice of the matrix should be carefully discussed before implementing it in a Salmonella surveillance programme; the ease of muscle sampling must be weighed critically against the probability of underestimating the actual seroprevalence when examining meat juice. A possible solution might be the sampling of blood at slaughter, which takes advantage of the ease of sampling at the slaughterhouse, and the correct estimation of the seroprevalence (cfr. monitoring in the Netherlands).

Anyhow, a serological sampling at the slaughterhouse (meat juice or blood) enables the examination of pigs from the same age class. This allows a standardised herd classification according to the Salmonella risk (high-medium-low level of antibodies). This is in contrast with the way that blood was sampled in Belgium (blood from different age categories at herd level was collected according the Aujeszky’s disease virus (ADV) programme).

Irrespective of the applied matrix, most serological testing in the EU is performed with indirect enzyme-linked immunosorbent assays (ELISA) that are based on the detection of antibodies against the somatic O-antigens 1, 4, 5, 6, 7 and 12. These O-antigens or lipopolysaccharides (LPS) are present on the cell surface of i.a. Salmonella Typhimurium and Derby (O: 1, 4, [5], 12), Salmonella Infantis and Mbandaka (O: 6, 7, 14), and Salmonella Enteritidis (O: 1, 9, 12). Consequently, the most prevalent serovars in the EU and Belgium are covered by these tests (EFSA and ECDC, 2013a; CODA, 2012). The
Salmonella LPS-Mix-ELISA was first developed in Denmark (Nielsen et al., 1995), and has been used in many countries as part of national Salmonella monitoring programmes (van der Wolf et al., 1999; Alban et al., 2002; Leontides et al., 2003). Various test kits have been developed and implemented in several national surveillance programmes so far (e.g. Herdchek® Swine Salmonella, IDEXX Laboratories; Salmotype® Pig Screen, Labor Diagnostik; Priocheck® Salmonella Ab porcine, Prionics). Caution is advisable however, when different ELISA kits are used within the same monitoring programme or herd, since several studies have demonstrated that the results of one same sample differed substantially after analysis with different test kits (Szabó et al., 2008; Vico et al., 2010). This may be due to the use of different cut-off values recommended in each of these kit instructions (Szabó et al., 2008). Vico et al. (2010) found that the Herdchek® Swine Salmonella kit showed the highest overall accuracy in comparison with the Salmotype® Pig Screen kit and the Priocheck® Salmonella Ab porcine kit.

The results of the indirect ELISA are expressed as optical density values (OD), since the level of antibodies present in a sample, determines the colouring extent during the assay, which is measured by a spectrophotometer (Fig. 7). Depending on the user’s (national) preference, these absorbance values can be presented as a sample-to-positive ratio (S/P) or as an optical density percentage (OD%), through the following formulae (Herdchek® Swine Salmonella, IDEXX Laboratories):

- S/P ratio = \( \frac{\text{OD}_{\text{Sample}} - \text{OD}_{\text{Negative Kit-Control}}}{\text{OD}_{\text{Positive Kit-Control}} - \text{OD}_{\text{Negative Kit-Control}}} \)
- OD% = S/P ratio ÷ 2.5 * 100%

Figure 7: A 96 ELISA well-plate during the last step of the indirect ELISA test, with the dark/light-coloured wells containing samples with a high/low level of Salmonella-specific antibodies, respectively.
(Based on Herdchek® Swine Salmonella, IDEXX Laboratories)
The determination of the cut-off values to define a sample as positive or negative, is depending on both the kit instructions and the national aims and regulations. For the IDEXX kit for instance, it is recommended to deviate from its suggested cut-off value of 10 OD% or 0.25 S/P for individual samples, when a stringent (20 OD% or 0.50 S/P) or general (40 OD% or 1.00 S/P) large-scale screening is performed. The first cut-off value is used for example in the surveillance programme of Denmark, the latter in Germany and the Netherlands. Belgium currently applies the cut-off value of 10 OD% or 0.25 S/P for individual samples, with however, a supplementary screening regulation for the classification of positive herds: if the mean S/P ratio of a herd exceeds 0.60 during three consecutive sampling rounds (i.e. within one year), that herd is designated as a ‘risk herd’. More details concerning this Belgian Salmonella Action Plan (SAP) can be found in paragraph 5.5.

In comparison with many bacteriological methods, serological testing with ELISAs has the advantage of being rapid and relatively cheap. In addition, it measures the pig’s response against previous Salmonella infection and is not dependent on the (intermittent) shedding of the pig at the time of sampling. Nevertheless, the actual presence of Salmonella bacteria can only be assessed by bacteriological examination, which remains of great importance from public health point of view.

Only few studies report a good-to-moderate correlation between bacteriology and serology (Lo Fo Wong et al., 2003; Sørensen et al., 2004; Korsak et al., 2006). More studies found generally a low correlation between both, whether the comparison was performed between samples from serum and faeces (Nielsen et al., 1995; Davies et al., 2003; Laevens and Mintiens, 2005; Farzan et al., 2007), serum and tissue (Davies et al., 2003; Nollet et al., 2005), meat juice and faeces (Davies et al., 2003; Casey et al., 2004; Rostagno et al., 2012b) or meat juice and tissue (Davies et al., 2003; Belsué et al., 2011). This finding can be explained by the temporal disassociation of approximately two months that exists between the prevalence peak of bacteriological excretion and serological antibody response (Kranker et al., 2003). Also the sample size for the monitoring at herd level will influence the level of agreement between the bacteriological and serological results: a sufficiently large sample is to be taken to allow an accurate insight into the Salmonella infection status of the herd (Anonymous, 2012). Most of the previous authors did agree however, that bacteriological testing is preferable for individual animal analysis (as this provides a 100% specificity, but is rather expensive for screening with large sample size), and that serologic testing, using a sufficiently large sample size, is more appropriate at herd level (as this method is rapid and more economical for screening numerous samples). A general comparison of bacteriology and serology is given in table 3.

In any case, for both methods the use of single point estimates should be dissuaded for evaluations of on-farm interventions and for monitoring purposes (Rostagno et al., 2012b).
Table 3: Comparison of bacteriological (isolation) and serological examination.

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Bacteriology (isolation)</th>
<th>Serology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak of detection post-infection a (influenced by re-infections!)</td>
<td>2 months (excreting pigs)</td>
<td>4 months (seropositive pigs)</td>
</tr>
<tr>
<td>Detection of recent infections, rare serotypes, infections with low antibody response</td>
<td>High detection rate</td>
<td>Low detection rate</td>
</tr>
<tr>
<td>Detection of carriers, infections with low excretion</td>
<td>Low positivity rate</td>
<td>High positivity rate b</td>
</tr>
<tr>
<td>Assay performance</td>
<td>Slow</td>
<td>Fast</td>
</tr>
<tr>
<td>Costs</td>
<td>Expensive</td>
<td>Inexpensive</td>
</tr>
<tr>
<td>Advisable level of <em>Salmonella</em> status determination</td>
<td>Individual (Herd) level</td>
<td>Herd (Individual) level</td>
</tr>
<tr>
<td>Monitoring infection dynamics</td>
<td>Less appropriate</td>
<td>More appropriate</td>
</tr>
<tr>
<td>Serotyping, antimicrobial resistance</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>

a Kranker et al., 2003  
b depending from cut-off value (Nollet et al., 2005)

5. *Salmonella* control on-farm

As pork is the most frequently consumed meat in the world (Narrod et al., 2011), the mitigation of any potential risk towards public health is justified. To increase food safety of pork products, many stages in the ‘farm-to-fork’, the ‘feed-to-fork’ or the ‘stable-to-table’ chain can be targeted, grouped in three levels: the pre-harvest level (i.e. on-farm), the harvest level (i.e. during transport to the slaughterhouse, in the lairage, at the slaughterline), and the post-harvest level (i.e. during processing, in retail, during preparation). Some authors focus on the importance of control measures in the slaughterhouse (Goldbach and Alban, 2006; Hurd et al., 2008; Bollaerts et al., 2010), while others prefer the optimization of interventions on-farm (Hotes et al., 2011; Wales et al., 2011). Most probably, the largest reduction of pork contamination may be expected when concurrent control measures are applied at every level of the pork production chain (Alban and Stark, 2005; EFSA 2008a).

However, *Salmonella* may also be transmitted directly from infected pigs to humans (Hendriksen et al., 2004), and may have an important economic impact through reducing the weight gain of infected pigs (Boyen et al., 2009; Davis et al., 2010; Farzan and Friendship, 2010; Gebru et al., 2010). These facts provide two additional arguments to focus on pre-harvest control measures. Reducing *Salmonella* prevalence in live pigs will however, demand the concurrent implementation of multiple measures at pre-harvest level. The potential interventions that are listed below are not likely to eliminate *Salmonella* presence on their own, but can achieve a significant *Salmonella* reduction when implemented together, as part of an overall control strategy on-farm.
5.1 Biosecurity

Biosecurity is the whole of preventive measures that can be taken to avoid the introduction of infections on a herd (external biosecurity) or the spread of infections within a herd (internal biosecurity) (Casal et al. 2007; Laanen et al., 2013). These measures are related to hygiene, pest control and pig-related management.

5.1.1 Hygiene

The European Union has set several legislations to guarantee consumers’ food safety. Regulation (EC) No 852/2004 for instance (a revision of the legislation on the hygiene of foodstuffs "Hygiene Package"), defines the food safety objectives to be achieved by the food operators, and points out the importance of adopting hygienic measures in pig herds (Anonymous, 2004).

Many studies have been conducted to identify risk factors for Salmonella prevalence in primary production, and all authors agreed that a great part of the identified risk factors were related to the hygienic actions on-farm (Lo Fo Wong et al., 2004a; Beloeil et al., 2007; Hautekiet et al., 2008; Hard et al., 2008; Fosse et al., 2009; Gotter et al., 2012, Rostagno et al., 2012a).

Two possible hygienic measures that should be implemented to improve the external biosecurity, are the use of boot baths (Hautekiet et al., 2008), and the presence of hygienic-lock facilities (Lo Fo Wong et al., 2004a). Efficient cleaning and disinfection procedures between batches is of paramount importance to minimize residual Salmonella contamination in the pens and thus improve internal biosecurity (Lo Fo Wong et al., 2004a; Erdman et al., 2005; Beloeil et al., 2007; Mannion et al., 2007; Hautekiet et al., 2008; Fosse et al., 2009; Hotes et al., 2011; Lurette et al., 2011; Rostagno et al., 2012a; Gautam et al., 2013). In addition, it is generally accepted that the cleaning step prior to disinfection must be applied very thoroughly to remove all organic material. Indeed, van der Wolf et al. (2001a) pointed out that using disinfectants without a preceding, adequate cleaning may even result in a higher Salmonella seroprevalence than when only cleaning is performed. McLaren et al. (2011) investigated various disinfectant products in a wet and dry experimental model. They demonstrated that chlorocresol-based disinfectants provided high bactericidal rates in both wet and dry tests, whereas formaldehyde-containing disinfectants were less effective in wet environment tests, and other chemical products (i.a. quaternary ammonium compounds, peroxygens) were only moderately effective. Further, not only the pens themselves and the stables should be considered, but also tools such as brooms, transport vehicles and footwear must be included in the cleaning and disinfection procedures (Twomey et al., 2010). Even the washing of the caretakers’ hands before tending to the pigs was shown to decrease Salmonella seropositivity (Lo Fo Wong et al., 2004a).
Increased efficiency of hygienic measures can thus reduce the prevalence of *Salmonella* shedding at slaughter. As mentioned previously however, these efforts alone are not capable of reducing the infection in the population satisfactory, and should be combined with other intervention methods (Gautam *et al*., 2013).

### 5.1.2 Pest control

The efficacy of proper hygienic procedures is often reduced by *Salmonella*-infected rodents or insects that are present in the farm environment after cleaning and disinfection (Davies and Wray, 1995). Unfortunately, many farmers only apply rodent control measures (not always efficient ones) after a certain subjective threshold is passed and mainly to prevent feed losses or infrastructural damage (Meerburg and Kijlstra, 2007).

The rodent-related zoonotic risks are thus frequently underestimated as a threat to both the external and internal biosecurity, as wild rodents – together with birds, flies and domestic pets – are the main reservoirs and vectors of *Salmonella* and various other pathogens causing disease in food animals and humans (*e.g.* *Campylobacter*, *Leptospira*, *Trichinella*, *Toxoplasma*). In a field survey on swine production systems, *Salmonella* was detected in samples from cats (12% of samples positive), birds (8%), flies (6%), and mice (5%), and a correlation was demonstrated with the number of *Salmonella*-shedding pigs (Barber *et al*., 2002).

Therefore, the importance of **pest control** for food safety purposes must be stressed on primary production sites. These control measures should be an integral part of the biosecurity package at a herd, and they should include the monitoring, prevention (*e.g.* sealing of farm buildings, removal of nesting material near stables, limiting access to feed and water) and control (rodenticides, insecticides, live traps, professional exterminator) of rodents, birds and flies (Meerburg and Kijlstra, 2007). This way, contact opportunities with pigs are limited as much as possible. Cats are not recommended as rodent exterminators in the stables, as their presence is positively correlated with Toxoplasma gondii seroprevalence in pigs (Meerburg *et al*., 2006) and they can act as *Salmonella* reservoir themselves (Barber *et al*., 2002).
5.1.3 Management

Although room decontamination efficacy had a greater impact on Salmonella prevalence in pigs at slaughter, when compared to pig management practices (Lurette et al., 2011), the effect of the latter biosecurity measures must not be underestimated. Numerous studies have indeed demonstrated a higher risk for Salmonella seropositivity and/or faecal prevalence when a number of management procedures, explained hereafter, were inadequate.

For instance, herds that implement proper entrance control, by the use of a fence enclosing the pig farm facilities and specific clothing before entering the facilities, also show a reduced risk for Salmonella seroconversion, in comparison with herds that lack these external biosecurity measures (Lo Fo Wong et al., 2004a; Beloeil et al., 2007; Hautekiet et al., 2008). Also a purchase policy with less than three supplier herds, was associated with lower average S/P-values (Lo Fo Wong et al., 2004a) and represents an important external biosecurity measure. This supports the generally accepted view that the herd immunity in closed farms is more stable than in open production systems that regularly purchase animals.

Respecting well-defined walking lines within the farm and applying a strict all in/all out procedure between batches represent valuable internal biosecurity measures, as they were related with lower average S/P-values (Lo Fo Wong et al., 2004a; Erdman et al., 2005; Hautekiet et al., 2008; Fosse et al., 2009). The pig stocking density (floor space per pig, as well as batch and herd size) was another internal biosecurity measure that was identified as an important risk factor for Salmonella seroconversion by Beloeil et al. (2007), Van der Wolf et al. (2001a) and Hautekiet et al. (2008). Likewise, a decreased seropositivity was reported when pigs had no or restricted contact with pigs from neighbouring pens or with slurry by the use of slatted floors (Lo Fo Wong et al., 2004a; Twomey et al., 2010; Hotes et al., 2011).

Also the reproductive management system may have an impact on Salmonella prevalence. In Belgium, approximately half of the sow herds use a more-week batch production system (Maes, 2012). Apart from better labour organization, such systems may also offer sanitary advantages (Vangroenweghe et al., 2012). Namely, the units in such a more-week batch system are used AI/AO and can be cleaned and disinfected more properly (with a longer sanitary transition), so that the risk for residual Salmonella is reduced.

When focussing on pig health, several studies have proven the association between infections with Salmonella and concurrent pathogens. Porcine Reproductive and Respiratory Syndrome virus (PRRSV) infection (Wills et al., 2000; Erdman et al., 2005; Beloeil et al., 2007), or intestinal damage caused by Ascaris suum larvae (van der Wolf et al., 2001a) lead for instance to a higher risk for Salmonella seropositivity.
Finally, **pig welfare** plays a major role in *Salmonella* spread as well, since a recrudescence of the bacteria in carrier pigs occurs in situations of stress, with the consequent shedding of the pathogen in the environment (‘intermittent excretion’). To avoid stress situations, various measures are possible, from providing environmental enrichment (De Jonge et al., 1996), to not mixing batches (Coutellier et al., 2007), and programming the room temperature in the zone of thermal neutrality (Hautekiet et al., 2008).

In conclusion, stringent cleaning and disinfection combined with an attentive pest control and the optimization of the herd management, will likely not eliminate *Salmonella* prevalence in commercial swine herds, but will certainly reduce the *Salmonella* burden and contribute to a better overall herd health status (Erdman et al., 2005). To improve biosecurity however, pig farmers must be prepared to make investments and especially change practices, which might militate against the voluntary adoption of these measures (Fraser et al., 2010). Nevertheless, the improvement of the pigs weight gain when reducing *Salmonella* infection (Balaji et al., 2000; Turner et al., 2002; Boyen et al., 2009; Davis et al., 2010; Farzan and Friendship, 2010; Gebru et al., 2010), forms a good incentive to pay for these expenses and adopt the necessary biosecurity measures.

### 5.2 Feed: contamination, characteristics and supplementations

#### 5.2.1 Salmonella-free feed

In the ‘feed-to-food’ consumer safety policy, the production of *Salmonella*-free feed is obviously the first critical control point, as a link between contaminated animal feed and human salmonellosis has been demonstrated (Crump et al., 2002). The impact of *Salmonella*-contaminated feed however, is considered greatest in countries with a low *Salmonella* prevalence, whereas in high prevalence countries, the breeding holdings represent the most important cause of *Salmonella* introduction (Hill et al., 2011).

The introduction of *Salmonella* into the feed can occur i) by using contaminated ingredients (Crump et al., 2002; Wierup and Häggblom, 2010), ii) during processing, iii) during transport to and storage at the farm, and iv) in the pig pens (Jones and Richardson, 2004). This may happen through contact with contaminated wildlife or rodent excreta, agricultural effluents and dust (Crump et al., 2002; Davies and Wales, 2010). In addition, low numbers of *Salmonella* present in the feed may multiply in warm, moist conditions such as feed troughs (Wales et al., 2010).

Producing *Salmonella*-free animal feed is possible by firstly preventing *Salmonella* bacteria from entering the feed facilities, and secondly by destroying the bacteria through heat or chemical treatment.
of the feed (Wales et al., 2010). Prevention can be achieved by purchasing *Salmonella-free ingredients* and by optimizing internal biosecurity in the feed manufacturing site. To check the intermittent or batch-specific contamination of the purchased ingredients, a thorough analysis of dust and aggregated spilled feed around mill machinery is preferable above direct feed sampling (Davies and Wales, 2010). Namely, these first sample types are a small subsample of a great deal of the material that passed through the feed manufacturing site and with a high surface area for the adherence of bacteria. This way, dust has proven to be a sensitive monitoring sample for *Salmonella* (Jones and Richardson, 2004). Therefore, to avoid contamination during manufacturing, feed producers should focus on an **adequate dust and rodent control** (Jones and Richardson, 2004). **Heat** treatment of the feed during pelleting or extrusion with a temperature above 80°C eliminates *Salmonella* in most cases (Himathongkham et al., 1996; Jones and Richardson, 2004). However, due to heat damage to the vitamins and other nutrients, and the absence of a residual antibacterial effect, most feed producers opt for chemical treatment, such as adding organic acids or formaldehyde, or for applying bacterial-membrane disrupting compounds. **Chemicals** do have a residual effect during storage, thereby reducing feed recontamination. Nevertheless, this effect might also interfere with (‘mask’) bacteriological detection of remaining, viable organisms (Wales et al., 2010). Mixtures of organic acids (e.g. formic, propionic, lactic and butyric acid) or their derivatives (e.g. potassium diformate) are commonly marketed, but the different acids appear to have highly variable antibacterial effects. Formaldehyde has strong disinfectant capacities against most bacteria (Wales et al., 2010), and therefore it is still a permitted additive in Belgium. However, as a genotoxicant, it is considered a carcinogenic hazard at concentrations above 1ppm (Arts et al., 2006). Bacterial-membrane disrupting compounds, such as terpenes or essential oils (plant-derived mixtures of terpenes), are often added to formaldehyde- and organic acid-containing products, as they improve their microbicidal effect (Trombetta et al., 2005; Carrique-Mas et al., 2007). This synergistic combination allows lower levels of formaldehyde and acids, and thus minimizes the corrosiveness and the operator risk.

### 5.2.2 Feed characteristics

Several publications reported associations between *Salmonella* prevalence in swine and different feed textures. Many have investigated the effect of pelleted feed versus **non-pelleted feed** and demonstrated a reducing effect on *Salmonella* (sero)prevalence in pigs by the latter feed (Kjeldsen and Dahl, 1999; Kranker et al., 2001; Lo Fo Wong et al., 2004a; Mikkelsen et al., 2004; Rajić et al., 2007; Garcia-Feliz et al., 2009; Hotes et al., 2010), even when the pelleting process rendered a less-contaminated product (Mikkelsen et al., 2004). A meta-analysis performed by Wilhelm et al. (2012) even ranked the efficacy of this intervention before feed supplementation with acids or vaccination. This texture effect is related to the
particle size (Kjeldsen and Dahl, 1999; Hedemann et al., 2005; Papenbrock et al., 2005). **Feed with large particles** (e.g. non-pelleted feed such as coarsely ground meal) increases the consistency of the stomach content, and thereby allows a prolonged gastric acidification. This decreases the number of *Salmonellae* entering the intestines more than is the case with small particle feed (e.g. pelleted feed and finely ground meal), that passes the stomach relatively rapid. Moreover, in coarse diets, less starch is accessible to the duodenal enzymatic digestion, rendering more unabsorbed starch and fibre towards the large intestine. This way, more bacterial degradation of starch is possible in this area, with consequently a higher production of fatty acids. These acids decrease *Salmonella* invasion in the intestinal epithelium (Boyen et al., 2008b) and promote epithelial growth (Mikkelsen et al., 2004). Hedemann et al. (2005) concluded in addition, that pigs fed pelleted diets secrete more mucins in the small intestinal area, which favour the binding and colonization of *Salmonella*.

When comparing studies which evaluated wet to dry feeding systems, a general conclusion is less evident. Various studies have shown a bacteriological or serological reducing effect on *Salmonella*, when liquid feed is administered in comparison with dry feed (van der Wolf et al., 1999, 2001a,b; Kranker et al., 2001; Lo Fo Wong et al., 2004a; Nollet et al., 2004; Farzan et al., 2006; Hautekiet et al., 2008; Hotes et al., 2010; Twomey et al., 2010; EFSA, 2011). This might be explained by the natural fermentation process in liquid feed, which lowers the pH, inhibiting or reducing the numbers of *Salmonella* bacteria. Likely, the same principle is applicable to a greater extent when administering ‘fermented liquid feed’ (FLF). To produce FLF, acidified or fermented by-products (e.g. whey or products containing lactic acid bacteria) are added to the liquid feed, which allow it to ferment under controlled conditions, reducing the pH level more intensely than liquid feed alone (van der Wolf et al., 2001c; Lo Fo Wong et al., 2004a). In contrast, some authors have reported no significant differences between liquid and dry feeding practices (Van Winsen et al., 2002), which might be caused by the hygienic risk when using trough feeding, as *Salmonella* bacteria from faecal contamination are able to multiply several hours in this moist environment (van der Wolf et al., 1999).

### 5.2.3 Feed supplementations

Although some studies in pigs have reported a reduced *Salmonella* shedding after antimicrobial treatment (Ebner and Mathew, 2000; Gebreyes et al., 2006), most studies have concluded the opposite, namely no (Rajić et al., 2007) or even an increased risk of *Salmonella* transmission in pigs when preventive group level antibiotic treatment is applied (Leontides et al., 2003; Beloeil et al., 2007; Funk et al., 2007). Wilhelm et al. (2012) concluded from their meta-analysis that in-feed medication with tetracyclines implies a significant hazard for *Salmonella* shedding. Most probably an antimicrobial selection pressure is
involved in such evaluated pig populations, causing a competitive advantage for less susceptible *Salmonellae*. In addition, a disruption of the normal microbial flora may have occurred in concert with the previous effect, which allows an increased *Salmonella* colonization of the gastrointestinal tract and associated tissues (Funk et al., 2007).

Anyhow, because of the emerging antimicrobial resistance worldwide and the consequent public health threat, the use of in-feed antibiotics has been restricted in many countries. Any prophylactic antimicrobial use – other than in very limited, clearly defined situations like atrophic rhinitis – should be actively discouraged (Callens et al., 2012).

In contrast, the majority of studies evaluating feed supplementation with **organic acids** describe a reduction of the *Salmonella* prevalence (O’Connor et al., 2008; Wilhelm et al., 2012). The effect of organic acids relies on dual mechanisms in reducing *Salmonella* contamination. First, they decrease the pH of the surrounding medium, and second, they have bactericidal effects when the acid is in its non-dissociated form. In that form, they can penetrate the bacterial cell and dissociate intracellularly, which lowers the cytoplasmic pH and consequently disrupts DNA synthesis (Kirchgessner et al., 1992). In addition, both mechanisms act synergistically, as a decreased pH implies a shift of dissociated acids to the non-dissociated form, which consequently intensifies the bactericidal effect (van der Wolf et al., 2001b).

However, these effects are highly variable, depending on the type of acid used. Short-chain fatty acids (like formic, lactic, sorbic, propionic and butyric acid) and medium-chain fatty acids (like fumaric, caprylic and caproic acid) have shown to decrease virulence gene expression and invasion of porcine intestinal epithelial cells by *Salmonella Typhimurium* (Boyen et al., 2008b; Messens et al., 2010). Formic and lactic acid for instance, have a stronger innate bactericidal effect on *Salmonella* than fumaric acid. Propionic and sorbic acid are also active against fungi and yeasts. Coating of fatty acids can result in an additional protection of the acids from metabolization by the gut microbiota, and allows them to reach the colonization sites (ileum, caecum and colon) in their active form (Van Immerseel et al., 2005; Boyen et al., 2008b; Messens et al., 2010). Besides this product-specific influence, also differences in the dosage, the age of the pigs, the treatment period, the management and *Salmonella* status of herds are responsible for the variable effect of organic acid treatments. Due to the many variations in these characteristics, previous studies showed both reducing (Papenbrock et al., 2005; van der Heijden et al., 2006; Creus et al., 2007; Taube et al., 2009; Tanaka et al., 2010) and non-reducing (Martín-Peláez et al., 2010; Walsh et al., 2012) effects on *Salmonella* excretion or antibody induction. Wilhelm et al. (2012) confirmed that these variable effects of acid-inclusion indeed suggest a context-sensitive treatment response. Therefore general recommendations are difficult to make, and pig producers should evaluate these tools in their own farm situation. To aid pig producers in doing so, companies should provide science-based proof of their products’ efficacy.
Another possible feed treatment is the supplementation of pro- or prebiotics. The main effects of these feed additives are the improved resistance of the animals against colonization with pathogenic bacteria and enhanced host mucosal immunity. This may result in a reduced pathogen load, an improved health status of the animals and a reduced risk of food-borne pathogens in foods, including *Salmonella* (Gaggià et al., 2010).

**Probiotics** comprise all live-microorganisms which improve the hosts’ health when administered as a feed supplement in adequate amounts (FAO/WHO, 2001). The probiotics currently approved for pigs in the EU, include *Lactobacillus* spp., *Bacillus* spp., *Lactococcus* spp., bifidobacteria spp., *Pediococcus* spp., *Enterococcus* spp. and *Saccharomyces* spp. (European Commission, 2012). The *Lactobacilli* for instance, producing lactic acid, have shown reducing effects in *Salmonella Typhimurium*-infected pigs (Casey et al., 2007). Most studies have reported beneficial effects on the gut microbiota in pigs, although some authors observed an alleviation of diarrhoea, according to the review of Gaggià et al. (2010). These inconsistent results, together with the many screening and selection procedures necessary to classify a probiotic (Friendship et al., 2009), make these products – although promising – a challenge to implement in a cost-effective way.

**Competitive exclusion products** can also contain various beneficial bacterial species, but in contrast with probiotics, these cultures are not added to the feed. They are in addition derived from caecal contents, and thus indigenous to a particular animal species. With this oral inoculation strategy, enteric pathogens are excluded from the intestinal tract by preferentially colonizing it with commensal or beneficial bacteria, hereby facilitating the natural establishment of a healthy gut microflora in young animals (Anderson et al., 1999). Several *Salmonella* challenge experiments have demonstrated a reduced shedding rate (Genovese 2003) or invasion of gut tissues (Fedorka-Cray et al., 1999; Nisbet, 2002; Genovese et al., 2003) after treating suckling piglets with competitive exclusion cultures.

**Prebiotics** are non-digestible feed ingredients that selectively stimulate the growth and/or activity of certain beneficial bacteria in the colon, and thus these ingredients indirectly improve host health as well (Gibson et al., 2005). Another potentially beneficial mechanism of prebiotics is the prevention of bacterial adherence to the gut epithelium (Roberts et al., 2013). Prebiotics include mainly medium- to long-chain carbohydrates (*i.e.* polysaccharides such as cellulose and starch), but also short-chain carbohydrates (*i.e.* oligosaccharides, like maltose, lactose and sucrose), proteins, peptides and certain lipids. As with probiotics, contradictory results were obtained (Gaggià et al., 2010). Although several human intervention studies on prebiotic consumption have already demonstrated a reduced epithelial adhesion of intestinal pathogens such as *Salmonella* (Robeरfroid et al., 2010; Roberts et al., 2013), prebiotics have not been studied intensively for the control of *Salmonella* in pigs.
Chapter 1

General Introduction

The restriction on in-feed antibiotics has also fueled the interest in a group of **plant-derived products**, known as botanicals, phytobiotics or phytogenetics (Windisch et al., 2008). These feed-additives include herbs, spices, essential oils, and oleoresins. Apart from having antioxidative effects (Jimenez-Alvarez et al., 2008), enhancing palatability (Janz et al., 2007), improving gut functions (Platel et al., 2004), or promoting growth (Windisch et al., 2008), some products have demonstrated *in vitro* efficacy against certain intestinal pathogens (Cowan, 1999; Jugl-Chizzola et al., 2005; Ruiz et al., 2010). However, the potential *in vivo* benefits of phytogenics have not been fully substantiated in pigs, and studies which evaluate the applicability as a *Salmonella* control measure are currently lacking.

Other, less well-known supplementations with less consistent results are chlorate salts, egg proteins and bacteriophages. The effect of **chlorate salt** administration is based on the respiratory nitrate reductase activity in Enterobacteriaceae (*e.g.* *Salmonella* spp.), which catalyzes the intracellular reduction of chlorate to cytotoxic chlorite (Burkey et al., 2004). As most beneficial anaerobic bacteria lack respiratory nitrate reductase, this treatment should theoretically not harm the gut flora (Friendship et al., 2009). In pigs, generally intestinal reductions in *Salmonella* counts have been reported after sodium chlorate supplementation (Anderson et al., 2001; Burkey et al., 2004; Patchanee et al., 2007), although the effect varied between different formulations and treated age-groups. Nevertheless, the use of chlorate is currently not allowed in the EU (European Commission, 2012).

Experiments have indicated that **egg yolk** inclusion in the feed reduced *Salmonella* colonization in poultry (Kassaify and Mine, 2004; Chalghoumi et al., 2009), which was attributed to the anti-adhesive granules and high-density lipoproteins in the egg yolk (Kassaify et al., 2005). In weaned piglets, Letellier et al. (2000) and Mathew et al. (2009) could not demonstrate an effect on *Salmonella* shedding after administrating *Salmonella*-immunized egg yolk powder, but more in-depth research in this matter is currently lacking.

Virulent **bacteriophages** are natural predators of their bacterial hosts, present in the gut of food animals, and they complete their lifecycle by lysis of the infected bacterium (Górski and Weber-Dabrowska, 2005). These bacteriophages are currently investigated as a potential intervention strategy against *Salmonella* in swine, and results seem promising (Callaway et al., 2011), especially when using phage cocktails to avoid the development of resistance in host cells (Wall et al., 2010). Recently, American authorities have approved a commercial bacteriophage-containing product as a ‘Generally Recognized As Safe’ (GRAS) food processing aid against *Salmonella* (SALMONELEX®, Micreos). Industrial scale projects in poultry are currently ongoing with this promising product, but the effect in pig industry remains to be investigated as well.
5.3 Drinking water supplementations

Supplementing the drinking water of pigs with e.g. organic acids or chlorate, has the advantage over feed supplementation that animals can be treated in a more flexible way and that they can be treated until the moment they leave the farm to be transported to the slaughterhouse. In most cases, feed withdrawal is applied 12 - 18h pre-slaughter, whereas Salmonella infection and excretion often increases prior to loading and during transport, due to stress (Loynachan et al., 2004; Wales et al., 2010). Mostly, investigations of organic acids or chlorate supplementations are reported for this drinking water administration.

Most of the characteristics of organic acids when supplemented in the feed, are also applicable when used in drinking water. The aimed pH decrease of the drinking water after supplementation depends on the innate pH and the buffering capacity of the water and the volume and form of organic acid administered. Hard, alkaline water needs to be supplemented with more acid than soft water to reach the same pH. Logically, a larger volume of acid obtains a lower pH of the drinking water, but when using organic acids with a low pKa value (e.g. formic, acetic and lactic acid), a smaller volume is required to reach a certain decrease in pH. Also the form of the administered acid is important, as the necessary bactericidal volume of certain acids cannot be added, due to a decreased pH level of the drinking water beyond palatability (i.e. a pH below 3.5 for pigs). Buffering these acids into a salt form (e.g. ammonium formate instead of formic acid) should allow the administration of more acid while reaching the same pH (van der Wolf et al., 2001b).

All these characteristics may explain the high variation in results between studies which investigated the efficacy of organic acid supplementation of the drinking water. Some studies showed beneficial effects on Salmonella seroprevalence (van der Wolf et al., 2001b; van der Heijden et al., 2006), whereas others could not show any improvements on seroprevalence (Hansen et al., 1999) or lymph node colonization (Letellier et al., 2000; De Busser et al., 2009). For assessing the efficacy of supplementation, it is therefore important to apply a correct acid mixture and dosage, and to monitor closely the pH level during treatment.

Apart from the efficacy, also some technical issues should be considered. First, acid-induced corrosion may occur. This problem can be alleviated by using buffered acids which are less corrosive, and by installing pipeline systems of PVC or stainless steel. Second, growth of fungi may be enhanced and may lead to the clogging of pipes and nipple drinkers. This problem can be (partly) prevented by adding propionic/sorbic acid, or copper/zinc to the water, and by cleaning the pipeline system thoroughly between batches (van der Wolf et al., 2001b). Consequently, a number of practical conditions (required pH of the drinking water, right acid volume, proper equipment) need to be fulfilled for a
successful supplementation. In chapter 4, these dose-related and practical conditions are discussed more detailed.

Several experimental studies with supplementation of chlorate salts in drinking water for pigs (Anderson et al., 2004) and poultry (McReynolds et al., 2005; Byrd et al., 2008) have shown beneficial effects as well. Anderson et al. (2004) showed a significant reduction of Salmonella Typhimurium in the caecum of weaned pigs, but the results were not satisfactory in finisher pigs.

5.4 Vaccination

Any invading pathogen like Salmonella, is firstly attacked by the innate immune system. Initially ‘sentinel cells’ like antigen presenting cells (APC), recognize pathogen-associated molecular patterns (PAMPs) on invading microorganisms. Thereafter, they produce several inflammatory cytokines for the chemo-attraction of phagocytic cells, which in turn eliminate the pathogen (Mastroeni et al., 2001; Tizard, 2009). As this innate immunity lacks memory capacity, it cannot ensure resistance against re-infection (Dougan et al., 2011). For this, the activation of the acquired immune system is necessary, in which mainly T- and B-lymphocytes and their products are involved. These lymphocytes are responsible for the so-called ‘cell-mediated’ immune response. This response is basically oriented towards intracellular pathogens, whereas immunoglobulins or antibodies from the B-cells mainly support the destruction of extracellular invaders and participate in the ‘humoral’ immune response (Tizard, 2009).

Since Salmonella is a facultative intracellular bacterium, both cell-mediated and humoral immune responses are essential for protection. Although cell-mediated immunity is generally accepted to be more effective against facultative intracellular bacteria (Robertsson et al., 1983; Haesebrouck et al., 2004), also antibodies and a mucosal immune response are important for protection during the typical intestinal colonization and the extracellular phases of the Salmonella infection (Dougan et al., 2011; Griffin and McSorley, 2011; Arguello et al., 2013).

Vaccination or immunization is defined as the administration of immunogenic substances to stimulate the acquired or adaptive immunity against a specific pathogen. Various vaccination strategies for inducing adaptive immunity against Salmonella infections will be described hereafter. For this, an active or passive pathway can be used, with or without certain ‘DIVA’ capacities (‘Differentiation between Infected and Vaccinated Animals’).
4.1 Active immunization

Active immunisation against *Salmonella* in pigs can be achieved by using: i) inactivated vaccines, such as whole killed bacteria (bacterins) and bacterial components (subunit vaccines); and ii) attenuated, live bacteria.

**Inactivated vaccines: bacterins and subunit vaccines**

**Bacterins** consist of whole bacteria that have been inactivated by heat, formaldehyde or genetic manipulation, so that replication is blocked while antigenic particles such as lipopolysaccharides (LPS), flagellae or fimbriae, are still present. Upon immunisation, these particles induce some degree of T-cell response (Mastroeni *et al.*, 2001), and stimulate B-cells to migrate and produce antibodies (Pape *et al.*, 1997).

The formalin-inactivated parenteral *Salmonella* Typhimurium bacterin of Farzan and Friendship (2010), applied to piglets at weaning, resulted in a decreased shedding of *Salmonella* Typhimurium during the fattening period. Another formaldehyde-inactivated *Salmonella* Typhimurium vaccine (DT104), administered to pigs at the beginning of the fattening period, also reduced *Salmonella* shedding and transmission during the fattening period, as well as at slaughter (Arguello *et al.*, 2013). An inexpensive alternative to heat or chemically inactivated bacteria was investigated by Szostak *et al.* (1996). They constructed a bacterial ‘ghost’ of *Salmonella* Typhimurium via ‘E-mediated lysis’: the gene ‘E’ was inserted into the bacterial DNA, which encodes for a transmembrane tunnel structure in the cell envelope, releasing the cytoplasmatic content from the cell. In contrast with the previous inactivation techniques, this ‘ghost’ construction results in an intact envelope, and thus in an improved antigen presentation. Specific humoral and cellular immune responses against these bacterial components were demonstrated in mice, which significantly prolonged survival after challenge with a virulent *Salmonella* Typhimurium strain. The same ‘E’ gene was targeted by Jawal and Lee (2013), but without using an antibiotic resistance gene marker during construction, and thus a biosafety enhanced *Salmonella* ghost vaccine was developed.

**Subunit vaccines** consist of purified bacterial components such as capsular antigens, proteins or exotoxins, which are presented to the immune system without introducing actual virulent particles. These vaccines however, often elicit weaker antibody responses than bacterins or live vaccines (Meeusen *et al.*, 2007).

The Vi capsular polysaccharide vaccine (ViCPS) against human typhoidal salmonellosis for example, has surely proven its efficacy, but offers only significant protection in the first two years after administration (Fraser *et al.*, 2007). The vaccine of Carnell *et al.* (2007), based on *Salmonella* Typhimurium type III secreted proteins, reduced faecal shedding and disease severity in pigs, although
it did not confer long-term protection. Exotoxins are produced within the bacterial cytoplasm and are mostly proteins with a high molecular mass and therefore good antigen capacities. Vaccines may contain the inactivated toxin or its antigenic derivative, providing a variable protection level (Haesebrouck et al., 2004). Rahman and Sharma (1995) found that formalin-treated enterotoxins of different Salmonella serotypes induced antibodies that neutralized the enterotoxic activity of crude enterotoxins. Also Chopra et al. (1999) showed enterotoxic and cytotoxic activities of the Salmonella enterotoxin from the Salmonella Typhimurium strain they examined, suggesting its role in acute gastroenteritis due to Salmonella. In contrast, Nakano et al. (2012) reported no association between the enterotoxin of Salmonella and its virulence. They demonstrated that the Salmonella enterotoxin regulates the localisation and levels of outer membrane protein A (OmpA) in Salmonella, and that it is involved therefore, in the maintenance of membrane integrity. As OmpA is one of the major immunogenic proteins, these authors concluded that the Salmonella enterotoxin might play an important role in the induction of Salmonella immune responses.

The abovementioned sub-optimal capacity of inactivated vaccines to evoke immune responses, can be improved by the addition of an adjuvant (indicated by its latin origin ‘adjuvare’ai). This adjuvant presents the antigen in such a way to APCs that, depending from the adjuvant product, the humoral or cell-mediated immunogenicity is enhanced, in comparison with the presentation of the simple aqueous antigen. Consequently, this results in a reduction of the necessary antigen dose in the vaccine (Aguilar and Rodriguez, 2007). Adjuvanticity can be obtained by many products, e.g. mineral salts, tensoactive compounds, bacterial DNA, emulsions, liposomes, nano-beads, virus-like particles, cytokines, polysaccharides (Aguilar and Rodriguez, 2007). Freund’s incomplete adjuvant (FIA), an emulsion of mineral oil and water enabling a slow release of antigen from the injection site, is probably the most well-known adjuvant in both human (Petrovsky and Aguilar, 2004) and veterinary (Stabel et al., 1991 (nursery piglets); Miyamoto et al., 1999 (laying hens)) vaccines.

**Attenuated vaccines**

Attenuated Salmonella vaccines contain live bacteria with very low virulence, which are able to replicate only locally or very slowly. These vaccines are generally considered superior to inactivated Salmonella vaccines, probably due to: i) their ability to induce a more pronounced cell-mediated immune response and the induction of mucosal IgA production (Robertsson et al., 1983; Haesebrouck et al., 2004; Meeusen et al., 2007); ii) their reproducing capacity beyond the initial vaccination, so that less boost immunizations are required or even single-dose protocols are possible; iii) the possibility of oral administration; iv) the low production cost and easy storage; and (v) the possible use as antigen carriers
for recombinant vaccines (Mastroeni et al., 2001). The attenuation of live vaccines can be acquired by several methods, as explained hereafter.

Traditional attenuation methods are empirical and involve the passage of bacteria into different cultures, chemical treatments or at suboptimal temperatures, allowing selection of less virulent strains without actually characterising the attenuation (Meeusen et al., 2007; Tizard, 2009). Foster et al. (2003) constructed this way an avirulent rough mutant of Salmonella Infantis, which provided in piglets a rapid protection against disease and a reduced tissue invasion after Salmonella challenge.

A second method is the manipulation or deletion of genes that are important for the bacterial metabolism (e.g. auxotrophic mutant strains) or for global regulating functions (Boyen et al., 2008a).

Auxotrophic Salmonella mutants require certain metabolites that are unavailable in mammalian tissues and have therefore a reduced ability to grow in vivo. Lumsden and Wilkie (1992), Barrow et al. (2001), and Trebichavsky et al. (2006) all investigated auxotrophic aromatic-dependent (aroA) mutant strains of Salmonella Typhimurium in piglets. Lumsden and Wilkie (1992) demonstrated a significant reduced disease severity and faecal shedding in weaned piglets after Salmonella challenge at one week post-vaccination, whereas Trebichavsky et al. (2006) could not establish any protection after their vaccination. In the latter study however, suckling piglets were vaccinated and then challenged already 24h later. Barrow et al. (2001) emphasised the need for adequate control of potential aroA vaccines, as their aroA mutant strains possessed some residual virulence in piglets. Other auxotrophic mutant strains tested in pigs, gained more success in reducing Salmonella infection: Springer et al. (2001), Lindner et al. (2007), Rösler et al. (2010) evaluated the same histidine-adenine auxotrophic mutant Salmonella Typhimurium strain (Salmoporc®, IDT Biologika). These authors have shown that the Salmoporc® vaccine may decrease both Salmonella colonization of intestinal mucosae and tissues and the shedding of Salmonella bacteria. Based on its auxotrophic feature, this vaccine can be distinguished from Salmonella field strains using a biochemical Salmonella Diagnostic Kit (Lindner et al., 2007; www.idt-biologika.de). This differentiation can also be achieved by molecular typing methods (Lindner et al., 2002).

Several mutations in global regulator genes have been explored in pig studies as well. Foss et al. (2013) used a mutant Salmonella Typhimurium strain ‘Δcrp’, lacking the cyclic AMP receptor protein gene, and proved that vaccines provide better efficacy in pigs that are challenged with a serogroup-matching Salmonella strain. A serovar-match between vaccine and challenge strain had less impact, as a significant reduction of Salmonella shedding was still obtained without a match. A mutant strain of Salmonella Typhimurium, unable to synthesize the zinc transporter ZnuABC ‘ΔznuABC’, prevented clinical symptoms and decreased faecal shedding in Salmonella-challenged fattening pigs in the study
of Gradassi et al. (2013). Similar beneficial results were observed by Roesler et al. (2004), who investigated a mutant gyrA-cpxA-rpoB of Salmonella Typhimurium in piglets.

Finally, also targeted deletions in virulence genes can be performed, which have the major advantage that the resulting vaccine strains are very unlikely to reverse into the wild-type strain (Haesebrouck et al., 2004). Clinical signs were prevented and no Salmonella was isolated from faeces of piglets vaccinated with the ΔcpxR-Δlon strain developed by Hur et al. (2011). A deletion of the poxA gene (encoding for a certain aminoacyl-tRNA synthetase) in Salmonella Typhimurium significantly diminished fever, reduced faecal shedding of Salmonella, and decreased Salmonella colonization of the tonsils in piglets (Bearson et al., 2011), indicating that this deletion exerts selective effects on stress survival and colonization capacities.

Notwithstanding all abovementioned experimental advances in the development of bacterial live vaccine candidates, only one vaccine against Salmonella Typhimurium is currently commercialized in the European Union (i.e. the Salmoporc® vaccine described above). Indeed, the construction of bacterial live vaccines is both difficult and expensive, and for licensing these vaccines at the European Medicines Agency (EMA), proof of the absence of zoonotic hazards must be provided. This strict licensing procedure is a very time-consuming process, as the guidelines demand well-defined and irreversible mutations, requiring extensive experimental testing (Sheridan and Coughlin, 2010).

### 5.4.2 Passive immunization

Passive immunization implies the transfer of antibodies from a donor animal to a receptor animal, in order to confer immediate protection to the latter susceptible animal (Tizard, 2009). Obviously, this requires first an active immunization of the donor animal. Some methods of passive immunization against Salmonella are available so far. However, not all of them are efficacious.

Vaccination of pregnant sows appears to be the most promising method to decrease slaughter pig prevalence of Salmonella (Hotes et al., 2011; Wales et al., 2011), and is even considered more effective than vaccinating suckling piglets by Ojha and Kostrzynska (2007). Maternal seropositivity to Salmonella protects piglets from excretion in the postweaning stage (Kranker et al., 2003; Nollet et al., 2003), and the theory behind this is plural. Vaccinating pregnant sows may result in i) a colostral or lactogenic transfer of antibodies and lymphocytes to piglets, which act locally in the piglets intestine and compensate to some extent for their immature intestinal immune system (Bailey and Haverson, 2006); and ii) a decreased excretion by these sows, and consequently in a diminished infection of their offspring (Roesler et al., 2006).
Several vaccination trials in sows led to the previous assumptions. Roesler et al. (2006) for instance, vaccinated pregnant sows antepartum in a Salmonella Typhimurium positive herd, using an inactivated herd-specific Salmonella Typhimurium vaccine. This resulted in a reduction of Salmonella excretion in these sows, and an elimination of excretion and colonization of Salmonella in their piglets. In addition, these piglets showed a significantly decreased IgA and IgG antibody level. Both findings indicated a lower Salmonella prevalence in the offspring of the vaccinated sows. A larger and longer study than the previous one was performed by Lindner et al. (2007), who combined passive and active immunization on a subclinically infected herd, by vaccinating both the sows and their offspring with a live-attenuated Salmonella Typhimurium vaccine (Salmoporc®). At slaughter age, the vaccinated pigs showed a significantly reduced lymph node colonization and seropositivity level. However, the control group was not monitored concurrently with the vaccinated group (historic control). Another attenuated Salmonella Typhimurium vaccine (ΔcpxR-Δlon), was administered orally to pregnant sows by Hur and Lee (2010). Colostral and serum IgA and IgG levels in the vaccinated sows and their piglets, respectively, were significantly increased as compared to the unvaccinated animals. In addition, no Salmonella was isolated from faecal samples of vaccinated piglets, in contrast with several unvaccinated piglets. When investigating the same vaccine strain by immunization of both the sows and their offspring (Hur et al., 2011), similar results were observed as in the previous trial. Recently, Matiasovic et al. (2013) demonstrated that vaccination of pregnant sows with an inactivated experimental Salmonella Typhimurium vaccine resulted in lower counts of a homologous Salmonella Typhimurium in the ileal and caecal mucosa of orally infected piglets.

Another possibility for applying passive immunization is the oral administration of egg yolk containing Salmonella-specific antibodies derived from Salmonella-challenged laying hens, which was successfully explored in experiments with poultry (Kassaify and Mine, 2004; Chalghoumi et al., 2009). When feeding pigs with egg yolk however, both Lettelier et al. (2000) and Mathew et al. (2009) could not demonstrate such a protective effect on Salmonella shedding. Lettelier et al. (2000) suggested that this might be due to an alteration of the egg antibodies by the pigs’ digestive tract, which may fail to recognize Salmonella bacteria before they penetrate the intestinal epithelium.

5.4.3 DIVA vaccines

A major disadvantage of vaccination is its interference with many current national Salmonella surveillance programmes, which rely on serology for the classification of herds according to their Salmonella risk (high, medium or low level of antibodies). In order to overcome this problem, several potential Salmonella ‘DIVA’ vaccines and their accompanying diagnostic tests have been developed,
which claim to allow a ‘Differentiation between Infected and Vaccinated Animals’ (van Oirschot, 1999). For several microorganisms, the DIVA strategy is applicable to both attenuated and inactivated vaccines (Pasick, 2004; Meeusen et al., 2007). In Salmonella-related research however, only few, live-attenuated DIVA strains in pigs have been constructed.

Selke et al. (2007) selected the outer membrane porine D (OmpD) as a putative suitable marker protein to construct a DIVA vaccine. They removed the OmpD gene from the DNA of a licensed live Salmonella Typhimurium vaccine (Salmoporc®), and immunized weaned piglets with this ‘SalmoporcΔompD’ strain before challenging them with a Salmonella Typhimurium DT104 isolate. Clinical disease and colonization of lymph nodes and intestinal mucosae were significantly reduced in the SalmoporcΔompD-vaccinated piglets. Additionally, a differentiation of the infected unvaccinated and vaccinated animals was possible using an OmpD-based ELISA, which cannot detect antibodies directed against the SalmoporcΔompD-strain. The fact that this vaccine candidate is based on a licensed parent strain, should facilitate the realization of future field studies and subsequent licensing procedures. However, two important drawbacks can be noticed. Firstly, Selke et al. (2007) found in their piglet experiment that the reduction of colonization was 10-fold lower upon vaccination with the SalmoporcΔompD strain than with the Salmoporc® parent strain. This might be explained by the importance of the OmpD protein in mediating protective antibody response (Gil-Cruz et al., 2009).

Secondly, the ELISAs used in most European Salmonella serosurveillance programmes are based on the detection of antibodies against the lipopolysaccharides (LPS) of Salmonella spp. (Cortiñas et al., 2009), since these outer membrane components are a major virulence factor of Salmonella enterica. Therefore, this DIVA vaccine is not directly broadly applicable as it necessitates a specific OmpD-based ELISA for differentiation.

Kong et al. (2011) investigated the deletion of LPS-synthesising genes, and found that a complete truncation of the LPS chain (ΔrfaG) is not a valuable option for developing an efficient live oral Salmonella Typhimurium vaccine. When one O-antigen unit (ΔrfaJ) was retained however, the authors did find an adequate stimulation of protective immunity after vaccination.

The same conclusion was drawn in the paper of Leyman et al. (2011), in which a ΔrfaJ Salmonella Typhimurium strain significantly protected mice against a subsequent infection with a virulent Salmonella Typhimurium strain. In piglets, the experimental vaccine induced a serological response that lacked detectable antibodies against LPS, but the effect on Salmonella excretion was not evaluated. With this vaccine, a differentiation of infected and vaccinated pigs was thus possible in the broadly-used LPS-based ELISA, without reducing the strain’s protective capacities in mice.
5.4.4 Conclusions

Overall, the available evidence from Salmonella vaccination studies suggests that Salmonella vaccines are associated with reduced Salmonella prevalence in swine. However, several arguments can be given why further research is necessary. Many studies were based on a limited number of animals and on a wide variety of target populations, sample sizes, types of vaccines, doses and dosing regimens (Denagamage et al., 2007). Also, no complete protection was obtained (Friendship et al., 2009; Wales et al., 2011), and the effect estimates in a meta-analysis study appeared very variable, which suggests a context-sensitive vaccination response and potential confounders (Wilhelm et al., 2012).

Therefore, in further research it is necessary to conduct transmission studies and large-scale trials to assess the effects of Salmonella vaccination under field conditions and to enable well-founded decisions concerning the potential use of vaccination in Salmonella control programmes.

5.5 Salmonella monitoring and control programmes

As pointed out in previous sections, it is of paramount importance to protect human health against Salmonella and other pathogens that are (in)directly transmissible between animals and humans. Therefore, the European Parliament published eleven years ago the Directive 2003/99/EC and Regulation (EC) No 2160/2003 for its Member States (MS), in order to set up targets for the monitoring and control of Salmonella and other food-borne zoonotic agents, respectively (Anonymous, 2003a,b).

When analysing the Salmonella policies in all 28 MS, 19 were applying an official Salmonella monitoring programme in 2010 (i.a. BE, DE, DK, NL), and nine were not (i.a. FR, UK) (Alban et al., 2002; Anonymous, 2007b; Merle et al., 2011; EFSA and ECDC, 2012a; Anonymous, 2013a,b). The Salmonella approach of Belgium and its five neighbouring MS (DK, DE, FR, UK, NL), belonging to both groups, is described below.

In Belgium, the Federal Agency for the Safety of the Food Chain (FASFC) implemented a national Salmonella Action Plan (SAP) in 2005, for farms with more than 30 fattening pigs. A herd evaluation was established based on the presence of Salmonella-specific antibodies in blood samples collected within the framework of the Aujeszky’s disease control programme (Anonymous, 1999). This serological examination is performed every four months, on 10-12 blood samples from randomly selected pigs of different weight categories (serological details are to be found in paragraph 4.2). If the resulting mean sample-to-positive (S/P) ratio exceeds 0.6 (=OD 24%) during three consecutive sampling occasions, the concerning herd is designated a ‘risk herd’ (Laevens and Mintiens, 2005). The SAP became compulsory in 2007 (Anonymous, 2007b). As from that moment, the ‘risk herds’ must allow a bacteriological
examination of pooled faecal samples from at least four different age groups. They must additionally complete a checklist and implement a Herd-specific *Salmonella* Action Plan (HSAP) together with the herd veterinarian, to detect and reduce present risk factors for *Salmonella* infection. Until now, no penalty consequences were attached to the result of the bacteriological examination. In case these ‘risk herds’ show again a mean S/P ratio above 0.6 in 3 consecutive sample events, they are mandatory visited by a veterinarian of Animal Health Care organisations (DGZ-ARSIA). In addition, since 2008, all farmers producing slaughter pigs have to report the herd health status, including their *Salmonella* risk herd designation, to the slaughterhouse 24h before arrival of the batch of slaughter pigs. This ‘Food Chain Information’ (FCI) document allows the slaughterhouse staff to apply a logistic slaughter procedure and/or to take other preventive measures against contamination. The slaughterhouses in their turn, must sample five randomly selected carcasses once a week (*i.e.* a carcass sponge, taken between two and four hours after slaughter for bacteriological analysis) for auto-control reasons and as part of the Hazard Analysis and Critical Control Points (HACCP) (Anonymous, 2007d). In case 5/50 samples or more are *Salmonella*-positive, the slaughter hygiene and process control must be improved.

Since the implementation of the HSAP in 2007 however, the *Salmonella* seroprevalence of the Belgian pig population has not significantly decreased (Méroc et al., 2012). Therefore, the sampling scheme and classification criteria of the current HSAP are currently under revision. Likely, the programme will shift from serological towards bacteriological monitoring, for which several reasons can be given. Firstly, the serological monitoring for Aujeszky’s disease has recently been reduced to one blood sampling per herd per year. Secondly, there is generally a moderate to low correlation between serology and bacteriology, as discussed above (Nielsen et al., 1995; Davies et al., 2003; Laevens and Mintiens, 2005; Nollet et al., 2005; Farzan et al., 2007; Belsué et al., 2011; Rostagno et al., 2012b). Finally, only bacteriological examination allows a determination of the present serovars and antimicrobial resistance.

The Scientific Committee of the FASFC has proposed several scenarios for adapting the monitoring of the present SAP (Anonymous, 2012):

i) bacteriological examination of pooled faecal samples at the farm, with a focus on breeding herds;

ii) examination of intestinal contents or ileo-caecal lymph nodes at the slaughterhouse, with feedback to the herds;

iii) concurrent examination of ileo-caecal lymph nodes and carcasses at the slaughterhouse, without feedback to the herds;

iv) a combination of scenarios i) and iii) to gather information at both the herd- and population-level.

Obviously, all scenarios have their own scientific and financial (dis)advantages (Chapter 4.3), which will be taken into consideration until a final decision is made by the Belgian Food Agency in 2014. Meanwhile, all blood samples within the framework of the Aujeszky’s disease programme remain to be
examined for *Salmonella*-specific antibodies as well. This implies blood sampling once a year for most herds, and three times for outdoor herds and breeding herds. The FCI document must contain the mean S/P ratio of the last blood sampling. All other former regulations on-farm remain in force (Anonymous, 2014). From June 2014 onwards, the slaughterhouses must in addition improve their slaughter hygiene and auto-control programme if more than 3/50 carcass samples turn out to be *Salmonella*-positive.

**Denmark** already launched a national *Salmonella* surveillance-and-control programme in 1995 (Mousing *et al.*, 1997). This programme has been revised several times since then (Alban *et al.*, 2002), and currently targets multiple steps in the pork production chain: the feed, herd and slaughterhouse level (EFSA and ECDC, 2012a).

Firstly, Denmark’s feed surveillance implies the examination of both feed ingredients and processed feed. Secondly, the herd surveillance consists of a monthly serological testing of breeding herds (blood samples) and fattening herds (meat juice samples). With these serological results of a mix-ELISA (with an OD cut-off value of 20%) a ‘serological *Salmonella* index’ is calculated to classify the herds into three levels (Alban *et al.*, 2002). This index is the weighted average of the results from the preceding three months, the immediate month counting three times as much as both months before (60%:20%:20%). A herd designated level 1 (index<40), 2 (40<index<70), or 3 (70<index) thus showed recently a low, moderate or unacceptably high *Salmonella* seroprevalence, respectively. Both penalties and rewards are associated with this classification. Breeding herds with an index higher than five must inform the purchasing herds. Level-2 and level-3 fattening herds must have faeces samples examined and face a reduced carcass price. Fattening pigs from level-3 herds are in addition, logistically slaughtered and these carcasses are subjected to specific hygienic precautions (e.g. hot water decontamination) (Alban *et al.*, 2010). In contrast, level-0 fattening herds (i.e. herds with no seropositive meat juice samples during the last three months) need to deliver less meat juice samples. Finally, slaughterhouse surveillance is performed by the daily examination of carcass swabs (Baptista *et al.*, 2010b), with the implementation of a strict control programme if certain levels are exceeded.

The **German** *Salmonella* monitoring programme became operational since 2002 (Blaha, 2004) and nationwide compulsory since 2007 (Anonymous, 2007c). It is based upon the serological examination of blood samples collected at the herd (within 14 days prior to slaughter) or meat juice samples from the slaughterhouse; the amount of samples depending on the herd size (Gotter *et al.*, 2012). These samples are analysed in the framework of the ‘Qualität und Sicherheit’ (QS) meat quality programme, via a mix-ELISA with a cut-off value of OD 40%. The percentage of yearly positive samples (re-calculated quarterly) is used to classify herds into three categories: Category I (0 - 20%), II (>20 - 40%), or III (>40%) implies a low, medium, or high *Salmonella* risk (Merle *et al.*, 2011). Category II herds need to
fill in a checklist to scrutinize and improve their hygienic status. Category III herds must have their fattening pigs logistically slaughtered and are obliged to implement on-farm intervention measures. When no improvement is obtained and interventions are lacking, these farmers risk financial consequences and an exclusion from the QS system. At slaughterhouse level, surface swabs are taken (EFSA and ECDC, 2012a) and a *Salmonella* reduction plan must be implemented.

In France, no official *Salmonella* monitoring is currently present (EFSA and ECDC, 2012a; Brossé DGZ 2014, personal communication). Several preparations to implement a programme were made in 2011, but are currently put on hold. These preliminary plans included the sampling of all pig herds with a capacity over 300 fattening pigs, and this nine times per quarter. Based on the results of every last four sampling occasions, the herds would be classified into three levels. Depending on the assigned level, herds would be encouraged to take preventive measures against *Salmonella*, or obliged to put sanitary measures into practice (such as a separated transport and slaughter) (Brossé DGZ 2014, personal communication). Yet, as mentioned above, these plans are not executed anno 2014.

In 2002, the British pig industry (British Pig Executive, BPEX) introduced a voluntary Zoonoses Action Plan *Salmonella* Monitoring Scheme (ZAP) for pig herds in the United Kingdom. This ZAP scheme was replaced in 2008, by the Zoonoses National Control Programme for *Salmonella* in pigs (ZNCP), to focus on a whole-chain risk-based *Salmonella* approach (Anonymous, 2013a). Like in Denmark and Germany, a categorization of the pig herds in three levels is made. This programme is based on monthly-collected meat juice samples that are analysed by a mix-ELISA with an OD cut-off value of 10%. Those herds with the greatest prevalence of seropositive pigs (*i.e.* ZAP-2 and ZAP-3 herds, with over 65% positive samples) must implement an action plan, guided by quarterly veterinary visits (Cook et al., 2003; Abrahantes et al., 2009). No true sanctions are applied, but herds with a *Salmonella* seroprevalence below 10% during the preceding year are rewarded with a Platinum Pig Award. Slaughterhouse surveillance is performed by the examination of five carcass swabs (sponges) per herd, with the frequency depending on the herd size.

Since the national average seroprevalence in British slaughter pigs had not significantly changed over the previous five years (Anonymous, 2013a), the meat juice testing was suspended in 2012. As from 2013, the ZNCP launched an on-farm *Salmonella* risk assessment tool to help farmers identify the most effective control methods for each unit, and is exploring bacteriological testing (Anonymous, 2013a).

Since 2005, the Netherlands apply a national monitoring programme somewhat similar to Belgium, as 12 blood samples are to be collected every four months, from herds with over 30 fattening pigs (Anonymous, 2013b). However, these blood samples, analysed with an OD cut-off value of 40%, may be
collected both at the herd (within three weeks prior to slaughter) or at the slaughterhouse. A scoring system is applied with this serological outcome, assigning the herds every four months a score 1 (OD<20%), 2 (20%<OD<40%), or 3 (40%<OD). Consequently, the sum of the scores of the preceding three trimesters determines the herd’s category: category 1 (sum is 3 or 4), category 2 (sum is 5 to 7) or category 3 (sum is 8 or 9) stands for a low, moderate or high Salmonella risk, respectively (Anonymous, 2013b). No penalties are involved so far, unless herds do not comply with the sampling obligations, for which they will be reprimanded or receive a fine. Concerning the Dutch slaughterhouse monitoring, bacteriological samples are examined from ten pooled carcasses per two weeks or from five individual carcasses per day in small or large slaughterhouses, respectively (Anonymous, 2013b).

Despite all efforts in the existing Salmonella surveillance programmes, it must be underlined that food safety is not a result of testing, but a result of management. Namely, pork that tested negative may still be contaminated and cause Salmonella outbreaks in importing EU countries with a very low national prevalence of Salmonella (Bruun et al., 2009). Every monitoring programme thus has an imperfect probability to assure the quality of a certain management. Therefore, every step in the pork production chain must implement proper Salmonella control measures, to aid surveillance in providing the consumer with a safe food product.
Chapter 2 - Aims
Salmonellosis remained one of the most important and widely distributed foodborne diseases in the European Union in the past 10 years. Since 2005, successful vaccination programmes in laying hens resulted in a substantial decrease in the number of human Salmonella Enteritidis infections across EU Member States. Therefore, a focus is put on the control of human Salmonella Typhimurium infections, which are mainly caused by contaminated pork. Salmonella can be introduced at many different levels of the pig production chain, such as the feed, the primary production, the slaughterhouse, and during pork processing. The primary production plays a key role in this chain, as a lower Salmonella infection level on-farm was associated with less carcass contamination at slaughterhouse level. Moreover, Salmonella infections may have an important negative impact on the weight gain of pigs. It is therefore important to optimize control measures at farm level. Unfortunately, given the many different risk factors, controlling Salmonella infections in pig herds is difficult.

The general aim of this thesis was to provide scientific underpinned advice to improve the control of Salmonella infections in pigs at pre-harvest level.

In previous challenge studies, both the supplementation of the feed or drinking water with organic acids and the use of vaccination showed promising, but inconsistent results in reducing Salmonella (sero)prevalence. The first objective of this thesis was therefore to conduct a transmission study in weaned piglets using a Salmonella Typhimurium challenge strain, to evaluate the effect of currently available organic acids and vaccination on the Salmonella spread, using bacteriological and serological techniques. The control measures included (1) feed with coated calcium-butyrate, (2) drinking water with a mixture of organic acids, and (3) vaccination with a live commercial Salmonella Typhimurium strain.

As most European surveillance programmes rely on serology using commercial LPS-based ELISAs, which do not allow differentiation between Salmonella-specific antibodies induced by vaccination or by infection, vaccine use may be compromised in these countries. Thus, the second objective was to investigate the effect of the previously evaluated commercial vaccine when modified to a DIVA (Differentiation of Infected and Vaccinated Animals) vaccine strain, in a similar transmission experiment with piglets. In addition, to assess the effect of the concurrent use of these intervention measures, this DIVA vaccination was evaluated with and without the administration of in-feed coated calcium-butyrate.

The third objective of this thesis was to verify the effect of one control measure used in the previous transmission studies under field conditions. Namely, the effect on the daily weight gain and on serological and bacteriological parameters was to be assessed in pigs from shortly after birth until
slaughter age in three *Salmonella* Typhimurium-positive farrow-to-finish pig herds. The commercial vaccine – currently not registered in Belgium – was used, as it was considered an easier and more practical intervention candidate to decrease the *Salmonella* infection pressure on-farm than the administration of organic acids.
Chapter 3 – Experimental Studies
1. Evaluation of three intervention strategies
to reduce the transmission of *Salmonella Typhimurium* in pigs

*(Transmission study 1)*


Abstract

Despite current control measures, *Salmonella* in pigs remains a major public health concern. In this in vivo study, the effect of three intervention strategies on *Salmonella* Typhimurium transmission in pigs was evaluated. The first intervention was feed supplemented with coated calcium-butyrate (group A); the second comprised oral vaccination with a double-attenuated *Salmonella* Typhimurium strain (group B) and the third was acidification of drinking water with a mixture of organic acids (group C). After challenge at eight weeks of age, animals were individually sampled for six weeks study (blood once per week; faeces twice per week) and were then euthanized at 14 weeks of age. Post-mortem ileum, caecum, ileocaecal lymph nodes, and tonsils were sampled, along with ileal, caecal and rectal contents, and were tested for the presence of *Salmonella* spp. Transmission was quantified by calculating an ‘adjusted’ reproduction ratio ‘R<sub>a</sub>’ and its 95% confidence interval (CI).

The proportion of pigs that excreted *Salmonella* via the faeces was significantly higher in group C (58%, \( P<0.01 \)) and the positive control group (41%, \( P=0.03 \)), compared to group B (15%). The proportion in group C was also significantly higher than in group A (23%, \( P=0.01 \)). Group A had the lowest proportion of positive post-mortem samples (18%), followed by group B (31%), the positive control group (41%) and group C (64%) (\( P<0.03 \)). The highest transmission was seen in the positive control group and group C (\( R_a=+\infty \) with 95% CI [1.88; +\( \infty \])), followed by group B (\( R_a=2.61 \) [1.21; 9.45]) and A (\( R_a=1.76 \) [1.02; 9.01]).

The results of this study suggest that vaccination and supplementation of the feed with coated calcium-butyrate limited *Salmonella* transmission and might be useful control measures.
Chapter 3.1

Experimental Study 1

Introduction

*Salmonella* infection in pigs is a major concern in the European Union (EU) [Regulation (EC) No. 2160/2003], as in the past ten years contaminated pork has been the second most important source of human salmonellosis in many EU countries (Hauser et al., 2010). Infection or contamination can occur at many different levels of the pig production chain such as via the feed, at the primary production site, in the slaughterhouse, and during pork processing. *Salmonella* infection and/or contamination at the primary production site plays a key role in this chain, as positive associations have been demonstrated between within-herd *Salmonella* seroprevalence and carcass contamination (Sørensen et al., 2004; Baptista et al., 2010b), and reducing pre-slaughter *Salmonella* infections increases pork safety, i.e. fewer infected lymph nodes and intestinal contents (Hurd et al., 2002).

Unfortunately, controlling *Salmonella* infections in pig herds is difficult. The pathogen is common, persists in the environment, and infections with most serovars occur without any obvious symptoms (Davies et al., 2004; EFSA, 2008a). Although hygiene and biosecurity on-farm are of paramount importance in decreasing *Salmonella* prevalence in slaughter pigs (Hotes et al., 2011), *Salmonella*-free housing cannot be obtained simply by cleaning and disinfection regimens at farm level (Mannion et al., 2007; McLaren et al., 2011). Hence, such regimens should be combined with other measures as part of an overall strategy to control *Salmonella* on-farm (Wales et al., 2009).

Several studies have evaluated the effect on *Salmonella* control of on-farm treatment of feed or water with acids (Letellier et al., 2000; van der Wolf et al., 2001b; Lo Fo Wong et al., 2004a; Farzan et al., 2006; Canibe et al., 2005; Creus et al., 2007; Poljak et al., 2008; De Busser et al., 2009; Taube et al., 2009; Tanaka et al., 2010). The results varied greatly between these studies, possibly because of the large differences in the acidification process and dosage used (O’Connor et al., 2008). Of these different acidification methods and products, the use of coated butyric acid appears promising, as it decreased *Salmonella* shedding significantly in several studies (Van Immerseel et al., 2005; Boyen et al., 2008b; Guilloteau et al., 2010).

Another possible method of on-farm control of *Salmonella* infections in pigs is vaccination. In most studies the use of *Salmonella* Typhimurium vaccines significantly decreased clinical signs and excretion of *Salmonella* (Springer et al., 2001; Roesler et al., 2004, 2006; Eddicks et al., 2009; Farzan and Friendship, 2010; Hotes et al., 2011; Hur et al. 2011). However, Denagamage et al. (2007) concluded in their review that the design and reporting deficiencies in many studies (e.g. little detail on population type, sample size, type of vaccine, dose and dosing regimens) meant that the association between vaccination and *Salmonella* reduction in finisher swine was promising, but not proven. Furthermore, currently available serological tests do not differentiate between commercial-vaccine-induced and infection-induced
antibodies, so vaccine use may be compromised in countries where serology is used for *Salmonella* surveillance (*e.g.* Denmark, Germany, UK, Belgium; EFSA and ECDC, 2011).

To our knowledge, the ability of such intervention measures to prevent the spread of *Salmonella* among pigs has not yet been investigated via transmission experiments. A great advantage of these experiments is that the reduction of both the infectivity and susceptibility of treated animals can be quantified, whereas traditional challenge studies only demonstrate the effect of reduced susceptibility (Springer *et al.*, 2001; Roesler *et al.*, 2004; Tanaka *et al.*, 2010). The aim of the present study was to evaluate, through the estimation of an adjusted reproduction ratio, the influence of three different intervention strategies, namely, (1) feed with coated calcium-butyrate; (2) vaccination; and (3) acidified drinking water, on the transmission in pigs of *Salmonella enterica* subspecies *enterica* serovar Typhimurium – the most prevalent *Salmonella* serotype in pigs in Belgium and Europe (CODA, 2010; EFSA and ECDC, 2012a).

**Materials and methods**

This experiment was approved by the ethical committee of the Scientific Institute of Public Health and the Veterinary and Agrochemical Research center IPH-VAR (100412-02).

**Herd selection**

For the initial survey, pig herds in the national *Salmonella* monitoring programme were selected, based on consistently low Sample-to-Positive (S/P) ratios (S/P<0.20) in three consecutive blood samples taken from grower-finishing pigs in the preceding year. These herds were then visited, bacteriological and serological samples were taken, and the supply farm was selected based on hygiene, management and sample results.

**Piglet selection**

In order to select *Salmonella*-negative piglets, six sows from each selected herd were chosen through a bacteriological and serological screening process, which was repeated three times. From each sow, three piglets were screened as well. Finally, the sows with the lowest S/P ratios were selected to provide the experimental piglets. The average S/P ratio for the sampled piglets (at the time of the third screening at the age of eight days) was 0.07 ± 0.10 (standard deviation).
In total, 69 piglets from six different sows were selected, weaned at 19 days of age and then transported to the experimental animal facilities of CODA-CERVA in a thoroughly cleaned and disinfected trailer.

**Study design**

Upon arrival, the piglets were randomly assigned into five groups: Group A (n=2x8) received feed supplemented with 0.3% w/w coated calcium-butyrate (Greencab-70®, Sanluc International) (Table 1), group B (n=2x8) was orally vaccinated at 22 and 43 days of age with 5x10^8-5x10^9 Colony Forming Units (CFU) of a double-attenuated histidine-adenine auxotrophic *Salmonella* Typhimurium vaccine (Salmoporc®, Impfstoffwerk Dessau-Tornau), and group C (n=2x8) received drinking water adjusted to pH 3.6-4.0 using 0.09% v/v of a mixture of formic, propionic, acetic and lactic acid (Agrocid Super®, CidLines) (Table 1). This water was checked daily with a pH-meter (pHep+ATC, Hanna Instruments). A positive control group (infected/untreated; n=2x8) and a negative control group (uninfected/untreated; n=5) were also included. All treatments were applied from arrival in the experimental facilities (three weeks of age) until the end of the experiment (14 weeks of age).

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>% in supplement</th>
<th>% in feed (1) or water (2)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>(1) Feed supplement in group A:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Butyrate anion</td>
<td>± 70.0</td>
<td>± 2.1</td>
</tr>
<tr>
<td>Calcium (organic)</td>
<td>± 14.0</td>
<td>± 0.4</td>
</tr>
<tr>
<td>Free Fatty Acids:</td>
<td>± 10.0</td>
<td>± 0.3</td>
</tr>
<tr>
<td>C16:0</td>
<td>7.5-9.0</td>
<td>0.23-0.27</td>
</tr>
<tr>
<td>C18:0</td>
<td>0.5-1.0</td>
<td>0.02-0.03</td>
</tr>
<tr>
<td>C18:1</td>
<td>0.5-1.0</td>
<td>0.02-0.03</td>
</tr>
<tr>
<td>C14:0</td>
<td>&lt;0.35</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td><strong>(2) Water supplement in group C:</strong> (pH 2.0-3.5)</td>
<td>(pH 3.6-4.0)</td>
<td></td>
</tr>
<tr>
<td>Formic acid</td>
<td>&gt; 50</td>
<td>&gt; 0.044</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>&gt; 10</td>
<td>&gt; 0.009</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>&gt; 10</td>
<td>&gt; 0.009</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>&lt; 5</td>
<td>&lt; 0.004</td>
</tr>
</tbody>
</table>

Group A was given deodorized calcium-butyrate coated with plant oils in the pig meal, while group C received a mixture of organic acids in the drinking water.

All animals were fed the same meal without antimicrobials throughout the study, except for group A where the feed was supplemented as described above. Every group was housed in a different room with two similar pens (2x8 pigs), which were separated with solid 1 m high partitions. The stocking density was 0.42 m² per pig.
At 57 days of age, two pigs in each pen - except in the negative control group - were moved to a separate room and were orally challenged (Day -1) with $10^8$ CFU of a nalidixic acid-resistant Salmonella Typhimurium strain 112910a, previously isolated from a pig without clinical signs of salmonellosis by Boyen et al. (2009). Twenty-four hours after this challenge, these ‘seeder’ pigs were replaced into their original pens (Day 0). All pigs were euthanized and autopsied at 95 days of age (Day 37).

**Sampling**

The sampling scheme is shown in table 2. From three weeks of age (Day -39) until euthanasia at 14 weeks of age (Day 37), blood samples were obtained from all 69 pigs once a week to detect Salmonella-specific antibodies via ELISA. From three weeks of age (Day -39) until challenge (Day -1) pooled faecal samples were taken weekly. After challenge, individual faecal samples were collected from the rectum of all pigs twice a week. At autopsy (Day 37), samples of ileocaecal lymph nodes, ileum, ileal content, caecum, caecal content, faeces and tonsils were taken, and examined bacteriologically.
Table 2: Experimental design with the taken samples/actions and the used diagnostics/products, in function of the age of the pigs.

<table>
<thead>
<tr>
<th>Sampling/action (frequency)</th>
<th>Age of the pigs (weeks)</th>
<th>Diagnostic method/product</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Pooled faeces per pen (once/week)</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Individual rectal faeces (twice/week)</td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>Blood (once/week)</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Supplementation in feed (A) and water (C) (ad libitum)</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Vaccination (B) (twice: primer + boost)</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Challenge (2 pigs/group)</td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>Rectal faeces, Ileum + content, Caecum + content, Ileocaecal lymph nodes, Tonsils (autopsy)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Isolation <sup>a</sup>

Feed: coated calcium-butyrate (Greencab-70, Sanluc International)
Water: mixture of organic acids (Agrocid Super, CidLines)

Commercial _Salmonella Typhimurium_ vaccine (Salmoporc, IDT)

Nalidixic acid-resistant _Salmonella Typhimurium_ strain 112910a

Isolation <sup>a</sup>

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<sup>b</sup> Commercial LPS-based ELISA kit (HerdChek Swine _Salmonella_, IDEXX Laboratories)
**Bacteriological examination**

Faecal examination was initiated within two hours of collection. *Salmonella* was isolated using the ISO 6579:2002/Amd1:2007 Annex D method (ISO, 2007). Briefly, the faecal samples (10-25g) were inoculated in buffered peptone water (BPW, Bio-Rad) in dilution 1:10 and incubated aerobically for 16-20 h at 37 °C. Of this solution, 0.1 mL was inoculated on a Modified Semi-solid Rappaport Vassiliadis plate (MSRV, Bio-Rad) and incubated aerobically at 41.5 °C for 48 h. Growth halos were plated on a Xylose Lysine Deoxycholate agar plate (XLD, Bio-Rad) and a Brilliant Green Agar plate (BGA, LabM) – the latter supplemented with 20 µg/mL nalidixic acid – and then incubated aerobically for 21-27 h at 37 °C. One *Salmonella*-suspected colony on these XLD plates (challenge or vaccine strain) or on the BGA plates (only the challenge strain) was inoculated in Triple Sugar Iron agar (TSI, Bio-Rad) and lysine decarboxylase bouillon (Oxoid) and incubated for 18-24 h at 37 °C for final identification.

For samples from group B, when growth of *Salmonella* bacteria was only observed on XLD and not on BGA, the sample was additionally tested with medium A and B (*Salmonella* Diagnostic Kit, IDT) to confirm that the isolated bacterium was the vaccine strain. Quantification of *Salmonella* was performed on faecal samples 3, 7 and 24 Days Post Infection (DPI) using standard enumeration protocols (Boyen et al., 2009).

The tissue samples taken at necropsy were first rinsed with phosphate buffered saline, then sliced, suspended in BPW (dilution 1:10) and homogenized in a stomacher (BagMixer, Interscience) for one minute. Further isolation was performed as for the faecal samples.

**Serological examination**

Blood samples were allowed to clot for 24 h at 15°C and were then centrifuged for 15 min at 1200 g. Serum was diluted twenty-fold and analysed for *Salmonella*-specific antibodies with a commercial ELISA kit based on lipopolysaccharide (LPS) antigens (*HerdChek Swine Salmonella*, IDEXX), following the manufacturer’s instructions. Optical densities (OD) were determined by photospectrometry with a 650 nm filter. The outcome was expressed as a ‘Sample to Positive’ ratio:

\[
\text{S/P} = \frac{\text{OD}_{\text{Sample}} - \text{mean OD}_{\text{negative kit control}}}{(\text{mean OD}_{\text{positive kit control}} - \text{mean OD}_{\text{negative kit control}})}
\]

Samples with an S/P ratio ≥0.25 (or OD% ≥10) were considered positive.
Statistical analysis

The number of positive faecal samples collected after challenge were compared between the groups using Generalized Estimating Equations (GEE) (PROC GENMOD and lsmeans; SAS version 9.2). A logit link function, a binomial distribution and an autoregressive first order correlation matrix was assumed with repeated measures. A one-way ANOVA test was used to assess differences between groups in the number of *Salmonella* spp. (log$_{10}$ normalized data) quantified in the faeces on Day 3, 7 and 24.

Bacteriological results from the tissue samples (ileum, ileal content, caecum, caecal content, ileocaecal lymph nodes, tonsils) were compared between groups using logistic regression analysis (SAS version 9.2). *P*-values below 0.05 were considered significant.

The S/P ratios obtained by the ELISA were analysed by repeated measures ANOVA (PROC MIXED and lsmeans; SAS version 9.2). Both pig and pen were considered random effects. An autoregressive first order correlation structure was used for the within-subject correlation. In all models the Bonferroni correction was used to account for the use of multiple comparisons.

The transmission of the *Salmonella* Typhimurium challenge strain in each group was estimated on the basis of the stochastic ‘Susceptible-Infectious’ (SI) infection model using an ‘adjusted’ reproduction ratio ‘$R_a$’. This ratio expresses the mean number of secondary infections caused by one typically infectious animal in a fully susceptible population during a defined time period (3–14 weeks of age in this study) (Meyns *et al.*, 2004). An $R_a$ value of for example 1.76 implies that an infected pig from a group infects on average 1.76 pen mates during the observed period. These $R_a$ values were calculated via the Maximum Likelihood Estimation (MLE), using each of the following definitions of infection. At least one positive sample was necessary to define an animal as *Salmonella*-positive based on: (1) individual faeces; (2) ileum and/or ileal content and/or caecum and/or caecal content; (3) ileocaecal lymph nodes and/or tonsils; (4) all tissue and/or all faecal samples. This was done to investigate the most suitable matrix for this estimation. For each of these four possible definitions the number of contact-infections was thus determined as variable ‘$X_i$’. The following equation was used for this estimation:

$$R_{a,mle} = \max_{R_a} \prod_{i=1}^{m} F(X_i, R_a \mid N, S_0, I_0)$$

in which $F(X_i, R_a \mid N, S_0, I_0)$ is the likelihood function for the observed $X_i$-value, if N (total number of piglets), $S_0$ (number of susceptible piglets), $I_0$ (number of infectious piglets) and m (number of experiments) are given (Meyns *et al.*, 2004).
Chapter 3.1

Experimental Study 1

Results

Bacteriological examination

All faecal samples taken prior to challenge were negative for *Salmonella* spp. No *Salmonella* spp. were isolated from the negative control group at any timepoint, supporting the conclusion that all other positive isolates were from infection with the introduced challenge strain. From challenge till euthanasia, the number of excreting pigs in group C (58%) was significantly higher than in group A (23%, \( P=0.01 \)) and B (15%, \( P<0.01 \)). Likewise, the number of shedding pigs in the positive control group (41%) was significantly higher than in group B (\( P=0.03 \)). No other significant differences between groups were observed for the faecal samples (Fig. 1). These results were consistent with the *Salmonella* bacterial counts on Day 3, 7, and 24. The mean \( \log_{10} \)-values (CFU/g faeces) were 2.5, 1.1, and 0.7 for group A, 1.0, 0.3, and 1.1 for group B, 3.6, 2.3, and 1.6 for group C and 2.1, 1.2, and 1.3 for the positive control group, with only significant differences at Day 3 and 7 when group C was compared with group A and B (\( P<0.01 \)).

![Figure 1: Isolation of the inoculated *Salmonella* strain in individual faeces (ISO 6579:2002/Amd1:2007 Annex D) twice a week: proportion of positive animals in function of the days post infection in Group A (feed with coated calcium-butyrate), Group B (vaccination), Group C (water acidified with organic acids to pH 3.6-4.0), and in the Positive and Negative Control Group. Day -1: Oral infection with \( 10^8 \) CFU *Salmonella* Typhimurium of two seeder pigs per group of eight pigs. Day 0: Reintroduction of seeders in their original pen. Day 38: Euthanasia and Necropsy.](image-url)
Of all tissue samples, 18%, 31%, 64% and 41% were found to be positive for *Salmonella* in group A, B, C and the positive control group, respectively (Table 3). Group C had significantly more infected tissue samples than all other groups (*P*<0.01). In group B, 31% of all tissue samples contained the vaccine strain, with the proportion of vaccine-positive tonsils being higher than all other tissues (*P*<0.01) (Fig. 2).

Table 3: Number of tissue and content samples positive for the *Salmonella* Typhimurium challenge strain, in the four groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Different necropsy samples</th>
<th>All necropsy samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ileum</td>
<td>Ileal Content</td>
</tr>
<tr>
<td>A) Coated calcium-butyrte (n = 16)</td>
<td>4/16 (25%)</td>
<td>2/16 (13%)</td>
</tr>
<tr>
<td>B) Vaccination (n = 16)</td>
<td>7/16 (44%)</td>
<td>4/16 (25%)</td>
</tr>
<tr>
<td>C) Water with organic acids (n = 16)</td>
<td>9/16 (56%)</td>
<td>5/16 (31%)</td>
</tr>
<tr>
<td>D) Positive control (n = 16)</td>
<td>9/16 (56%)</td>
<td>7/16 (44%)</td>
</tr>
<tr>
<td>All groups (n=64)</td>
<td>29/64 (45%)</td>
<td>18/64 (28%)</td>
</tr>
</tbody>
</table>

* A= feed with 0.3% coated calcium-butyrte; B= vaccination at 3 and 6 weeks of age; C= water with 0.09% organic acids; D= positive control; 
* significant difference between group C and the other groups (*P*<0.03).
The adjusted reproduction ratios $R_a$ for the different infection definitions are shown in table 4. Overall $R_a$ values were lower in group B ($R_a=2.6\ [1.21;\ 9.45]$) and A ($R_a=1.76\ [1.02;\ 9.01]$) than in group C and the positive control group D (both $R_a=+\infty\ [1.88;\ +\infty]$). No significant differences were observed between groups, except for the comparison of the lymph nodes and tonsils. In this category, the $R_a$ value in group A was significantly lower than in C ($P<0.03$).

Table 4: The adjusted reproduction ratio $R_a$ for the three intervention groups A, B, C and the positive control group D.

<table>
<thead>
<tr>
<th>Group</th>
<th>All individual Faeces</th>
<th>Ileum/content/ Caecum/content</th>
<th>Ileocaecal Lnm/ Tonsils</th>
<th>All Organs/ All Faeces</th>
</tr>
</thead>
<tbody>
<tr>
<td>A) Coated calcium-butyrate (n = 16)</td>
<td>1.62 [0.85; 8.43]</td>
<td>0.77 [0.35; 3.80]</td>
<td>0.32 [0.12; 2.94]</td>
<td>1.76 [1.02; 9.01]</td>
</tr>
<tr>
<td>B) vaccination (n = 16)</td>
<td>1.16 [0.56; 3.95]</td>
<td>1.03 [0.56; 3.95]</td>
<td>1.19 [0.70; 7.18]</td>
<td>2.61 [1.21; 9.45]</td>
</tr>
<tr>
<td>C) Water with organic acids (n = 16)</td>
<td>3.53 [1.88; 11.70]</td>
<td>2.52 [1.67; 24.87]</td>
<td>3.53 [1.88; 11.65]</td>
<td>$+\infty\ [1.88;\ +\infty]$</td>
</tr>
<tr>
<td>D) Positive control (n = 16)</td>
<td>3.53 [1.88; 11.70]</td>
<td>1.91 [1.22; 24.78]</td>
<td>0.89 [0.47; 4.23]</td>
<td>$+\infty\ [1.88;\ +\infty]$</td>
</tr>
</tbody>
</table>

$P$-value (A, D) 0.22 0.15 0.41 0.22
$P$-value (B, D) 0.11 0.20 0.44 0.29
$P$-value (C, D) 1.00 0.38 0.11 1.00
$P$-value (A, B) 0.37 0.12 0.07 0.45
$P$-value (A, C) 0.22 0.06 0.03 $^c$ 0.22
$P$-value (B, C) 0.11 0.10 0.16 0.29

$^a$ A= feed with 0.3% coated calcium-butyrate; B= vaccination at 3 and 6 weeks of age; C= water with 0.09% organic acids; D= positive control.

$^b$ Using the ISO 6579:2002/Amd1:2007 Annex D, a pig was considered as infected, if at least one of the samples of four marked categories in every column was found positive during the transmission period.

$^c$ significant difference between group A and C ($P<0.03$).

**Serological examination**

All pigs were seronegative before the start of the study. The evolution of *Salmonella*-specific antibody titres is illustrated for all groups in Fig. 3. During the entire experiment, titres in the negative control group remained very low (S/P ratios below 0.01). In group B, an increasing mean *Salmonella*-specific antibody response was observed from Day -19 until Day -1 (day of challenge) and also subsequently until the end of the experiment. In groups A and C and the positive control group, *Salmonella*-specific antibodies increased from two weeks after challenge; no significant differences in titre were detected between these groups. The mean antibody titre of group B was significantly higher than of all other groups.
Figure 3: Weekly *Salmonella*-specific antibody detection in serum (ELISA): Mean S/P ratio in function of the days post infection in group A (feed with coated calcium-butyrate), group B (vaccination), group C (water with organic acids), and in the positive and negative control group. Day -36 and Day -15: primer and boost vaccination in group B, respectively. Day -1: oral infection with $10^8$ CFU *Salmonella Typhimurium* of two seeder pigs per group of eight pigs. Day 0: reintroduction of seeders in their original pen. Day 38: euthanasia and necropsy.

**Discussion**

Current control measures are not sufficient to prevent the impact of pig-related *Salmonella* on public health. The present study investigated three intervention strategies to reduce the transmission of *Salmonella Typhimurium* in pigs. Supplementation of feed with coated calcium-butyrate and oral vaccination with the current attenuated strain both limited *Salmonella Typhimurium* transmission; however, water acidified with the current organic acid mixture was shown non-effective in this study.

Using the present ‘transmission’ design, where treatment was given prior to challenge (treated seeders were brought together with treated contact pigs), an adjusted transmission ratio $R_a$ was calculated which quantified the effects of the treatments on both infectiveness and susceptibility. This is an important contrast to efficacy studies in which principally the susceptibility is tested, and not the combined effect (Springer *et al.*, 2001; Roesler *et al.*, 2004; Tanaka *et al.*, 2010). A similar approach has been used in previous transmission experiments with other pathogens (Laevens *et al.*, 1998; Dewulf *et al.*, 2001; Velthuis *et al.*, 2003; Heres *et al.*, 2004; Meyns *et al.*, 2004).

When observing the obtained transmission ratios, higher $R_a$ values were found in the group treated with acidified water (C) and in the untreated positive control group (D), independently of the definition of infection. This suggested a lower transmission of *Salmonella* in the treated groups A and B when compared to groups C and D. Yet, the $R_a$ value in both group A and B – based on the detection of
Salmonella in faeces – was above one, indicating that there was still spread of the infection during the study period.

In the present trial, the coated calcium-butyrate supplement (Greencab-70, Sanluc International) was chosen as one strategy, since this has been shown to be beneficial in previous experiments (Boyen et al., 2008b; Guilloteau et al., 2010), though not with the current transmission design. In the digestive tract, butyrate can accelerate the renewal of necrotic areas, down-regulate Salmonella virulence, suppress intestinal inflammation and diminish pathogen invasion (Hamer et al., 2008). However, to reach their active form the intestinal sites that Salmonella spp. use to colonize the pig (ileum, caecum and colon), supplemented short-chain fatty acids need protection or ‘coating’ from the intestinal environment (Van Immerseel et al., 2005; Boyen et al., 2008b), because they are generally quickly metabolized and then absorbed by gastro-intestinal epithelial cells (Louis et al., 2007). The results of our study showed a tendency towards reduced Salmonella transmission in this group, in comparison with the acidified water group and the positive control group. Therefore, we conclude that coated calcium-butyrate may be helpful in the reduction of Salmonella infections in pigs.

In contrast, the mixture of formic, acetic, propionic, and lactic acid (Agrocid Super, CidLines) that was added to the drinking water in this study did not reduce the transmission rate of Salmonella Typhimurium in comparison with the positive control group. A possible explanation is that this acid shock induced a stress response system, enabling Salmonella Typhimurium to survive in an extreme low acid environment (an acid tolerance response or ‘ATR’ (Foster and Spector, 1995; Baik et al., 1996)). Previous reports on the use of organic acids via drinking water have produced inconsistent results. Van der Wolf et al. (2001b) observed a reduction in Salmonella when a 0.2% v/v organic acid mixture was administered during the entire finishing period. In contrast, no reductions were observed with the same concentration applied for a shorter time (two weeks pre-slaughter) (De Busser et al., 2009), or using a lower concentration administered to weaners (0.02% v/v in Letellier et al., 2000). However, the comparison of these study results is hindered not only by the different duration of application or concentration, but also by the fact that different organic acid mixtures were used in all these studies.

The vaccine used in this study (Salmoporc, IDT) has been demonstrated to reduce both faecal shedding and colonization of the intestinal tract of piglets (Springer et al., 2001). In the present transmission study, this vaccination resulted in the lowest number of shedding pigs and the lowest $R_a$ (faeces results). However, vaccination also increased the Salmonella-specific antibodies and resulted in a proportion of tissue samples colonized by the Salmonella challenge strain comparable to that in the positive control group (31% vs. 41%, respectively). Although the vaccine and wild type strains can be distinguished using the Salmonella Diagnostic Kit provided by the manufacturer of the vaccine, the commercial LPS-
based ELISA cannot differentiate between vaccination- and infection-induced antibodies in serum or meat juice. Vaccination may thus hamper the use of serology for *Salmonella* monitoring programmes, if blood samples are collected before 14 weeks of age. Programmes that are based on bacteriology, new diagnostic tests that do not detect vaccine-induced antibodies (Selke *et al.*, 2007) or new vaccines that are not detected by current ELISA methods (Leyman *et al.*, 2011) are to be considered if vaccination is going to become a routine part of *Salmonella* control.

**Conclusions**

Both feed supplementation with coated calcium-butyrate and particularly vaccination with an attenuated vaccine decreased *Salmonella* Typhimurium transmission in pigs. Further studies are needed to assess the practical issues related to the implementation of these promising interventions. For example, more data are needed to determine the best age groups and treatment regimens for the coated calcium-butyrate and on to learn how to overcome the problem of *Salmonella*-specific antibodies in vaccinated pigs. Alternative bacteriology-based monitoring programmes, diagnostic tests that do not detect vaccine-induced antibodies or new vaccines that are not detected by current antibody detection methods are required.

**Acknowledgements**

We thank the Federal Public Service of Health, Food Chain Safety and Environment for the funding of this project (contract RT 09/5 SalmoSu). The sampling assistance of Willem Van Campe and the technicians of the experimental centre is gratefully appreciated. We also thank Heidi Vander Veken, Andy Lucchina and Florence Crombé for their help in processing the samples at the necropsy, and Wannes Vanderhaeghen and Leon Oosterik for their critical review.
2. Effect of a DIVA vaccine with and without in-feed use of coated calcium-butyrate on transmission of Salmonella Typhimurium in pigs

(Transmission study 2)


Abstract

For satisfactory *Salmonella* control, good biosecurity along the pork production chain is crucial, although additional control measures on-farm need to be considered. This study evaluated the effect of two potential control measures against the spread of *Salmonella Typhimurium* via a transmission experiment with 56 piglets (3 - 15 weeks of age): two groups were orally vaccinated with $10^7 - 10^8$ Colony Forming Units (CFU)/2 mL of a new attenuated *Salmonella Typhimurium* vaccine ‘Salmoporc-ΔrfaJ’ with DIVA capacities (Differentiation between Infected and Vaccinated Animals) ($n = 2 \times 16$); the feed of one group was additionally supplemented with coated calcium-butyrate. Two weeks post vaccination, four pigs per group were orally challenged with $10^7$ CFU/2 mL of a *Salmonella Typhimurium* strain 112910a. Both groups were compared with a positive (challenged/untreated; $n = 16$) and negative (unchallenged/untreated; $n = 8$) control group. Until six weeks post challenge, blood, individual faecal and finally tissue samples were examined. Adjusted transmission ratios ‘$R_a$’ were estimated, based on the challenge strain isolation from faecal and/or tissue samples.

In both intervention groups, $R_a$ values were lower compared to the positive control group, although these differences were not significant. In the combination group DIVA vaccine + coated butyrate less non-challenged contact animals excreted *Salmonella* and less tissue samples were found *Salmonella*-positive in all pigs, when compared to the positive control group ($P < 0.01$). Seroconversion was detected in none of the vaccinated animals before challenge, when using commercial lipopolysaccharide (LPS) ELISAs targeting only *Salmonella* O-antigens, deleted in this vaccine. This was in contrast with an in-house whole-cell ELISA testing for various *Salmonella* antigens, in which *Salmonella*-specific antibodies were found pre-challenge in the serum of the vaccinated pigs.

Both interventions showed a limited, non-significant reduction of *Salmonella* transmission between piglets. They may have applications towards *Salmonella* control and surveillance. Firstly, the number of *Salmonella* excreting contact pigs was significantly lower in the group where vaccination was combined with coated calcium-butyrate in the feed; secondly, the new vaccine confirmed its DIVA capacity. Therefore, these interventions merit further research with larger sample sizes, to optimize their use for *Salmonella* programmes.
Chapter 3.2

Experimental Study 2

Background

*Salmonella* infections are one of the most important and widely distributed foodborne diseases in the European Union. Contaminated pork has been linked to 34.5% of the human outbreaks of *Salmonella enterica* subspecies *enterica* serovar Typhimurium (*Salmonella Typhimurium*) in the EU (EFSA and ECDC, 2013). Therefore, any reduction of the *Salmonella* risk by pork products would contribute to the protection of human health. However, the control of *Salmonella* in pork production remains a significant challenge in the preharvest sector. Implementing good biosecurity and operating a high standard of hygiene is crucial in every link of the pork production chain (Hotes et al., 2011), but additional control measures on-farm are indispensable (Mannion et al., 2007).

Vaccination is one possible supplementary measure that can be implemented in *Salmonella* control programmes. It is currently used successfully in the poultry industry of several European countries (Collard et al., 2008; EFSA and ECDC, 2012). Also in pigs, various vaccine studies have demonstrated a significant decrease in clinical signs and excretion of *Salmonella* (Hotes et al., 2011; Roesler et al., 2004; Farzan and Friendship, 2010; De Ridder et al., 2013). Most European serosurveillance programmes however, rely on commercial lipopolysaccharide (LPS) ELISAs, which do not allow differentiation between *Salmonella*-specific antibodies induced by vaccination or by infection (Abrahantes et al., 2009). Several DIVA vaccines (Differentiation of Infected and Vaccinated Animals) have recently been developed (Selke et al., 2007; Leyman et al., 2011), of which one vaccine, a *Salmonella Typhimurium* strain without the 'rfaJ' gene, induces antibodies undetectable with LPS-based ELISA (Leyman et al., 2011). Another control measure with promising features against *Salmonella* infections, is the oral administration of butyrate, especially in its coated form (Van Immerseel et al., 2005; Boyen et al., 2008). Not only does this organic acid enhance pig performance by improving gut function, it also down-regulates *Salmonella* virulence gene expression (Guilloteau et al., 2010). None of these studies however, have investigated the effect on the actual transmission rate of *Salmonella* between pigs.

The aim of the present study was to evaluate the effect of a commercial vaccine that was modified to a DIVA vaccine strain, and its combination with coated calcium-butyrate in the feed on the transmission of *Salmonella Typhimurium* in weaned pigs.
Methods

**Study design**

At three weeks of age, 56 *Salmonella*-negative piglets were randomly assigned to four different stables in the experimental animal facilities of CODA-CERVA, with two separated pens each (Table 1). Group A (n = 2x8) was orally vaccinated at four and seven weeks of age with $10^7 - 10^8$ Colony Forming Units (CFU)/2 mL of a live LPS-mutant *Salmonella Typhimurium* strain, named the ‘Salmoporc-$\Delta rfaJ$’ strain, in reference to the deletion of the ‘$rfaJ$’ gene (Leyman *et al.*, 2011) in a commercial vaccine (Salmoporc®, IDT Biologika); group B (n = 2x8) was vaccinated similarly and received additionally feed supplemented with 0.3% coated calcium-butyrate throughout the experiment (Globamax Performant, Sanluc International); a positive control group C (challenged/untreated; n = 2x8) and a negative control group D (unchallenged/untreated; n = 8) were included as well. Each group, except the negative control group, consisted of two replicates to increase the power of the experiment.

<table>
<thead>
<tr>
<th>Age of pigs in weeks</th>
<th>Sampling/action</th>
<th>Frequency (Number of samples n)</th>
<th>Diagnostic method/product</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 - 8</td>
<td>Floor faeces (group A, B, C, D)</td>
<td>Once/week (1 pool/pen; n = 7)</td>
<td>Isolation (ISO 6579:2002/Amd1:2007 Annex D)</td>
</tr>
<tr>
<td>3 - 15</td>
<td>Blood (group A, B, C, D)</td>
<td>Once/week (n = 56)</td>
<td>ELISA $^a$</td>
</tr>
<tr>
<td>3 - 15</td>
<td>Feed supplementation (group B)</td>
<td>Ad libitum (n = 15)</td>
<td>Coated calcium-butyrate (Globamax Performant, Sanluc International)</td>
</tr>
<tr>
<td>4 + 7</td>
<td>Oral vaccination (group A, B)</td>
<td>Twice: primer + boost (n = 16(A); n = 15(B))</td>
<td>$10^7 - 10^8$ CFU of DIVA vaccine ‘Salmoporc-$\Delta rfaJ$’</td>
</tr>
<tr>
<td>9</td>
<td>Oral challenge (group A, B, C)</td>
<td>Once; in a separate stable, with replacement 24 h later (2 pigs/pen; n = 12)</td>
<td>$10^7$ CFU of nalidixic acid-resistant <em>Salmonella Typhimurium</em> strain 112910a</td>
</tr>
<tr>
<td>15</td>
<td>Rectal faeces, ileum + content, caecum + content, ileocaecal Lnn, tonsils (group A, B, C, D)</td>
<td>At necropsy (n = 56)</td>
<td>Isolation (ISO 6579:2002/Amd1:2007 Annex D)</td>
</tr>
</tbody>
</table>

$^a$ Two ELISA tests were performed: (1) commercial LPS-based ELISA (HerdChek Swine *Salmonella*, IDEXX); (2) In-house whole-cell ELISA (Leyman *et al.*, 2011).
The pens were separated with solid concrete partitions and had a semi-solid concrete floor (half grid, half rubber mats). The pig stocking density was 0.42 m$^2$ per animal. In all groups, the same starter and pig meal without antimicrobials was administered throughout the study, (i.e. from arrival in the experimental facilities at three weeks of age until euthanasia at 15 weeks of age), except in group B for which the feed was supplemented as described above. The pigs were housed at natural day-night rhythm with ad libitum access to water and feed. Each group of eight pigs was provided with a rubber ball as environmental enrichment.

With the exception of the negative control group, two pigs per pen were orally challenged at nine weeks of age with 10$^7$ CFU/2 mL of a nalidixic acid-resistant Salmonella Typhimurium strain 112910a (Boyen et al., 2009) in a separate pen. Twenty-four hours later, these challenged ‘seeder’ pigs were replaced with the naive, non-challenged contact pigs (0 Days Post Contact or ‘DPC’), and all pigs were monitored until 15 weeks of age (42 DPC). This day (0 DPC) was considered as the start of the transmission experiment. Before challenge at nine weeks of age, individual blood samples and pooled faecal pen samples were taken once a week. After challenge, blood samples were obtained once a week, whereas individual rectal faeces were collected twice a week from all pigs. At necropsy, rectal faeces, ileum, ileal content, caecum, caecal content, ileocaecal lymph nodes and tonsils were sampled. The experimental design was approved by the Animal Care and Ethical Committee of the IPH-VAR (Approval number 100412–02).

**Sample analysis**

All faecal samples were examined using the ISO 6579:2002/Amd1:2007 Annex D method (ISO, 2007). Firstly, the samples (10-25g) were diluted 1:10 in buffered peptone water (BPW, Bio-Rad) and aerobically incubated for 16-20 h at 37°C. Of this solution, 0.1 mL was inoculated on a modified semi-solid Rappaport-Vassiliadis plate (MSRV, Bio-Rad) (one sample per plate) and aerobically incubated for 46-50 h at 41.5 °C. After this period, a loopful of the growth area in this MSRV enrichment must be plated out on a xylose lysine deoxycholate agar plate (XLD, Bio-Rad) and another agar for choice. In this study was opted for a brilliant green agar plate (BGA, Lab M), known to allow satisfactory growth of both the vaccine and challenge strain. The latter plate was supplemented with 20 μg/mL nalidixic acid for differentiating these Salmonella Typhimurium strains. For 21-27 h, both plates were given an aerobic incubation at 37 °C. A Salmonella-suspected colony on the BGA plates was expected to be only the nalidixic acid-resistant challenge strain, whereas on the XLD plates, colonies of both the challenge and vaccine strain were able to grow. From these XLD or BGA plates, one Salmonella-suspected colony was inoculated in triple sugar iron agar (TSI, Bio-Rad) and lysine decarboxylase
bouillon (Oxoid) and incubated for 18-24 h at 37 °C for final confirmation. After preparation, the tissue samples were investigated similarly to the faecal samples.

The blood samples were allowed to coagulate at room temperature and were then centrifuged for 15 min at 1200 g. The serum collected thereafter, was diluted twenty-fold and analysed with two ELISA tests: (1) a commercial ELISA kit based on LPS O-antigens of serogroup B, C1 and D (HerdChek Swine Salmonella, IDEXX); (2) an in-house whole-cell ELISA, based on a variety of surface-antigens on the Salmonella Typhimurium strain 112910a (Leyman et al., 2011). Consequently, antibodies against the ‘Salmoporc-ΔrfaJ’ vaccine strain, which expresses no O-antigens, should only be detectable with this whole-cell ELISA. Other Salmonella strains on the other hand, will be detected in both ELISA tests. Optical densities (OD) in both ELISAs were determined by photometry with a 650 nm filter. Samples with a Sample-to-Positive (S/P) ratio ≥0.25 (= OD% ≥10) were defined as positive.

Statistical analysis

Based upon all faecal samples, the numbers of excreting pigs were compared via generalized estimating equations (GEE), using the proc genmod procedure in SAS 9.2 (SAS Institute Inc., USA). For this, a binomial distribution and logit link function were used. Bacteriological results from the tissue samples (ileum, ileal content, caecum, caecal content, ileocaecal lymph nodes, tonsils) were compared between groups (DIVA vaccine, DIVA vaccine + Coated Butyrate, Positive Control) using logistic regression analysis in SAS 9.2. P-values below 0.05 were considered significant.

The transmission of Salmonella Typhimurium in each group was estimated on the basis of the stochastic ‘SI’ infection model (Susceptible-Infectious), using an ‘adjusted’ transmission ratio ‘R_a’ for the observed period of six weeks, which is derived from the basic reproduction ratio R_0 for the entire infectious period (de Jong and Diekmann, 1992). In this study, each intervention group contained n = 8 piglets, of which initially two challenged animals were infectious (I_0 = 2) and six contact animals were susceptible (S_0 = 6). An R_a value below or above one means respectively, that each infected animal will pass the infection on to less or more than one naive contact animal during the observed period of six weeks. This R_a value was estimated via the Maximum Likelihood Estimation (MLE) based on the final size of the study, which represents the total number of contact infections (Meyns et al., 2004). A contact animal was considered infected, when at least one sample was positive in: i) individual faeces, or ii) ileum, caecum and/or their content, or iii) ileocaecal lymph nodes and/or tonsils, or iv) all tissues and/or all faeces collected. R_a values were calculated for each of the previous four categories, and significant differences between groups were assessed (Kroese and de Jong, 2001).
Results

**Bacteriological examination**

In each group, all pooled faecal samples taken prior to challenge were *Salmonella*-negative. All individual faecal samples from the negative control group remained negative during the whole experiment. One contact pig of group B died at -1 DPC due to a polybacterial bronchopneumonia and was excluded from the study (no respiratory pathogens could be identified at necropsy).

After challenge, no significant differences between the groups were observed in the numbers of *Salmonella*-excreting animals, when considering all challenged and non-challenged pigs (Fig. 1). If the challenged seeder pigs were not taken into account for all 11 sampling points however, significantly fewer *Salmonella*-positive samples were obtained in group B (9/121), when compared to group C (30/132) ($P < 0.01$). At the end of the trial, respectively 8/12 (67%), 7/11 (64%) and 12/12 (100%) non-challenged contact pigs from group A, B and C had excreted *Salmonella* in their feces on at least one sampling occasion (Table 2). From all necropsy samples of both challenged and non-challenged pigs, 27, 13 and 41% were found positive for the challenge strain in group A, B and C, respectively, with a significant difference between group B and C ($P < 0.01$) (Table 3). Respectively 6/12 (50%), 2/11 (18%) and 10/12 (83%) non-challenged contact pigs from group A, B and C had one tissue sample or more colonized with *Salmonella*. Both the faecal shedding and tissue colonization are presented individually in Table 2.

![Figure 1: Proportion of *Salmonella* challenge strain positive faecal samples per groups, as a function of time (DPC: days post contact). □ DIVA vaccine; • DIVA vaccine + Coated calcium-butyrate; ✗ Positive Control; ✖ Negative Control. DPC = -1: Replacement and oral challenge with $10^7$ CFU/2 mL *Salmonella* Typhimurium of 2 seeder pigs per group of 8 pigs, with reintroduction of seeders in their original pen 24 h later.](image-url)
Table 2: Presentation of the individual *Salmonella*-positive faecal and necropsy samples (grey) per sampling occasion and tissue, respectively.

<table>
<thead>
<tr>
<th>Group</th>
<th>Pig</th>
<th>Faeces</th>
<th>Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Days Post Challenge</td>
<td>Excreting pig</td>
</tr>
<tr>
<td></td>
<td>-1</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>A) DIVA vaccine</td>
<td>S</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>(n = 16)</td>
<td>S</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>Yes</td>
<td>Yes</td>
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<tr>
<td></td>
<td>C</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>B) DIVA vaccine + Coated Butyrate</td>
<td>S</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>(n = 15)</td>
<td>S</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>Yes</td>
<td>Yes</td>
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<tr>
<td></td>
<td>C</td>
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<td>No</td>
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<td></td>
<td>C</td>
<td>Yes</td>
<td>Yes</td>
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<td>Yes</td>
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<td>C</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>C) Positive control</td>
<td>S</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>(n = 16)</td>
<td>S</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>Yes</td>
<td>Yes</td>
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<td>Yes</td>
<td>Yes</td>
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<tr>
<td></td>
<td>C</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

*a* = Seeder pig (in italics); *C* = Contact pig.


§ A) oral vaccination at four and seven weeks of age with $10^7 - 10^8$ CFU/2 mL of the live ‘Salmoporc-ΔrfαJ’-strain; B) vaccination of (A) plus feed supplemented with 0.3% coated calcium-butyrate; C) positive control that was challenged without being treated.
Table 3: Results of the descriptive and logistic regression analysis of the necropsy samples positive for the *Salmonella* challenge strain in the three groups.

<table>
<thead>
<tr>
<th>Group §</th>
<th>Parameters</th>
<th>Different necropsy samples</th>
<th>All necropsy samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ileum</td>
<td>Ileal Content</td>
</tr>
<tr>
<td>A) DIVA vaccine (n = 16)</td>
<td>Number (Proportion)</td>
<td>4 a</td>
<td>4 ab</td>
</tr>
<tr>
<td></td>
<td>OR*</td>
<td>0.66</td>
<td>0.10</td>
</tr>
<tr>
<td>B) DIVA vaccine + Coated Butyrate (n = 15)</td>
<td>Number (Proportion)</td>
<td>2 a</td>
<td>1 a</td>
</tr>
<tr>
<td></td>
<td>OR*</td>
<td>0.25</td>
<td>0.03</td>
</tr>
<tr>
<td>C) Positive control (n = 16) (ref)</td>
<td>Number (Proportion)</td>
<td>5 a</td>
<td>8 b</td>
</tr>
</tbody>
</table>

* a, b Numbers in a column, not sharing the same letters in superscript, are significantly different: ileal and caecal content (P < 0.02) and all necropsy samples (P < 0.01).

* Odds Ratios (OR >1 or <1, define the intervention of group A or B as a risk or protective factor, respectively, for having *Salmonella*-positive tissue samples, when compared to the positive control group.

§ A) oral vaccination at four and seven weeks of age with $10^7 - 10^8$ CFU/2 mL of the live ‘Salmoporc-$\Delta$rfaf’ strain; B) vaccination of (A) plus feed supplemented with 0.3% coated calcium-butyrate; C) positive control that was challenged without being treated.

The adjusted transmission ratios $R_a$, based on the faecal and/or tissue results of all challenged and non-challenged pigs, were lower in all four categories in both vaccinated groups A and B, when compared to the unvaccinated group C (Table 4). These differences between groups were not significant (P > 0.05).

Table 4: Adjusted reproduction ratio $R_a$ [95% confidence interval] in group A/B/C, as a function of four different categories.

<table>
<thead>
<tr>
<th>Group §</th>
<th>Individual faeces a</th>
<th>Ileum / content / Caecum / content a</th>
<th>Ileocaecal Lnn / Tonsils a</th>
<th>All tissues / Individual faeces a</th>
</tr>
</thead>
<tbody>
<tr>
<td>A) DIVA vaccine (n = 16)</td>
<td>1.76 [1.02; 9.01]</td>
<td>1.03 [0.56; 3.95]</td>
<td>0.77 [0.35; 3.80]</td>
<td>2.37 [1.46; 10.02]</td>
</tr>
<tr>
<td>B) DIVA vaccine + Coated Butyrate (n = 15)</td>
<td>2.52 [0.99; 9.62]</td>
<td>0.50 [0.17; 5.82]</td>
<td>0.37 [0.14; 4.64]</td>
<td>2.52 [0.99; 9.62]</td>
</tr>
<tr>
<td>C) Positive control (n = 16)</td>
<td>+∞ [1.88; +∞] *</td>
<td>2.52 [1.02; 9.01]</td>
<td>1.19 [0.70; 7.18]</td>
<td>+∞ [1.88; +∞] *</td>
</tr>
</tbody>
</table>

* Using the ISO 6579:2002/Amd1:2007 Annex D, a pig was considered as infected, if at least one of the samples of four marked categories in every column was found positive during the transmission period.

§ A) oral vaccination at four and seven weeks of age with $10^7 - 10^8$ CFU/2 mL of the live ‘Salmoporc-$\Delta$rfaf’-strain; B) vaccination of (A) plus feed supplemented with 0.3% coated calcium-butyrate; C) positive control that was challenged without being treated.

* +∞ = plus infinity

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Serological examination

In the commercial LPS-based ELISA, all pigs were seronegative for *Salmonella*-specific antibodies before challenge (Fig. 2). In contrast, the in-house whole-cell ELISA showed that the mean S/P ratio increased already one week before challenge in both vaccinated groups A and B, whereas the increase occurred two weeks after challenge in the positive control group C (results not shown).

![Graph showing weekly detection of *Salmonella*-specific antibodies](image)

Figure 2: Weekly detection (LPS-based ELISA, IDEXX) of *Salmonella*-specific antibodies (mean S/P ratio), as a function of time (DPC: days post contact). ■ DIVA vaccine; ○ DIVA vaccine + Coated calcium-butyrate; × Positive Control; □ Negative Control. DIVA vaccine; DIVA vaccine + Coated calcium-butyrate; Positive Control; Negative Control. DPC = -1: Replacement and oral challenge with $10^7$ CFU/2 mL *Salmonella Typhimurium* of 2 seeder pigs per group of 8 pigs, with reintroduction of seeders in their original pen 24 h later.

Discussion

The increase of global meat consumption during the next decades is expected to be largely due to increases in pork (Kearney, 2010). The reduction of any pig-related public health risk is therefore of considerable importance. *Salmonella Typhimurium* represents a most relevant pig-associated risk despite current control measures (EFSA and ECDC, 2013a), and was therefore selected for the present transmission experiment in weaned pigs. This study aimed to assess the effect of a DIVA vaccine (Leyman *et al.*, 2011), with and without coated calcium-butyrate in the feed.

As a consequence of the study design (transmission experiment), in which the treatment was applied to all pigs before challenge, the combined treatment effect on both the excretion of the inoculated pigs and the susceptibility of the contact animals is evaluated. This is an important difference with efficacy studies in which mainly the susceptibility is assessed and not the combined effect (Roesler *et al.*, 2004;
Taube et al., 2009; Tanaka et al., 2010). This study concept also allows a better simulation of the field situation, namely infection of a treated pig which may subsequently infect other treated pigs in the area. Several other bacterial diseases have been investigated in similar experiments (Meyns et al., 2004; Velthuis et al., 2003; Heres et al., 2004; Dewulf et al., 2004; Bohez et al., 2008; Vrancken et al., 2009; Villarreal et al., 2011; Crombé et al., 2012; Claes et al., 2013). As the $R_a$ values were higher than one in this experiment, the present interventions will not eradicate infection with Salmonella Typhimurium during the studied period. However, the treated groups demonstrated statistically non-significant lower $R_a$ values for every matrix, in comparison with the positive control group. Therefore, a more distinct reducing effect might be expected when observing pigs for a longer time, as was demonstrated in previous efficacy studies (Farzan and Friendship 2010; van der Wolf et al., 2001b; Canibe and Jensen, 2003), which investigated the effect of vaccination, acidified drinking water, and fermented liquid feed during the entire finishing period, respectively. This expected enlarged difference between groups might be explained by a prolonged potential spread of Salmonella in the untreated control group, while no further spread is expected in the treatment groups.

Concerning vaccination, different studies have demonstrated a reduction of Salmonella shedding and/or Salmonella seroprevalence in pigs (Roesler et al., 2004; Farzan and Friendship 2010; Selke et al., 2007; Springer et al., 2001; Hur et al., 2011). Vaccine-induced antibodies should be distinguishable however, from those induced by infection in order not to hamper monitoring programmes based on serology. Selke et al. (2007) developed a DIVA vaccine of which the induced antibodies were not detectable in their in-house ELISA, but the DIVA vaccine of Leyman et al. (2011) induced antibodies undetectable with a commercial LPS-based ELISA that is commonly used in serosurveillance programmes (HerdChek Swine Salmonella, IDEXX). Consequently, the latter vaccine would be suitable for use in European serosurveillance programmes, whereas the first vaccine would not. In the present study, the method of Leyman et al. (2011) was therefore applied on the commercial vaccine Salmoporc®, resulting in the ‘Salmoporc-ΔrfaJ’ strain. The commercial vaccine Salmoporc® has proven its effectiveness previously (Springer et al., 2001) using the same administration procedure as the current study, and Leyman et al. (2011) demonstrated a similar protection after immunization with either a wild-type Salmonella Typhimurium strain or its DIVA variant. Therefore, the ‘Salmoporc-ΔrfaJ’ strain used in this study was expected to have the same protective effect as the Salmoporc® parent strain. Whereas the in vivo study of Leyman et al. (2011) involved four unchallenged vaccinated piglets, this new DIVA strain was evaluated in 16 challenged piglets. Although the ‘Salmoporc-ΔrfaJ’ strain as single intervention resulted in a limited statistically non-significant reduction of transmission, serological surveillance using the commercial ELISA tests remains applicable. Therefore, it may be considered a promising tool in future Salmonella surveillance, worthy of further investigation for optimization.
Previous studies with organic acids in the feed showed both reducing (Tanaka et al., 2010; Creus et al., 2007) and non-reducing (Martín-Peláez et al., 2010; Walsh et al., 2012) capacities on Salmonella excretion or antibody induction. In this study, we opted for supplementing the feed with coated calcium-butyrate, as earlier studies (Van Immerseel et al., 2005; Boyen et al., 2008) have shown that coating of fatty acids is needed to let them reach the colonization sites (ileum, caecum and colon) in their active form. In addition, the in-feed coated butyrate was combined with the current DIVA vaccine, as these two control strategies are believed to be complementary. Namely, both strategies have a different working mechanism to combat Salmonella transmission, i.e. the vaccination enhancing the host’s immune response and the coated butyrate targeting Salmonella bacteria in the gut environment and improving intestinal epithelial growth. In the current study, Salmonella transmission in the DIVA vaccine + butyrate group was not significantly different from the one in both other challenged groups. In this group however, significantly less non-challenged contact animals excreted Salmonella and significantly less organ samples of all challenged and non-challenged pigs were found Salmonella-positive, when compared to in the positive control group. An additional beneficial effect was thus observed in the combination group, in comparison with the DIVA vaccine intervention on its own, which might be explained by this dual approach to Salmonella infection. However, the combination of both strategies would also incur considerable additional costs, in comparison with the single interventions. Therefore, more research including a cost-benefit analysis and while monitoring pigs for a longer period (e.g. from weaning till market age), is warranted.

Conclusions

Both interventions in this study did not show a significant reduction of Salmonella Typhimurium transmission. Significantly less contact pigs excreted Salmonella however, in the group where vaccination was combined with coated butyrate in the feed, and the vaccine itself confirmed its ‘DIVA’ capacity. Therefore, these interventions merit further research to improve their applicability in Salmonella control programmes.

Acknowledgements

We thank the Federal Public Service of Health, Food Chain Safety and Environment for the funding of this project (RT-09/05). The assistance in sampling of Willem Van Campe and the technicians of the experimental centre is gratefully appreciated, as is the help of Heidi Vander Veken, Andy Lucchina, Florence Crombé, Wannes Vanderhaeghen, and Leon Oosterik when processing the necropsy samples.
3. Usefulness of a live *Salmonella* Typhimurium vaccine to control *Salmonella* infections on farrow-to-finish pig herds

*(Field study)*


Adapted from: *The Veterinary Journal* (submitted)
Abstract

Efficient control programmes for *Salmonella* in pig herds are important to reduce the risk for salmonellosis in humans and to limit performance losses incurred by *Salmonella*-infected pigs. Vaccination against *Salmonella* may constitute an interesting control strategy at farm level.

In this study we investigated the usefulness of an attenuated *Salmonella* Typhimurium strain to control *Salmonella* infections in three *Salmonella* Typhimurium-positive, farrow-to-finish pig herds. Within each herd, 120 piglets were orally vaccinated at three and 24 days of age, and 120 piglets were left unvaccinated as controls. At specific time points, all pigs were weighed and faeces, ileocaecal lymph nodes, caecal contents and carcass swabs were examined for *Salmonella* by isolation; serum was analysed for *Salmonella*-specific antibodies via ELISA. Prior to slaughter, a significantly higher mean daily weight gain (546.8g), significantly less *Salmonella*-excreting pigs (8.9%) and a significantly lower *Salmonella*-specific mean antibody titre (S/P 1.07) were observed over all vaccinated animals of the three herds, compared to the controls (509.3g; 27.8%; S/P 1.51). Overall, no significant differences in the proportions of *Salmonella*-positive slaughter samples were found between the vaccinated vs. the control group for ileocaecal lymph nodes (36.3% vs. 40.0%), caecal contents (30.6% vs. 42.6%), and carcass swabs (29.0% vs. 28.6%). In the vaccinated vs. the control group of two herds however, significantly less *Salmonella*-positive lymph node samples (57.8% vs. 77.0%) and caecal contents samples (18.4% vs. 54.5%) were present. From 3.0%, 2.0% and 8.3% of all faecal, lymph node and caecal contents samples from the vaccinated pigs respectively, a *Salmonella* strain was isolated with the same auxotrophic characteristics and genotype as the vaccine strain.

Our findings indicate that the currently used vaccination protocol improves daily weight gain and reduces *Salmonella* shedding on *Salmonella* problem herds, however without consistent reduction of *Salmonella* loads and possible persistence of the vaccine strain in tissues.
Introduction

The largest number of reported food-borne outbreaks in the EU is currently caused by Salmonella (26.6% of all outbreaks). In 2011, approximately 56.8% of the human salmonellosis cases could be attributed to pigs (EFSA and ECDC, 2013a). Although most infected pigs do not show clear clinical signs, Salmonella infections may reduce the weight gain significantly (Boyen et al., 2009; Davis et al., 2010; Farzan and Friendship 2010). Therefore, interventions that decrease Salmonella prevalence at farm level are of utmost importance for both humans and swine.

Biosecurity measures, such as all in/all out management, pest control, cleaning and disinfection may help to decrease the Salmonella infection level (Lo Fo Wong et al., 2004a; Fosse et al., 2009; Baptista et al., 2010a), but they are often insufficient as stand-alone measures. Therefore, additional control strategies on-farm are indispensable.

Numerous studies have described the effect of feed or water supplementation with organic acids on Salmonella infection in pig herds, but with inconsistent results (Letellier et al., 2000; van der Wolf et al., 2001b; Creus et al., 2007; Walsh et al., 2007, 2012; Boyen et al., 2008b; Tanaka et al.2010; De Ridder et al., 2013a,b). Also the feed characteristics can influence Salmonella prevalence in pigs, e.g. coarsely versus finely ground feed, and liquid against dry feed (Canibe et al., 2005; Farzan et al., 2006; Visscher et al., 2009; Wilhelm et al., 2012). However, these feed or water interventions are not readily applicable in every farm, as substantial additional investments may be required, like extra pumps and corrosion-resistant materials.

Vaccination of pigs against Salmonella could represent an interesting control measure, which does not need such infrastructure changes. A Salmonella Enteriditis vaccine is currently successfully used in laying hens in several European countries (Collard et al., 2008; EFSA and ECDC, 2012a), and in pigs most vaccination studies against Salmonella generally report beneficial effects (Springer et al., 2001; Roesler et al., 2004; Lindner et al., 2007; Selke et al., 2007; Rössler et al., 2010; Leyman et al., 2012; Arguello et al., 2013). However, most studies in pigs were conducted with a limited number of suckling (Rössler et al., 2010) or weaned piglets (Springer et al., 2001; Roesler et al., 2004; Selke et al., 2007; Leyman et al., 2012; De Ridder et al., 2013a,b), and used a challenge infection protocol. Their relevance for field conditions needs to be verified with a large number of finisher pigs (Lindner et al., 2007; Farzan and Friendship 2010; Arguello et al., 2013).

At the moment, only one Salmonella vaccine is commercially available for use in pigs in the EU (Salmoporc®, IDT Biologika). It is a live Salmonella Typhimurium vaccine, authorized only in Germany and Poland, that has been evaluated on large-scale in sows and weaned piglets at three and six weeks of age (Lindner et al., 2007). In a small-scale infection experiment, the double administration of this vaccine to suckling piglets at three and 28 days of age significantly reduced organ colonization and faecal
excretion (Röslер et al., 2010). This early vaccination scheme at three and 28 days of age minimizes farmers work load, but its efficacy needs to be confirmed by large-scale studies while monitoring the pigs until slaughter age. In addition, the possible interference of this vaccination protocol with Salmonella serosurveillance, requires investigation.

Therefore, we evaluated the weight gain, the Salmonella-specific antibody titre and Salmonella presence in faeces and organ tissues of pigs, after oral immunisation at 3 and 28 days of age with this commercially available live Salmonella Typhimurium vaccine.

Materials and methods

Herd selection and experimental animals

Three pig herds (A, B, C) were selected based on the following inclusion criteria: i) a high Salmonella-specific antibody level in finishing pigs as determined by the Belgian national Salmonella Surveillance Programme, in accordance with the EU Regulation No 2160/2003 (Van der Stede et al., 2008), ii) being a closed farrow-to-finish herd, iii) the possibility to monitor piglets from birth till slaughter, iv) the farmer’s agreement to apply the same management during the experiment as before. The presence of Salmonella Typhimurium on these herds was confirmed prior to the study, by analysing pooled faecal samples from sows and fattening pigs, and ileocaecal lymph nodes from slaughter pigs. The size of the pens in the nursery units ranged from 25 to 30, and in the fattening units from 10 to 15 pigs; both units had fully slatted floors in the three herds. The biosecurity level of herd A and C were comparable, while this level was markedly lower in herd B (considering pest control and hygiene). All pigs received non-medicated feed during the trial. The weaned piglets were fed dry meal in all herds; the fattening pigs were given meal in herd A, and pellets in herd B and C.

In spring 2012, 20 pregnant sows (Topigs20) in each farm were selected three days after farrowing, and their piglets were randomly allocated at litter-level to either a vaccinated or unvaccinated group. All suckling piglets of each sow were included in the study and monitored until slaughter age. The sows from the piglets in each group had the same parity distribution. The study was approved by the ethical committee of the Faculty of Veterinary Medicine, Ghent University (EC 2011/196).

Vaccination and sampling design

The details of the trial design are summarized in figure 1. Briefly, in each herd, approximately 120 piglets from 10 litters received an oral vaccination at three and 28 days of age with an attenuated histidine-adenine auxotrophic Salmonella Typhimurium strain (Salmoporc®, Lot nr. 0130311, IDT Biologika).
Vaccination group), while 120 piglets from 10 other litters were left unvaccinated (C=control group). Transfer of piglets from one sow to another was not allowed after three days of age. All piglets were weaned at 28 days of age. From weaning until slaughter age, the pigs of the vaccinated and control group were kept separately on the farm, during transport to the slaughterhouse and in the lairage area. In the fattening period, the degree of separation differed between herds as follows: it was most strict in herd C (separated rooms), followed by herd A (separated pens, vaccinated pens grouped together) and herd B (separated pens, vaccinated pens dispersed among unvaccinated ones). All pigs were individually weighed to calculate the daily weight gain (DWG) between 3 days and 29 weeks of age. Within each herd, 30 randomly selected pigs per group were sampled serially at different time points for individual rectal faeces and blood. In all herds, the pigs were transported to the slaughterhouse in two times, with both groups equally represented at every occasion. The pigs of herd A and C were all slaughtered in slaughterhouse 1 (2x morning) and 2 (2x noon), respectively; herd B pigs were slaughtered in slaughterhouse 2 (noon) and 3 (morning) at the first and second occasion, respectively. At slaughter, the following samples were collected from each group: caecal contents from these same 30 pigs, ileocaecal lymph nodes from these 30 pigs together with 40 other randomly selected slaughter pigs, and swabs from every 10th carcass at the slaughter line.

**Sample analysis**

*Salmonella* was isolated from the faecal samples (rectal and caecal; ca. 10-25g) using the ISO 6579:2002/Amd1:2007 Annex D (ISO, 2007). Briefly, the samples were diluted 1:10 in buffered peptone water (BPW, Bio-Rad) and incubated for 16 - 20 h at 37°C. From every pre-enrichment solution, 100 μl was spotted onto one modified semi-solid Rappaport-Vassiliadis plate (MSRV, Bio-Rad) and incubated for 46 - 50 h at 41.5°C. After this period, a loopful from the edge of a typical migration zone on each MSRV plate was streaked onto a xylose lysine deoxycholate agar plate (XLD, Bio-Rad) and a brilliant green agar plate (BGA, Lab M). Both plates were incubated at 37°C for 21 - 27 h. When *Salmonella* presumptive colonies were present on both the XLD and BGA plates, one colony was inoculated in triple sugar iron agar (TSI, Bio-Rad), sorbitol agar (Becton Dickinson) and lysine decarboxylase broth (Oxoid) and incubated for 18 - 24 h at 37°C for final confirmation.

Before bacteriological examination of the ileocaecal lymph nodes (ca. 10-13g), they were flamed briefly to decontaminate the surface, sliced, diluted 1:10 in BPW and then homogenized with a stomacher blender (BagMixer, Interscience) for one minute. The carcass swabs were immersed directly in BPW without preparation. Further isolation was performed identically as for the faecal samples.
Isolates from samples from the vaccinated pigs, were additionally tested using the *Salmonella* Diagnostic Kit®, produced by the manufacturer of the vaccine (IDT Biologika). This kit distinguishes wild-type *Salmonella* strains from the vaccine strain through the use of two fluid media A and B. As the vaccine strain is histidine-adenine auxotrophic, it only grows in medium B which contains histidine and adenine, whereas wild-type strains show growth in both media. Additionally these strains were typed by Multiple-Locus Variable-number tandem repeat Analysis (MLVA) (Larsson *et al.*, 2009) and analysed by a Pulsed-Field Gel-Electrophoresis (PFGE) (Peters, 2009).

Blood samples were centrifuged after coagulation for 5 min at 1500 g to collect serum. This serum was diluted twenty-fold and analysed for *Salmonella*-specific antibodies under blinded conditions with a commercial ELISA kit based on lipopolysaccharide (LPS) O-antigens of serogroup B, C1 and D (HerdChek Swine *Salmonella*, IDEXX Laboratories). The results were expressed as a ‘Sample to Positive’ ratio; samples with an S/P ratio \( \geq 0.25 \) (\( = \) OD% \( \geq 10 \)) were defined as positive.
Chapter 3.3

Experimental Study 3

Figure 1: Flowchart of the study design in the three farrow-to-finish herds.

- **n= 3 herds** with *Salmonella Typhimurium*
- **n= 62 sows**
- **n= 709 piglets**

**Randomization**

**Vaccination**
- **n= 30 litters**
- **n= 347 piglets**

**Sampling occasions**
- **Enrolment**
- **3D of age**
- **28D of age**
- **10W of age**
- **16W of age**
- **29W of age**
- **31W of age** (slaughter)

**Control**
- **n= 32 litters**
- **n= 362 piglets**

**Mortality**
- **n= 15 died (4%)**
- **n= 10 died (3%)**
- **n= 6 died (2%)**
- **n= 7 died (2%)**
- **n= 5 died (14%)**

- **n= 201 lymph nodes**
- **n= 121 caecal content**
- **n= 34 carcass swabs**

<table>
<thead>
<tr>
<th>Protocol for 3 herds together:</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>n= 300 pigs per group</strong> (Vaccination+Control)</td>
</tr>
<tr>
<td>- vaccination ³: all in vacce group</td>
</tr>
<tr>
<td>- weighing ³: all group</td>
</tr>
<tr>
<td>- blood ² and faeces ³: 90/group</td>
</tr>
<tr>
<td>- ileocaecal lymph nodes ³: 210/group</td>
</tr>
<tr>
<td>- caecal content ³: 105/group</td>
</tr>
<tr>
<td>- carcass swab ³: random 10%/group</td>
</tr>
</tbody>
</table>

- **3 oral administration of 5x10⁹ – 5x10⁸ CFU (1 mL) of a double-attenuated histidine-adenine auxotrophic *Salmonella Typhimurium* strain (Salmonar, IDT Biologika), at 3 and 28D of age.**
- **³ The weight at 3 days and 29 weeks of age was used to calculate the daily weight gain (DWG).**
- **² ELISA was performed with HerdChek Swine Salmonella (IDEXX Laboratories).**
- **³ Isolation was performed according to ISO 6579 annex D.**
- **⁴ Extra data were obtained initially to account for mortality losses thereafter.**
- **⁵ Data were missing due to an impossible identification (lost eartag) or sampling (empty rectum).**
**Statistical analysis**

The daily weight gain between three days and 29 weeks of age of each pig (excl. pigs that died before slaughter), was normally distributed and was compared between the vaccinated and control group using an independent-samples t-test in SAS 9.2 (SAS Institute Inc., Cary, NC, USA).

The number of positive faecal samples was compared between vaccinated and control animals by means of generalized estimating equations (GEE) (Proc Genmod in SAS 9.2). A logit link function, a binomial distribution and an exchangeable correlation structure was used (correlation is the same among any two observations from the same animal). Mortality and isolation data at slaughter were analysed using the Pearson Chi-square test in SAS 9.2. In these analyses, samples containing the vaccine strain were considered *Salmonella*-negative.

The differences in S/P ratios between vaccinated and control animals were analyzed by repeated measures ANOVA, using the Proc Mixed procedure with lsmeans and slice options in SAS 9.2. Time and group (vaccinated and control) were included in the model as fixed effects and the herd as random effect. An unstructured covariance structure was selected, based on the lowest AIC value (Akaike Information Criterion). To adjust for the multiple comparisons in this procedure, the Ryan-Einot-Gabriel-Welch (REGWQ) test was used.

*P*-values below 0.05 were considered significant (two-sided tests).

**Results**

Some animals in both groups were lost for follow-up, due to mortality (runts, physical abnormalities or idiopathic causes) or lost ear tags (Fig. 1). There was no significant difference in mortality (*P* = 0.90) between the vaccinated (V) (50/347) and control (C) group (59/362).

**Daily Weight Gain (DWG)**

For the three herds, a significantly higher DWG (*P < 0.01) was observed in group V (*n* = 289; DWG = 546.8g, 95% CI = [537.7-555.8]), when compared to group C (*n* = 302; DWG = 509.3g, 95% CI = [499.4-519.2]) (Table 1). This result was consistent in each herd of the study, since the following differences in the mean DWG were found between the V and C groups (*P<0.01): 29.6g (herd A), 37.2g (herd B), and 38.9g (herd C) (Table 1).
Table 1: Daily Weight Gain (DWG; gram) between 3 days and 29 weeks of age. Presented for the vaccination and control group, for the three farrow-to-finish pig herds A, B, C separately and over all herds.

<table>
<thead>
<tr>
<th>Herd</th>
<th>Mean DWG [95% CI]</th>
<th>Difference DWG (P &lt; 0.01)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vaccination</td>
<td>Control</td>
</tr>
<tr>
<td>A</td>
<td>593.0 [578.8-607.3]</td>
<td>563.4 [549.0-577.9]</td>
</tr>
<tr>
<td>B</td>
<td>504.3 [488.9-519.8]</td>
<td>467.1 [452.5-481.7]</td>
</tr>
<tr>
<td>C</td>
<td>535.6 [520.9-550.4]</td>
<td>496.7 [482.3-511.0]</td>
</tr>
<tr>
<td>A+B+C</td>
<td>546.8 [537.7-555.8]</td>
<td>509.3 [499.4-519.2]</td>
</tr>
</tbody>
</table>

**Bacteriological analysis of faecal samples taken in the herd**

The proportions of *Salmonella*-positive faecal samples collected on-farm, are shown for the three sampling occasions in figure 2. Some data are missing, due to an empty rectum or lost ear tags (Fig. 1).

Over all herds, the proportions of *Salmonella*-excreting pigs in group V and C did not differ significantly at the first two sampling occasions: 9/99 (9.1%) (V) vs. 11/98 (11.2%) (C) at 10 weeks of age (P = 0.46), and 9/89 (10.1%) (V) vs. 11/82 (13.4%) (C) at 16 weeks of age (P = 0.50). Two weeks prior to slaughter however, at 29 weeks of age, this proportion was significantly different between both groups: 7/79 (8.9%) (V) vs. 22/79 (27.8%) (C) (P < 0.01).

When analysing each herd separately, the proportion of *Salmonella*-excreting pigs was also lower in herd B (1/29 (3.4%) (V) vs. 12/29 (41.4%) (C) (P < 0.01)) and in herd C (0/22 (0.0%) (V) vs. 6/22 (27.3%) (C) (P < 0.01)), whereas this was not the case in herd A (6/28 (21.4%) (V) vs. 4/28 (14.3%) (C) (P = 0.49).
Figure 2: *Salmonella*-excreting pigs (%) and *Salmonella*-specific antibody levels in blood (mean S/P ratio) per group and herd. Presented for four sampling occasions: 1) 3 days of age (3 D), 2) 10 weeks of age (10 W), 3) 16 weeks of age (16 W), and 4) 29 weeks of age, i.e. 2 weeks prior to slaughter (29 W). The error bars represent the 95% confidence interval. The asterisks (*) below the graphs indicate a significant difference between the control (black) and vaccination (grey) animals per herd (A, B, C).
**Bacteriological analysis of samples taken in the slaughterhouse**

The proportions of *Salmonella*-positive samples from ileocaecal lymph nodes, caecal contents and carcass swabs, collected in the slaughterhouse, are presented in table 2. Some data are missing, due to lost ear tags (Fig. 1).

Over all herds, no significant differences in the proportions of *Salmonella*-colonized samples were found between the V and C group for ileocaecal lymph nodes: 73/201 (36.3%) (V) vs. 72/180 (40.0%) (C) \(P = 0.46\); for caecal contents: 37/121 (30.6%) (V) vs. 49/115 (42.6%) (C) \(P = 0.06\); and for carcass swabs: 9/31 (29.0%) (V) vs. 8/28 (28.6%) (C) \(P = 0.97\).

When analysing each herd separately, the following significant differences were found in the proportions of isolation-positive samples between the V and C group of two herds: in the lymph nodes from herd B: 37/64 (57.8%) (V) vs. 47/61 (77.0%) (C) \(P < 0.03\), and in the caecal contents from herd C: 9/49 (18.4%) (V) vs. 24/44 (54.5%) (C) \(P < 0.01\).

---

**Table 2:** Proportion of slaughter samples positive for *Salmonella* wild-type strains, presented per herd and group. Presented for each vaccination (Vacc) and control (Contr) group of herd A, B and C, in function of three sampled matrices: (1) ileocaecal lymph nodes (Lnn); (2) caecal content (Cc); and (3) carcass swabs (Sw).

<table>
<thead>
<tr>
<th>Sampled matrix</th>
<th>A Vacc</th>
<th>A Contr</th>
<th>B Vacc</th>
<th>B Contr</th>
<th>C Vacc</th>
<th>C Contr</th>
<th>A+B+C Vacc</th>
<th>A+B+C Contr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lnn</td>
<td>25/66 (38%)</td>
<td>14/63 (22%)</td>
<td>37/64 (58%)</td>
<td>47/61 (77%)</td>
<td>11/71 (16%)</td>
<td>11/56 (20%)</td>
<td>73/201 (36%)</td>
<td>72/180 (40%)</td>
</tr>
<tr>
<td>Cc</td>
<td>6/39 (15%)</td>
<td>5/37 (14%)</td>
<td>22/33 (67%)</td>
<td>20/34 (59%)</td>
<td>9/49 (18%)</td>
<td>24/44 (55%)</td>
<td>37/121 (31%)</td>
<td>49/115 (43%)</td>
</tr>
<tr>
<td>Sw</td>
<td>0/10 (0%)</td>
<td>1/10 (10%)</td>
<td>9/10 (90%)</td>
<td>7/8 (88%)</td>
<td>0/11 (0%)</td>
<td>0/10 (0%)</td>
<td>9/31 (29%)</td>
<td>8/28 (29%)</td>
</tr>
</tbody>
</table>

1 Proportions in a row, underlined and with different letters in superscript, are significantly different between the Vaccination (Vacc) and Control (Contr) group: Ileocaecal lymph nodes in herd B \(P < 0.03\); Caecal content in herd C \(P = 0.02\).
**Detection of the vaccine strain**

In the biochemical kit (Diagnostic Kit®, IDT Biologika), 3/99 (3.0%), 2/89 (2.2%) and 3/79 (3.8%) of the faecal samples from the vaccinated pigs tested histidine-adenine auxotrophic at 10, 16 and 29 weeks of age, respectively. Similarly, the kit suggested the presence of a histidine-adenine auxotrophic strain in 4/201 (2.0%), 10/121 (8.3%) and 0/31 (0.0%) samples of the lymph nodes, caecal contents and carcass swabs, respectively. In the MLVA, the strains from all kit-positive samples belonged to the same MLVA type as the vaccine strain (Table 3). The PFGE (Table 3) demonstrated in addition, that all kit-positive samples shared the same PFGE-profile as the vaccine strain, with the exception of one isolate (lane 13) (Figure 3). The proportion of samples that tested positive for the *Salmonella* vaccine strain, are presented per herd in table 4.

Figure 3: Pulsed-field gel electrophoresis (PFGE) images of the 22 strains derived from the vaccinated pigs, which tested positive for the vaccine strain in the biochemical Diagnostic Kit (IDT Biologika). Lanes ‘1-8’ contain isolates from faeces from herd A (1,2), B (3,4), and C (5-8); Lanes ‘9-12’ contain isolates from lymph nodes from herd A (9), and C (10-12); Lane ‘V’ contains the vaccine strain Salmporc® and lane ‘+’ a *Salmonella* Braenderup strain (PulseNet control strain; Lanes ‘13-22’ contain isolates from caecal contents from herd A (13,14), B (15,16), and C (17-22).
Table 3: Results of the different analyses, performed on the 22 strains derived from the vaccinated pigs, which tested positive for the vaccine strain in the biochemical Diagnostic Kit (IDT Biologika), presented per herd.

<table>
<thead>
<tr>
<th>Herd</th>
<th>Samplea</th>
<th>Kitb</th>
<th>MLVA (New nomenclature alleles)c</th>
<th>PFGEd profile</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>STTR9 STTR5 STTR6 STTR10 STTR3</td>
<td></td>
</tr>
<tr>
<td>SSI1 (Control)</td>
<td>-</td>
<td>6 9 13 10 0211</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>Salmoporc®(Vaccine)</td>
<td>+</td>
<td>2 17 5 14 0212</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>F10W-Pig564</td>
<td>+</td>
<td>2 17 5 14 0212</td>
<td>B</td>
</tr>
<tr>
<td></td>
<td>F10W-Pig572</td>
<td>+</td>
<td>2 17 5 14 0212</td>
<td>B</td>
</tr>
<tr>
<td></td>
<td>Lnn-Pig512</td>
<td>+</td>
<td>2 17 5 14 0212</td>
<td>B</td>
</tr>
<tr>
<td></td>
<td>Cc-Pig512</td>
<td>+</td>
<td>2 17 5 14 0212</td>
<td>B?</td>
</tr>
<tr>
<td></td>
<td>Cc-Pig529</td>
<td>+</td>
<td>2 17 5 14 0212</td>
<td>B</td>
</tr>
<tr>
<td>B</td>
<td>F10W-Pig344</td>
<td>+</td>
<td>2 17 5 14 0212</td>
<td>B</td>
</tr>
<tr>
<td></td>
<td>F17W-Pig326</td>
<td>+</td>
<td>2 17 5 14 0212</td>
<td>B</td>
</tr>
<tr>
<td></td>
<td>Cc-Pig316</td>
<td>+</td>
<td>2 17 5 14 0212</td>
<td>B</td>
</tr>
<tr>
<td></td>
<td>Cc-Pig383</td>
<td>+</td>
<td>2 17 5 14 0212</td>
<td>B</td>
</tr>
<tr>
<td>C</td>
<td>F17W-Pig199</td>
<td>+</td>
<td>2 17 4 14 0212</td>
<td>B</td>
</tr>
<tr>
<td></td>
<td>F29W-Pig150</td>
<td>+</td>
<td>2 17 5 14 0212</td>
<td>B</td>
</tr>
<tr>
<td></td>
<td>F29W-Pig179</td>
<td>+</td>
<td>2 17 5 14 0212</td>
<td>B</td>
</tr>
<tr>
<td></td>
<td>F29W-Pig199</td>
<td>+</td>
<td>2 17 5 14 0212</td>
<td>B</td>
</tr>
<tr>
<td></td>
<td>Lnn-Pig130</td>
<td>+</td>
<td>2 17 5 14 0212</td>
<td>B</td>
</tr>
<tr>
<td></td>
<td>Lnn-Pig137</td>
<td>+</td>
<td>2 17 5 15 0212</td>
<td>B</td>
</tr>
<tr>
<td></td>
<td>Lnn-Pig151</td>
<td>+</td>
<td>2 17 5 14 0212</td>
<td>B</td>
</tr>
<tr>
<td></td>
<td>Cc-Pig127</td>
<td>+</td>
<td>2 17 5 14 0212</td>
<td>B</td>
</tr>
<tr>
<td></td>
<td>Cc-Pig143</td>
<td>+</td>
<td>2 17 5 14 0212</td>
<td>B</td>
</tr>
<tr>
<td></td>
<td>Cc-Pig150</td>
<td>+</td>
<td>2 17 5 14 0212</td>
<td>B</td>
</tr>
<tr>
<td></td>
<td>Cc-Pig152</td>
<td>+</td>
<td>2 17 5 14 0212</td>
<td>B</td>
</tr>
<tr>
<td></td>
<td>Cc-Pig178</td>
<td>+</td>
<td>2 17 5 14 0212</td>
<td>B</td>
</tr>
<tr>
<td></td>
<td>Cc-PigRV37</td>
<td>+</td>
<td>2 17 5 14 0212</td>
<td>B</td>
</tr>
</tbody>
</table>

a SSI= Salmonella Typhimurium strain; Salmoporc®=histidine-adenine auxotrophic Salmonella Typhimurium strain; F10W/17W/29W= Faeces sampled at 10/17/29 weeks of age; Lnn= Lymph nodes; Cc= Caecal content;
b Diagnostic Kit®, IDT Biologika;
c MLVA= Multiple-Locus Variable-number tandem repeat Analysis;
d PFGE= Pulsed-Field Gel-Electrophoresis

Table 4: Proportion of samples from the vaccinated pigs, which tested positive for the Salmonella vaccine strain, presented per herd. Presented per herd A, B, C and over all herds, in function of four sampled matrices: 1) faeces at 10 (F 10W), 16 (F 16W) and 29 (F 29W) weeks of age, 2) ileocaecal lymph nodes (Lnn), 3) caecal content (Cc), and 4) carcass swabs (Sw).

<table>
<thead>
<tr>
<th>Herd</th>
<th>F 10W</th>
<th>F 16W</th>
<th>F 29W</th>
<th>Lnn</th>
<th>Cc</th>
<th>Sw</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2/35 (5.7%)</td>
<td>0/32 (0.0%)</td>
<td>0/28 (0.0%)</td>
<td>1/66 (1.5%)</td>
<td>2/39 (5.1%)</td>
<td>0/10 (0.0%)</td>
</tr>
<tr>
<td>B</td>
<td>1/30 (3.3%)</td>
<td>1/28 (3.6%)</td>
<td>0/29 (0.0%)</td>
<td>0/64 (0.0%)</td>
<td>2/33 (6.1%)</td>
<td>0/10 (0.0%)</td>
</tr>
<tr>
<td>C</td>
<td>0/34 (0.0%)</td>
<td>1/29 (3.4%)</td>
<td>3/22 (13.6%)</td>
<td>3/71 (4.2%)</td>
<td>6/49 (12.2%)</td>
<td>0/11 (0.0%)</td>
</tr>
<tr>
<td>A+B+C</td>
<td>3/99 (3.0%)</td>
<td>2/89 (2.2%)</td>
<td>3/79 (3.8%)</td>
<td>4/201 (2.0%)</td>
<td>10/121 (8.3%)</td>
<td>0/31 (0.0%)</td>
</tr>
</tbody>
</table>
Serology

The *Salmonella*-specific antibody levels (mean S/P ratio) are shown for each group, herd and sampling occasion in figure 2. At three days of age, just prior to vaccination, the mean S/P ratio over all herds was comparable between both groups: 1.50 ±0.87 (STDEV) in group V (n = 120) and 1.53 ±0.79 in group C (n = 119) (P = 0.73). However, in herd A the mean S/P ratio of V group (S/P =2.11 ±0.68; n = 40) and C group (S/P =1.75 ±0.58; n = 40) differed significantly (P < 0.03).

At ten weeks of age, the mean S/P ratio in both groups was three- to five-fold lower than at three days of age. The mean S/P ratio over all herds was significantly higher in the V group (mean S/P 0.49 ±0.51; n = 100) compared to the C group (mean S/P 0.31 ±0.46; n = 102) (P < 0.01). This is mainly due to the significant difference between both groups in herd A and herd C: the mean S/P ratio was 0.43 ±0.33 (n = 35) (V) vs. 0.18 ±0.21 (n = 36) (C) for herd A (P < 0.02), and 0.71 ±0.70 (n = 33) (V) vs. 0.09 ±0.21 (n = 37) (C) for herd C (P < 0.01). The opposite was true for herd B with a mean S/P ratio of 0.32 ±0.36 (n = 32) (V) vs. 0.66 ±0.66 (n = 29) (C) (P < 0.01).

At 29 weeks of age, the S/P ratio over all herds was significantly higher in the C group (P < 0.01): a mean S/P ratio was detected of 1.07 ±0.55 (n = 89) (V) vs. 1.52 ±0.68 (n = 87) (C). This outcome was mainly attributable to the results obtained in herd A and herd C, as a mean S/P ratio of 0.94 ±0.39 (V) vs. 1.53 ±0.55 (C) was detected in herd A (P < 0.01), and 0.86 ±0.54 (V) vs. 1.69 ±0.71 (C) in herd C (P < 0.01). No significant difference was observed in the mean S/P ratios of herd B: 1.39 ±0.58 (V) vs. 1.34 ±0.75 (C) (P = 0.71).

Discussion

In this study, a live vaccine applied orally in pigs at 3 and 28 days of age yielded overall beneficial results towards daily weight gain. *Salmonella* excretion in faeces and serum antibody levels were also lower in the vaccinated animals than in the controls, prior to slaughter. Apart from these beneficial effects, some limitations of the vaccine to be used under field conditions were found as well, and are discussed below.

The significantly higher daily weight gain of the vaccinated pigs implies a clear incentive for pig producers to control the *Salmonella* infection pressure in their herd. Because most *Salmonella* infections are subclinical in pigs, they might not have realized the full impact of *Salmonella* on pig performance so far. The feed intake could not be recorded for both groups separately, however. Consequently, the feed conversion ratio, an economically important parameter, could not be measured.
Additional research addressing all economically important performance parameters is warranted for a more accurate insight in the vaccine’s influence on this.

The overall bacteriological and serological results of this study were promising, but there was a considerable variation between the three herds. Over all herds, significantly less vaccinated pigs excreted *Salmonella* prior to slaughter, in comparison with the control pigs. This is an important finding, as finishing pigs may introduce *Salmonella* this way into the slaughterhouse environment (Letellier et al., 2009). Also a significantly lower *Salmonella*-specific antibody level was observed before slaughter among the vaccinated animals over all herds, suggesting a decreased *Salmonella* infection pressure in the preceding period (Lo Fo Wong et al., 2004b; Baptista et al., 2009). The variable individual results between the three herds might be due to several reasons. Firstly, in field conditions pigs are infected at different points in time, with a herd- and even batch-dependent variability in both infection pressure and host response (Beloeil et al., 2003; Lo Fo Wong et al., 2004b; Rostagno et al., 2012b). Secondly, the presence of herd-specific *Salmonella* strains might have influenced the vaccination effect as well (Van Parys et al., 2013). In addition, some study-specific variation may have altered the outcome, since the degree of separation of the vaccinated from the control groups was not identical in the three farms. Unlike herd A and B (with the vaccinated and control pens in one room), both the antibody titre and faecal excretion in herd C (with all vaccinated pens in a room separated from the control pens) were significantly reduced in the vaccinated finishing pigs. This may indicate that vaccination will attain its most distinctive effect when all pigs in a stable or herd are vaccinated. This corroborates with another recent vaccination study, in which faecal excretion and organ colonization were reduced the most, when all pigs in the barn were vaccinated instead of the pigs in certain pens only (Arguello et al., 2013). Finally, a different maternal antibody level in the piglets at the moment of vaccination on the three herds might have contributed to the herd variation as well. In our study, we chose to vaccinate the pigs early at three and 28 days of age, in order to induce protection against possible infections occurring already in the nursery period. Vaccination at these time-points has also some practical advantages as the pigs can be taken up easily to apply the vaccine orally, and because piglets are handled anyway at weaning before moving them to the nurseries. No interference by maternal antibodies was expected upon this early prime vaccination, as Rössler et al. (2010) could not demonstrate a negative effect of maternal antibodies on the *Salmonella* vaccination using the same vaccine in suckling pigs. In the present study however, the herd with the highest maternal *Salmonella*-specific antibody levels in pigs at first vaccination was the only herd without a significant decreased *Salmonella* excretion in the vaccinated pigs at finishing stage. This observation suggests that maternal immunity might have interfered with the oral vaccination. Further field studies on the role of maternal immunity on the efficacy of this vaccine are therefore needed before recommending the current vaccination scheme.
No persistence of the vaccine strain was expected in the vaccinated groups of this study, as a previous experiment with the current vaccine could not detect the vaccine strain in the lymph nodes of slaughter pigs that had been vaccinated at 21 and 42 days of age (Lindner et al., 2007). In contrast, this study suggested the presence of the vaccine strain in the faeces throughout the fattening period in all three herds, and in the lymph nodes and caecal content at slaughter. No clear explanation is available for this persistence of the vaccine strain or occurrence of a vaccine-like strain. Anyhow, this finding might have important implications for bacteriological monitoring programmes, since supplementary bacteriological testing would be necessary to avoid *Salmonella*-positive results in vaccinated herds. Although the histidine-adenine auxotrophic *Salmonella* Typhimurium strain used here is most probably attenuated in humans too, further studies are necessary to address possible public health implications of our findings.

In conclusion, the early vaccination scheme with the current live *Salmonella* Typhimurium vaccine in herds with a high *Salmonella* infection level, improved daily weight gain, reduced faecal excretion, and induced a lower *Salmonella*-specific antibody titre at slaughter age, when compared to non-vaccinated pigs. This vaccine could therefore, be a helpful tool in the control of *Salmonella* infection. However, the effects were variable between herds, and a possible persistence of the vaccine strain until slaughter age requires further studies.

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Chapter 4 – General Discussion
1. Introduction

Directive 2003/99/EC and Regulation (EC) No 2160/2003 demanded targets for the monitoring and control of food-borne zoonotic agents in all EU Member States (MS). Currently, many MS have already implemented national control programmes to reduce *Salmonella* prevalence in poultry (Anonymous, 2007a; EFSA and ECDC, 2012a) or pigs (Alban et al., 2002; Anonymous, 2007b; Merle et al., 2011; EFSA and ECDC, 2012a; Anonymous, 2013a,b). For poultry, most MS met the *Salmonella* reduction targets thanks to a successful *Salmonella* control programme, and in particular by vaccinating laying hens. This vaccination became mandatory in Belgium in 2007 (Anonymous, 2007a). It is assumed that the vaccination of laying hens was the main reason for the decrease in the number of *Salmonella* Enteritidis infections in humans, reported in the EU over the period 2008-2011 (EFSA and ECDC, 2013a). The number of *Salmonella* Typhimurium cases in humans however, has remained constant in the same period, or has even increased if the monophasic *Salmonella* Typhimurium cases are taken into account (EFSA and ECDC, 2013a; Fig. 8). As *Salmonella* Typhimurium is by far the most common serovar isolated from pigs and pork (EFSA and ECDC, 2013a), this might indicate that the control programmes of several reporting MS are currently insufficient to reduce *Salmonella* prevalence in pig production.

![Figure 8: Number of reported confirmed human salmonellosis cases in 25 EU Member States, shown per month in 2008-2011 (EFSA and ECDC, 2013a).](image-url)
In Belgium, a national pig Herd-specific Salmonella Action Plan (HSAP) was implemented in 2007, but since then no significant improvement was observed of the Salmonella seroprevalence of the pig population (Méroc et al., 2012), neither was there a reduction recorded in the human Salmonella Typhimurium cases (NRSS, 2012).

This might be explained partially by the fact that highly efficacious measures are currently not available. As such, there is little confidence in any measure, which may lead to poor compliance. In addition to that, Salmonella control measures were not mandatory, but merely recommended for herds that were assigned ‘at risk’ in the HSAP, which also hinders significant improvements.

Apart from the control measures, also the Salmonella monitoring and follow up was inefficient in Belgium. Méroc et al. (2012) recently pointed out that the observed serological improvement of one-third of the risk herds from the HSAP, was probably due to random variation in the serological response. The different pig weight categories that were sampled, as demanded for the Aujeszky’s disease programme, may have led to variable mean S/P ratios within a herd from one sampling round to the next. Moreover, farms that should be assigned as ‘high risk’ might be missed, as these authors showed that the current sample size (12 serum samples per farm visit) is too small to attain a herd-sensitivity above 95%. Consequently, these facts may have resulted in an erroneous determination of a herd’s risk status. As Belgium is one of the five largest exporting countries of pig meat in the EU (Anonymous, 2010), the need for more efficacious control measures and a more accurate monitoring programme is urging.

This general discussion elaborates on the control measures that were evaluated during the experimental work performed in this thesis and on the possible monitoring scenarios that are considered for the future SAP.

2. *Salmonella* control in primary production

To investigate the effect of possible control measures, different study designs are available. In this thesis, two experimental transmission studies in piglets and one field study in fattening pigs were performed to evaluate such control measures.

The design of the transmission studies implied that treatment (‘the control measure under study’) was applied to all pigs before some of them (the ‘seeder’ pigs) were experimentally infected. With this study design, the seeder pigs in each ‘treatment’ group transmitted different amounts of bacteria towards the contact animals (Chapter 3.1).

On the one hand, this design allowed investigating the combined effect of the treatment on both the excretion of the challenged pigs and the susceptibility of the naive contact animals. This is for instance not the case in more commonly used efficacy studies, in which all treated pigs are challenged and thus
mainly the susceptibility is assessed (Roesler et al., 2004; Taube et al., 2009; Tanaka et al., 2010). Transmission studies may therefore provide a better simulation of the field situation, since control measures do have an effect in the field on both the excretion of and susceptibility to *Salmonella*. In other words, a treated pig may indeed be infected and subsequently infect other treated pigs in the same area. Only considering the reduction of the susceptibility of the contact animals may thus underestimate the treatment effect. This has also been demonstrated in the past with the vaccination against Aujeszky’s disease virus in pigs. Based on classical efficacy studies, the vaccine appeared insufficiently effective (Baskerville, 1981; Wittmann, 1986, 1991), whereas in transmission experiments, it was shown that the vaccine reduced transmission and could eventually result in an elimination of the virus (Stegeman, 1995; Bouma et al., 1996; van Nes, 2001). Afterwards, this turned out to be correct in many European countries. Since then, transmission studies have been conducted for many different pathogens (Velthuis et al., 2003; Dewulf et al., 2004; Heres et al., 2004; Meyns et al., 2004, 2006; Bohez et al., 2008; Vrancken et al., 2009; Villareal et al., 2011; Crombé et al., 2012; Claes et al., 2013).

On the other hand, these different amounts of bacteria excreted by the treated seeders in transmission studies provide an unpredictable challenge level towards the contact animals, whereas in efficacy studies exactly definable challenge doses are administered. Therefore, the results of the latter studies are generally more reproducible.

Anyhow, the experimental conditions used in both transmission and efficacy studies warrant some caution when extrapolating the results towards field conditions. In commercial herds, pigs are infected with many different pathogens and the housing, nutrition and management conditions may not be optimal. These factors may all influence the treatment effect. Therefore, the experimentally evaluated interventions in piglets need to be verified under field conditions, using a large number of animals monitored until slaughter. Because of time and financial restrictions, only one promising intervention of both transmission studies (vaccination) was further evaluated in this thesis, using a large field study.

As pointed out in the general introduction (Chapter 1), many control measures to decrease the *Salmonella* infection pressure at primary production are described, such as improved biosecurity, nutritional changes and vaccination. As biosecurity measures alone are not sufficient in reducing infection levels in the population, they should be combined with other intervention methods (Gautam et al., 2013). The intervention strategies studied in this thesis will be discussed hereafter, namely the supplementation of organic acids (two in-feed products and one drinking water additive), as well as the oral vaccination with a live commercial non-marker vaccine (early- and older-age protocol) and a live experimental marker vaccine.
2.1 In-feed organic acids

The first in-feed organic acid evaluated, butyric acid, is a short-chain fatty acid and a main end-product of intestinal microbial fermentation of dietary fibres, celluloses, starches and sugars. Butyrate (the salt form of butyric acid) was proven to reduce the Salmonella load ex vivo in the feed, and to exert potent effects in vivo on pathogen control and on a variety of intestinal mucosal functions (Guilloteau et al., 2010). Similar to other organic acids, non-dissociated butyrate enters the bacterial cell and dissociates due to the higher intracellular pH. This in turn lowers the pH in the bacterial cell and prevents DNA synthesis and hence replication (Kirchgessner et al., 1992). Besides this bactericidal effect, butyrate can also down-regulate the expression of invasion genes that are located on the Salmonella Pathogenicity Island 1 (SPI-1) (Lawhon et al., 2002; Gantois et al., 2006). At the time of our transmission studies, an uncoated and coated formulation of butyrate was available on the market. Only the coated products, ‘microencapsulated’ by mineral or lipid carriers, are able to reach the intestinal invasion site of Salmonella. Uncoated organic acids are resorbed in the upper part of the intestinal tract (Van Immerseel et al., 2005; Boyen et al., 2008b). In addition, coated butyric acid has proven superior in controlling Salmonella colonization, compared to coated formic and acetic acid (Van Immerseel et al., 2004; Boyen et al., 2008b). Therefore, coated calcium-butyrate – the salt form being less odorous and volatile than the free form – was used in our transmission experiments.

In the first transmission study (Chapter 3.1), the number of piglets with Salmonella-positive faeces and tissues was reduced in the group treated with 0.3% in-feed coated calcium-butyrate (Greencab-70®, currently named Globamax Performant®, Sanluc International), resulting in a lower adjusted transmission ratio $R_a$ (although statistically not-significant), when compared to the positive control group. The treated piglets in this group also had a significantly higher daily weight gain than the positive control piglets. In the second transmission experiment (Chapter 3.2), piglets were treated with the same product, but they were additionally orally vaccinated with an experimental Salmonella Typhimurium marker vaccine. In this second study, the treated group had again a significantly lower number of contact animals excreting Salmonella and a significantly lower number of Salmonella-positive tissue samples at necropsy, when compared to the positive control group. The adjusted transmission ratios $R_a$ in the treated group were lower than in the positive control group, but the difference was not statistically significant. The daily weight gain was not significantly improved in this group. This indicates that the combination of the coated calcium-butyrate and the current vaccination may not be an optimal combination. The butyrate might have inhibited the orally administered live vaccine strain, resulting in a lowered efficacy of the vaccination. It is however also possible that more pronounced effects would have been obtained if more pigs were monitored for a longer period, e.g. until slaughter age. Another way to achieve a more distinct effect might be the administration of butyrate at an even earlier age. Le
Gall et al. (2009) have demonstrated that a more retarded gastric emptying is induced by feeding sodium butyrate pre-weaning instead of post-weaning. Consequently, this prolonged gastric presence also implies an increased feed digestibility at pre-weaning age and thus a better performance.

Similarly, Boyen et al. (2008b) found a reduced Salmonella excretion in a challenge experiment with piglets, by administering 0.2% coated butyric acid. Biagi et al. (2007), using 0.1% to 0.4% uncoated butyrate in the feed, could not demonstrate an influence on intestinal counts of Enterobacteriaceae in piglets, nor on their growth. Similar to both previous findings, Salmonella-reducing effects were observed in piglets after adding other coated organic acids to the feed (0.4% microencapsulated fumaric, sorbic, malic, and citric acid in piglets by Piva et al., 2007), whereas this was not the case when administering uncoated organic acids (0.4% fumaric, lactic, propionic, citric, benzoic acid by Walsh et al. (2007) and 0.4% formic, lactic acid by Martín-Peláez et al. (2010)). In some studies uncoated organic acid products in the feed did yield beneficial effects, but these authors used a higher dose (1.2% K-diformate by Papenbrock et al. (2005) and Taube et al. (2009); 2.8% lactic acid by Tanaka et al. (2010)), or treated older pigs for a longer period (0.4 to 0.6 % formic, lactic acid during the fattening period by Creus et al., 2007) than was the case in the above mentioned experiments.

Another organic acid feed-additive that was investigated in the second transmission experiment, was a commercial, non-coated premix of 0.5% benzoic acid and 0.3% citric acid (results not published). No significant reduction in Salmonella shedding or transmission was observed in this premix group when compared to the positive control group, and the daily weight gain was even significantly lower.

Similar non-beneficial effects on faecal Salmonella prevalence and weight gain were seen in challenged piglets that were treated with a lower dose of only benzoic acid (0.3%) via the drinking water (Walsh et al., 2012). The fact that both additives were not microencapsulated, as described above, might partially explain the lack of effect in our experiment and the one of Walsh et al. (2012). Another study in non-infected growing pigs, fed with a 1% benzoic acid diet, could not demonstrate an improved weight gain or feed conversion ratio either (Bühler et al., 2006). In contrast, both Guggenbuhl et al. (2007) and Papatsiros et al. (2011) have shown that a similar in-feed dosage of benzoic acid (0.5%) to non-infected weaned piglets improved their growth performance significantly. Since these three studies showed that growth was enhanced in weaned piglets and not in growing pigs, this suggests that an early administration might be more efficient to improve pig performance. However, as Bühler et al. (2006), Guggenbuhl et al. (2007) and Papatsiros et al. (2011) did not challenge the pigs with Salmonella, the impact of this feed-additive on Salmonella-infection could not be assessed. Further challenge studies with benzoic acid, preferably in the coated form, are thus recommended to judge its applicability in Salmonella-infected herds.
In conclusion, our results and those published in literature showed that microencapsulation improves the effect of in-feed organic acids on *Salmonella* shedding and on growth performance of pigs, irrespective of the acid type. However, many other parameters may influence their effect, *e.g.* the dosage, the treatment period and the age of the treated pigs.

### 2.2 Organic acids in drinking water

The third organic acid product examined in our transmission study was added to the drinking water. This organic acid mixture was applied at a dosage of 0.09% v/v, resulting in a final concentration of 0.05% formic, 0.01% propionic, 0.01% acetic and 0.005% lactic acid in the drinking water, and a drinking water pH of 3.6 - 3.8. Unfortunately, the number of *Salmonella*-excreting piglets in this group was higher significantly higher than the number in the positive control group. Also significantly more tissue samples were *Salmonella*-positive in this group than in the positive control group. This suggests that the addition of this organic acid mixture via water even enhanced the excretion of *Salmonella* spp.

Similar poor results with acidifying the drinking water were found in weaned piglets by Letellier *et al.* (2000). These authors observed no reduced *Salmonella* colonization of mesenteric lymph nodes after challenge, when administering drinking water with 0.02% formic acid. A rather low amount of acids was supplemented in both our study and the one of Letellier *et al.* (2000), which probably led to an insufficient bactericidal effect. No higher dosage was possible with both supplemented products however, as adding more product would further decrease the pH of the drinking water below 3.5 and affect palatability (De Busser *et al.*, 2008). This might explain the lack of effect in the study of Hansen *et al.* (1999), in which the pH of the drinking water of fattening pigs was 3.1 after adding a higher dosage of 0.1% formic acid. This low pH of 3.1 might have caused adaptive acid responses inside the *Salmonella* bacteria (de Jonge *et al.*, 2003), and/or an insufficient water intake by the pigs. Both Van der Wolf *et al.* (2001b) and van der Heijden *et al.* (2006) demonstrated however, that the administration of approximately 0.1% formic acid was possible to reach a drinking water pH of only 3.8, if half of the acids were buffered as ammonium formiate. Their studies have shown a decreased seroprevalence at slaughter when treating pigs during the entire fattening period. Therefore, the application of organic acids in their buffered salt form might be a solution for attaining a sufficiently bactericidal effect without the palatability problems or the induction of *Salmonella* adaptive responses to a low pH. De Busser *et al.* (2009) could not find a decreased *Salmonella*-colonization of slaughter samples unfortunately, although they provided drinking water with an acceptable pH of 3.6 - 4.0 and with a satisfactory amount of formic acids (*i.e.* 0.1% formic acid, along with 0.05% acetic, 0.03% propionic, and 0.002% sorbic acid). This might have been due to the short treatment period of two weeks prior to slaughter, or to cross-contamination in the slaughterhouse.
2.3 Vaccination with a live commercial non-marker vaccine

Another intervention method investigated in this thesis through *in vivo* studies, was the oral vaccination against *Salmonella*. Two different live vaccines were evaluated for the first time in Belgium.

The first vaccine, the only commercially available *Salmonella* vaccine for pigs in the EU (*Salmoporc*®, IDT), is a histidine-adenine auxotrophic vaccine. It was used in the first transmission experiment (Chapter 3.1) and in the field trial (Chapter 3.3). The transmission trial was performed according to the initial vaccination protocol recommended by the manufacturer (*i.e.* oral vaccination at three and six weeks of age), whereas the currently recommended protocol was applied in the field study (*i.e.* early oral vaccination at three days and three weeks of age). The latter vaccination protocol was not validated by the manufacturer at moment of the transmission study, but it is the preferred protocol, since it minimizes the farmers’ work load at the moment of vaccination.

The transmission study demonstrated that the vaccine was capable of decreasing the number of *Salmonella*-excreting piglets and the adjusted transmission ratio $R_a$, when compared to the unvaccinated positive control group. The mean *Salmonella*-specific serum antibody titre of the vaccinated group however, was significantly higher throughout the experiment until D38 after inoculation, compared to the positive control group. The proportion of tissue samples colonized with the *Salmonella* challenge strain, did not differ significantly from the one in the positive control group at euthanasia, six weeks post challenge (*i.e.* 14 weeks of age). Moreover, as many *Salmonella* vaccine strain-positive as *Salmonella* challenge strain-positive tissue samples were found, when using a differentiating biochemical kit provided by the manufacturer of the vaccine (*Salmonella* Diagnostic Kit®, IDT). In other words, without performing this additional differentiation, the vaccinated group would have shown twice as much *Salmonella*-positive tissue samples 52 days post vaccination, when compared to the positive control group. According to the manufacturer, the maximum persistence of the vaccine strain is 42 days after vaccination (Eddicks et al., 2009), so this period was slightly exceeded in the first transmission experiment (Chapter 3.1). Springer et al. (2001) also administered the current vaccine to weaned piglets before challenging them with *Salmonella Typhimurium*. Their study ended already one week post challenge and no conclusions were reported concerning the persistence of the vaccine strain. These authors also observed a significantly higher antibody response in the vaccinated animals, but found a significantly lower number of challenge strain organisms per gram of lymph nodes, ileal and caecal contents in the vaccinated than in the unvaccinated group.

Despite the promising effect of this commercial vaccine of reducing *Salmonella* excretion in our transmission experiment, the presence of vaccine-induced antibodies and the longer persistence of the
vaccine strain raised questions concerning the possible use of the vaccine in the current HSAP. As the transmission experiment and also most previous vaccination studies were based on a limited number of animals, these issues needed further investigation on a larger scale. This led to the conduction of our field trial, in which the vaccine was tested in three Salmonella problem herds, and pigs were monitored from shortly after birth until slaughter age.

At the end of the fattening period, a significantly lower Salmonella-specific antibody titre and significantly less Salmonella-excreting pigs were observed in the vaccinated group over all herds, compared to the control group. The vaccinated animals of one herd showed additionally significantly less Salmonella-positive samples in the lymph nodes, while this was the case for caecal contents in another herd. These results might indicate that the current vaccination strategy decreased the overall Salmonella infection pressure. Lindner et al. (2002; 2007) implemented the same vaccine in one herd, according to the initial vaccination protocol (vaccination at three and six weeks of age). They also found a significant decreased number of Salmonella-colonized lymph nodes in the vaccinated slaughter pigs. In the vaccinated group of each herd, the daily weight gain (DWG) between three days and 29 weeks of age of the vaccinated group was significantly higher than the non-vaccinated control group. A similar outcome in finisher pigs was reported in the vaccination trial of Farzan and Friendship (2010). The better DWG among the vaccinated fattening pigs supports the hypothesis that the vaccination yielded a lower Salmonella infection pressure, as Salmonella infections cause a reduction of the weight gain (Balaji et al., 2000; Turner et al., 2002; Boyen et al., 2009; Davis et al., 2010).

However, our serological and bacteriological results differed largely between the three herds. Prior to slaughter, a significantly lower Salmonella-specific antibody titre in the vaccination group of two herds was associated with either a significantly lower faecal Salmonella prevalence (herd C), or a slightly higher faecal prevalence (herd A), when compared to the control group. Also the opposite situation was observed, as a slightly higher antibody titre in the vaccination group (herd B) was found together with a significantly lower faecal prevalence. Similar variations were observed between the herds at earlier stages of the trial. This finding underlines that the effect of Salmonella vaccination may differ largely between pig herds. This may be due to different factors, such as the infection course and infection level (Lo Fo Wong et al., 2004b), the presence of herd-specific Salmonella strains (Van Parys et al., 2013), the maternal antibody levels (Chapter 3.3) and possible indirect contacts with non-vaccinated animals (Chapter 3.3; Arguello et al., 2013). Also differences in the cell-mediated response, which is generally recognized to be more important than humoral responses in protection against Salmonella (Meeusen et al., 2007), might have influenced the vaccination effect. The cellular response was however, not investigated in our trial. Hence, this herd variability indicates that different measures, targeting all components of the farm system, are indispensable, as they are mutually supportive with vaccination (Hotes et al., 2011). In addition, it is advisable to vaccinate all pigs in a compartment or herd instead of
certain pens, in order to improve the overall vaccination effect (Our field trial, Chapter 3.3; Arguello et al., 2013).

Another finding that needs attention in further research, was the isolation of a *Salmonella* Typhimurium strain with the same auxotrophic characteristics and genotype as the vaccine strain, until slaughter. This strain was found in all three herds, in faecal samples as well as in samples of lymph nodes and caecal contents. Surprisingly, none of the lymph node samples of slaughter pigs tested positive for the vaccine strain in the trial of Lindner et al. (2007). Therefore, a thorough investigation of the isolated strain from our field study is essential, since the possible persistence of the vaccine strain might have important implications for bacteriological monitoring programmes. In that case, supplementary bacteriological testing would be necessary to avoid *Salmonella*-positive results in vaccinated herds. This would evidently increase the costs and prolong the time for diagnostic confirmation. Although the auxotrophic vaccine was found genetically-stable after five passages in piglets, and it was not detected in human isolates investigated by the Roberts Koch Institute in the last ten years (Springer, personal communication), the potential public health risk (e.g. the risk for YOPI) should in addition be investigated.

Overall, our results indicated that vaccination decreased the *Salmonella* infection pressure in the herds. However, it is not clear yet whether vaccination in pig production with the current *Salmonella* live vaccine would attain the same spectacular drop of human salmonellosis, as did vaccination in the laying hen industry in the EU. The described variability of the vaccination effect between herds might be reduced overtime if vaccination would be applied on large-scale in primary production, and if cross-protection between *Salmonella* serovars could be provided by a multivalent *Salmonella* vaccine (Farzan and Friendship, 2010).

### 2.4 Vaccination with a live experimental marker vaccine

As the Salmoporc® vaccine-induced antibodies are not distinguishable in LPS-based ELISAs from antibodies induced by natural *Salmonella* infections, they might interfere with most current European serological monitoring programmes (Osterkorn et al., 2001; Alban et al., 2002; Laevens and Mintiens, 2005; Anonymous 2013a,b). Indeed, in our first transmission experiment, vaccination with Salmoporc® induced a higher seropositivity rate in the vaccinated than in the unvaccinated piglets, until D38 after inoculation. The same outcome was seen in slaughter pigs in the vaccination study of Lindner et al. (2007), using Salmoporc® as well. That is, when applying a cut off level of OD% 40 (i.e. S/P 1.00) (Lindner et al., 2007) or OD%10 (i.e. S/P 0.25) (Chapter 3.1). Although a lower seropositivity rate was observed prior to
slaughter in the vaccinated than in the unvaccinated pigs of our field study (Chapter 3.3), the mean S/P ratio of all vaccinated pigs (S/P = 1.07) then exceeded the OD% 40 as well.

To overcome this antibody distinction issue in serosurveillance programmes, several DIVA vaccines (Differentiation of Infected and Vaccinated Animals) have recently been developed (Selke et al., 2007; Nagy et al., 2008; Kong et al., 2011; Leyman et al., 2011). These last three studies describe the useful deletion of certain LPS-encoding genes in the Salmonella genome, resulting in candidate-vaccine strains that induce antibodies undetectable with LPS-based ELISAs. Protective serological responses were induced in mice by the ArfaJ Salmonella Typhimurium strains of Kong et al. (2011) and Leyman et al. (2011). The last authors also developed a whole-cell ELISA, which could detect antibodies induced by the candidate-vaccine strain.

The protective effect of such a vaccine in weaned piglets was assessed in our second transmission experiment (Chapter 3.2). The same deletion described by Leyman et al. (2011) was applied to the Salmoporc® parent strain, resulting in the ‘Salmoporc-ArfaJ’ vaccine candidate. When comparing the unvaccinated group and the two vaccinated groups after challenge with a Salmonella Typhimurium strain, less tissue samples were colonized and a lower transmission ratio was observed in both vaccinated groups. In the vaccinated group without in-feed coated calcium-butyrate, these results were not significant though, and the daily weight gain was not significantly improved. Also in our field study with the parent strain of the marker vaccine, no reducing vaccination effect was seen in serum at 10 weeks of age nor in faeces at 16 weeks of age. By the time of slaughter however, the vaccinated pigs did gain more weight and were less likely to excrete Salmonella when compared to the non-vaccinated pigs. It is therefore reasonable to speculate that the piglets in the DIVA vaccination groups of this transmission study might have demonstrated a more distinct beneficial effect, when monitored for a longer period than the first 14 weeks of life (e.g. the entire fattening period). When measuring the Salmonella-specific antibody titres by an LPS-based ELISA, also no significant difference was observed between both vaccinated groups and the positive control group. After vaccination and before challenge however, Salmonella-specific antibodies were absent in the LPS-based ELISA and present in the whole-cell ELISA. This finding confirmed the DIVA capacities of the Salmoporc-ArfaJ vaccine strain, and merits further research with larger sample sizes to further investigate the efficacy and safety of the vaccine.
2.4 Feasibility of the evaluated interventions in practice

Supplementing the drinking water is a more flexible and controllable way for the farmer to administer organic acids, than is the case if the feed is supplemented by the feed company. For the farm equipment however, the use of organic acids has greater implications when administrating acidified drinking water rather than acidified feed. Firstly, installations of stainless steel are required to avoid corrosion of the pipe lines and damage to the concrete under the drinkers. Together with the purchase of a dosing pump, the costs for the farmer may be substantial, unless this equipment can be used for other administration purposes as well. Secondly, clogging of the drinking nipples due to fungal growth might occur. This can be partly circumvented by adding sorbic or propionic acid to the mixture. Nevertheless, serious clogging problems were still observed in the study of van der Wolf et al. (2001b), despite the use of sorbic acids. Although Salmonella seroprevalence can be reduced by acidifying the drinking water with efficacious acid components and a correct dosing (van der Wolf et al., 2001b; van der Heijden et al., 2006), it might be a less expensive and more practical solution to supplement the feed with organic acids. The calculated cost for our acid mixture in the drinking water was €1.60 per slaughter pig, when applied during the entire fattening period (equipment costs not included however). Van der Wolf et al. (2001b) estimated in their field trial that the costs per slaughtered pig would be 2.49€ for their product, and 0.41€ for the equipment (2.90€ in total). Van der Heijden et al. (2006) calculated these total costs for acidifying the water to be 2.53€ per pig. The coated calcium-butyrate used in our study would require €3.96 per pig at the administered dose of 3kg/ton feed (incl. mixing costs). For this in-feed product, no administration problems are to be expected and thus no additional material costs on-farm are to be added. Lowering the doses and shortening the periods of acid administration can reduce costs, but this may have a negative impact on the efficacy (Creus et al., 2007). It must be noted however, that the faecal concentration of Salmonella in naturally infected pigs is usually $10^5$ times lower than the experimental infection dose used in the transmission studies (Pires et al., 2013). Therefore, it is reasonable to expect that an administration dose of the coated calcium-butyrate of 2kg/ton, resulting in 2.64€ per slaughtered pig, would obtain similar reducing results in practice as in our transmission studies. In contrast, the commercial vaccine that was implemented in our transmission and field trial does not demand any of the previous equipment expenses. It would cost the farmer €1.60 per slaughter pig (excl. veterinariion costs). Similar to the commercial vaccine, the evaluated DIVA vaccine would not request notable extra equipment or production costs. Even though the method to construct such a DIVA strain is patented, no price per slaughter pig is available yet, as validation procedures are currently still ongoing.

Anyhow, without governmental financial support, these costs may be considered too high by pig producers. Since pig producers already bear many of the costs of Salmonella control, while the benefits
are mainly reaped by the pork consumers, they have little incentive to invest in Salmonella control (Goldbach and Alban, 2006). However, a lower Salmonella infection level may also lead to a better growth of the pigs (Balaji et al., 2000; Turner et al., 2002; Boyen et al., 2009; Davis et al., 2010). This indicates that the costs of efficacious intervention strategies might be compensated by the benefits of an improved weight gain (Farzan and Friendship, 2010) and consequently may be cost-efficient. To clarify this, further economical evaluations are necessary, which take into account all costs and benefits of the interventions.

Obviously, not only the reduced pig productivity is of concern when discussing Salmonella-related economic losses. The total annual human health losses for instance (i.e. costs due to healthcare and loss of labour), caused by Salmonella in pigs, were estimated to amount €90 million in the EU (Anonymous, 2010). Given the scale of these costs, it is important to investigate how the human health risks by consuming pork can be minimized. Therefore, a consortium of the Federal Communications Commission (FCC) (comprising Food Control Consultants Ltd. and Agri-Livestock Consultants Ltd.) developed a cost-benefit analysis to test the economic profitability of targeting Salmonella reduction in slaughter pigs (Anonymous, 2010). In this analysis, four intervention scenarios were evaluated: i) the establishment of a support unit and increased sampling, ii) the first scenario plus improved feed practices and farm-level biosecurity, iii) the first scenario plus interventions targeting the purchase of Salmonella-free pigs or feed in high- or low-prevalence countries, respectively, and iv) the third scenario plus all transport and slaughterhouse measures. Unfortunately, the analysis did not show an economic benefit in any of these scenarios. Additional sensitivity analyses did suggest however, that there is some economic rationale for a gradual development of Salmonella control at farm level. The consortium concluded that it is still worthwhile continuing the investigations for possible Salmonella-reducing interventions (Anonymous, 2010). In this matter, it is imperative to investigate companies’ claims that their products are able to reduce Salmonella prevalence. Governmental directives should be developed, demanding Salmonella-specific in vitro, in vivo and field trials which demonstrate the efficacy of these products. Although it is widely accepted that individual products are not equally effective across all pig farms, this certification of products would aid pig producers in choosing the most applicable product for their specific herd. This tailored combination of interventions is indispensable for an effective Salmonella approach, as no universal control measure has been identified so far and all components of the farm-system can be targeted this way.
3 The future Salmonella Action Plan

Pig producers are dependent on the advice from public and private veterinary services for the implementation of management strategies that limit both Salmonella introduction and spread in a farm. Therefore, the implementation of an accurate monitoring and efficient control programme at farm-level is critical for reducing Salmonella prevalence in finishing pig units. The current herd-Specific Salmonella Action Plan (HSAP) in Belgium however, did not lead to a significant decrease of the Salmonella seroprevalence in the Belgian pig population, since it entered into force in 2007 (Méroc et al., 2012). This lack of improvement in the Salmonella status together with several additional reasons as described in the general introduction, led to the suggestion of four adaptation scenarios by the Scientific Committee of the FASFC, which each have certain advantages and disadvantages (Anonymous, 2012).

The first suggested scenario, describing the bacteriological examination of pooled faecal samples at the farm, has the advantage that the presence of certain Salmonella serovars can be demonstrated unambiguously. A clear determination of the herd infection pressure would be impeded however, since the intermittent excretion of Salmonella (van Winsen et al., 2001) might cause false-negative results if the sample size is too small. Neither would it be possible to evaluate the evolution of the Salmonella prevalence, as there would be little certainty about the age of the sampled pigs. In addition, this sampling strategy at herd-level would have considerable financial implications, since many samples are required to attain a statistical significant evaluation of the herd prevalence. These monitoring costs would be augmented even more if the evaluated current commercial vaccine (Salmoporc®, IDT) would be used (Chapter 3.3), since in that case, bacteriological confirmation testing might be necessary to avoid a misclassification at herd level.

The examination of intestinal contents at the slaughterhouse, described in the second scenario, would allow a relevant determination of the risks for carcass contamination. Moreover, as all sampled pigs have approximately the same age at slaughter, the evolution of Salmonella prevalence within herds could be evaluated as well. The intended feedback to the herds might not be reliable however, since cross contamination is possible during transport and in the lairage (Hurd et al., 2001; Loynachan et al., 2004; Rostagno et al., 2005; Wales et al., 2010; De Busser et al., 2011), due to a stress-induced recrudescence and excretion of Salmonella (Verbrugghe et al., 2011). The sampling of the intestines would in addition imply major logistic difficulties along the slaughter line, particularly the risk of direct carcass contamination. Boughton et al. (2007b) have shown that the caecum may represent a major source of Salmonella contamination at slaughter in the absence of careful evisceration techniques. Also, similar financial efforts as for scenario one would be required, as a result of the necessary sample size.
The examination of the ileocaecal lymph nodes would allow a better – though no absolute – indication of the *Salmonella* prevalence on a herd. Namely, a rapid *Salmonella* infection of both intestinal and associated lymphoid tissues was observed after exposing pigs to a contaminated environment, although this effect was much more distinct in caecal contents than in lymph nodes (Boughton et al., 2007b). The logistic difficulties are less critical in this scenario, as the risk for carcass contamination when sampling lymph nodes is less evident in comparison with the collection of fluid intestinal content. The other advantages of this third scenario are similar to the previous one, as are the financial disadvantages.

Scenario four aims at evaluating both the *Salmonella* prevalence in slaughter pigs and the cross contamination at the slaughterline. For this, the ileocaecal lymph nodes and the surface of the same carcass would be examined concurrently. As no feedback to the individual herds is intended for this scenario, but only an evaluation of the *Salmonella* prevalence at population level, a simulation model showed that 1000-times less samples would be required (Anonymous, 2012). Consequently, this could redirect resources towards prevention and sensitization at both the pre-harvest and harvest level. On the other hand, the withdrawal of the herd classification might decrease the motivation of pig producers to implement *Salmonella* control measures. The Scientific Committee therefore suggested a combined approach of scenario one and four: an annual examination of pooled faecal samples at herd level, plus an extensive investigation of lymph nodes and carcasses at slaughterhouse level. This would obviously increase the examination expenses again, with in addition the issue of false-negative results (especially if only one herd sample per year would be required).

Scenario four appears most promising for national implementation in terms of the practical and financial aspects. The results obtained in this thesis indicate that better prevention measures and an increased sensitization at herd level are indeed important targets to decrease the *Salmonella* burden. However, the combined approach of scenario one and four would imply considerable financial consequences. An increased awareness of pig producers with relation to the negative impact of *Salmonella* on pig performance could remain an incentive and could compensate partially for a possible decreased motivation due to the withdrawal of the herd classification. Instead of the previous combined approach, a concurrent serological examination of blood collected at the slaughterhouse (after stunning) could be applied. Firstly, this is logistically a better way of sampling when compared to pooled faecal samples at farm level, and secondly, it is also a cheaper diagnostic method than bacterial isolation. In addition, a more accurate follow up of the seroprevalence than in the previous years is expected, since only one age category is sampled at the slaughterhouse. In the long term, the comparison of the obtained data from lymph nodes, carcass swabs and blood might provide a better insight in the *Salmonella* epidemiology at population level, and might determine the most cost-efficient diagnostic method for the SAP.
Besides the suggested adaptation of the monitoring for the future *Salmonella* Action Plan, also the current control should and probably will be modified. The focus will not only be on the primary production, but also on other links of the pork production chain. Firstly, the introduction of *Salmonella* via contaminated feed could be avoided through an intensified feed control program. This is of lesser importance in high-prevalence MS like Belgium however, since pig feed mainly becomes an important *Salmonella* source once the environmental contamination by sows or slaughter pigs is reduced to low levels (Hill *et al.*, 2011). Further, the on-farm interventions, such as vaccination, should be implemented primarily on the breeding holdings to decrease the introduction and spread of *Salmonella*-positive pigs in the purchasing herds. Minimising cross contamination during transport (Loynachan *et al.*, 2004; Rostagno *et al.*, 2005) by decreasing stress factors (e.g. high pig density, suboptimal temperatures, commingling of pig batches or herds) and increasing hygiene of the vehicles, is another important point in the chain to be targeted. An intensification of the control efforts at harvest level (i.e. in the lairage (Hurd *et al.*, 2001; De Busser *et al.*, 2011) and at the slaughterline) is also of major significance, since several studies concluded that interventions at the harvest level are more economically profitable than those at herd level (Miller *et al.*, 2005; Goldbach and Alban, 2006; Bollaerts *et al.*, 2010).

In general, the ‘farm-to-fork’ concept implies that the responsibility of controlling *Salmonella* in pork must be shared among the different partners of the entire pork production chain, and should not only lie with the pig producers. Belgium is currently one of the major pork exporters in the EU (next to Denmark, Germany, The Netherlands and Spain) (Anonymous, 2010), and a more integrated approach of the *Salmonella* Action Plan of the whole pork production chain is indispensable to meet with the demands of the market.

### 4 Perspectives for further research: *Salmonella* interventions in pigs.

Several intervention strategies for on-farm use were evaluated in this thesis, which yielded a better insight of their effect on the *Salmonella* infection pressure among pigs, and their applicability in practice. However, remaining questions or new concerns need further investigation.

First of all, relevant data on the costs and benefits associated with the implementation of on-farm interventions against *Salmonella* in pigs are generally lacking. The consortium of the Federal Communications Commission (FCC) published a report in this matter (Anonymous, 2010), in which no significant economic benefit was found from any of the four investigated intervention scenarios. However, several measures that were included in these scenarios, did not have a (reported) beneficial impact on pig performance. Such measures are indeed unlikely to show an economic benefit, unless the costs are shared by different partners of the pork production chain (Friendship *et al.*, 2009). Hence, to convince farmers to invest in *Salmonella* intervention methods, emphasis must be put on the improved
weight gain of the pigs and their resistance to other pathogens, when *Salmonella* prevalence is reduced. Additional cost/benefit analyses with the products of this thesis that did improve the pigs’ weight gain (*i.e.* coated calcium-butyrate in the feed and the commercial vaccine) are therefore advisable.

Further research is also necessary to investigate the potential persistence of the vaccine strain in slaughter pigs and pork. Moreover, the interaction between host and vaccine strain should be determined and safety studies should evaluate the consequences for human health. Whether the occurrence in the field of *Salmonella* wild-type strains with similar characteristics, or the reversion of the vaccine strain’s auxotrophic features might be possible, needs to be the subject in further research.

Once the previous questions become clarified without any remaining concerns for public health, more field trials are recommended to determine the most efficient administration strategy within the framework of the *Salmonella* Action Plan. It should be investigated whether vaccination of both the sows and their offspring obtains the best *Salmonella* reducing effects, or if an approach of vaccinating solely the sows or the piglets, would be satisfactory. As passive immunity against *Salmonella* disappears progressively in piglets (*Schwarz et al.*, 2011), the vaccination of the sows and also their piglets seems a valuable approach to compensate for this infection vulnerability window in weaned piglets. Rösler *et al.* (2010) already showed promising results with this vaccination scheme in a small-scale sow-piglet experiment, indicating the usefulness of field trials in this matter. However, a combination of vaccinating sows and administrating organic acids to weaned piglets, might be a more practical solution on-farm.

As this commercial *Salmonella* Typhimurium vaccine provides little cross-protection against other *Salmonella* serovars (Sven Springer, personal communication 2013), also the investigation of the effect and applicability of autovaccines could be an interesting avenue worth exploring (*Farzan and Friendship*, 2010).

Finally, various feed and water additives are marketed with the claim to reduce *Salmonella* infections in pigs. As described in this thesis, the effect of such products on *Salmonella* prevalence is greatly depending on *i.a.* the formulation, the dosage and the duration of application. Therefore, companies should provide science-based proof of efficacy. For this, legislative directives should be established to specify the requirements for such certificates (*e.g.* *Salmonella*-specific *in vitro, in vivo* and field trials), as is already the case for new vaccines.
Chapter 5 – Summary
1. Summary

*Salmonella enterica* subspecies *enterica* is one of the leading causes of bacterial foodborne disease in the world. Annually, approximately 93.8 million, 95500 and 3200 human salmonellosis cases are reported in the world, in the EU and in Belgium, respectively. The real salmonellosis occurrence in humans is most probably even 10 - 100 times higher, since only a fraction of all infections is officially recorded. Ten years ago, EU Member States were asked to set out targets for *Salmonella* reduction by implementing national control programmes in livestock. The *Salmonella* control programme in poultry (especially the vaccination of the laying hens) has been proven successful in Europe, as a spectacular drop of human *Salmonella* Enteritidis cases has occurred in the EU since its implementation. Recently, 57% and 74% of the reported human cases were estimated to originate from pigs in the EU and Belgium, respectively. This indicates that the main *Salmonella* source for human illness has shifted from poultry to pig production. No less than 8.3% and 18.8% of the pig carcasses in the EU and Belgium were indeed found *Salmonella*-positive, respectively. Chapter 1 describes the occurrence, the epidemiology and symptoms associated with *Salmonella* infections in both humans and pigs.

Despite the many research on *Salmonella* and the implementation of the *Salmonella* Action Plan (SAP) during the past years, little improvements were attained in the *Salmonella* seroprevalence in Belgian pig herds. As Belgium is one of the largest pork-exporting countries in the EU, the pressure is high to meet with the *Salmonella* reduction targets of the importing countries. *Salmonella* infections have in addition an important negative impact on the weight gain of pigs. Therefore, *Salmonella* monitoring and control procedures in the Belgian pig production must urgently be improved. In Chapter 1 an overview is given of current diagnostic methods for *Salmonella* in pigs, of possible control measures on-farm, and of the current *Salmonella* control programme in Belgium and other EU countries.

The general aim of this thesis (Chapter 2) was to investigate and optimize control measures against *Salmonella* infections in pigs at pre-harvest level.

In Chapter 3.1, the effect of three intervention strategies on *Salmonella* Typhimurium transmission in three- to 14-week old piglets was evaluated. The first intervention in this transmission experiment included the use of feed supplemented with 0.3% w/w coated calcium-butyrate. The second intervention strategy comprised oral vaccination with a commercial, attenuated histidine-adenine auxotrophic *Salmonella* Typhimurium strain at three and six weeks of age. The third included the acidification of the drinking water with 0.09% v/v of a mixture of organic acids, to a pH of 3.6-3.8. At eight weeks of age, several piglets of each intervention group were orally challenged with a *Salmonella* Typhimurium strain. To monitor the challenge strain’s transmission towards the other naive contact
animals in the six weeks thereafter, individual blood samples and faecal and tissue samples from all pigs were analysed with an LPS-based ELISA and with the ISO 6579:2002/Amd1:2007 Annex D method, respectively. Transmission was quantified by an ‘adjusted’ reproduction ratio 'R\text{a}' for the bacteriological results.

The mean serum antibody titre of the vaccinated group was significantly higher throughout the experiment, compared to the challenged/untreated positive control group. The lowest proportions of challenge strain-positive faecal and tissue samples were found in the vaccination group and in the group with in-feed coated calcium-butyrate, when compared to the positive control group and the group with acidified water. The vaccination group however, counted as many vaccine strain-positive as challenge strain-positive tissue samples, when using a differentiating biochemical kit provided by the manufacturer of the vaccine. The lowest transmission ratio R\text{a} over all bacteriological matrices was seen in the feed-supplemented group, closely followed by the vaccination group, whereas the positive control group and the acidified water group showed a much higher R\text{a}. The results of this study suggested that vaccination with the commercial *Salmonella* Typhimurium live vaccine and the feed supplementation with the used dosage of coated calcium-butyrate limited *Salmonella* transmission in pigs and might be useful control measures. The acidification of the drinking water with the current acid components and at the applied dosage however, did not appear to provide any protection to the piglets during the supplemented period.

The antibodies induced by the commercial vaccine used in the first transmission experiment were not distinguishable in LPS-based ELISAs from antibodies induced by natural *Salmonella* infections. Therefore, they might interfere with most current European serosurveillance programmes. A second, similar transmission experiment was conducted (Chapter 3.2) to evaluate the protective capacities of an oral ‘DIVA’ vaccine candidate against *Salmonella* Typhimurium infection. This live ‘Salmoporcs*-\text{ΔrfaJ}’ vaccine candidate ‘Differentiates Infected from Vaccinated Animals’, since it lacks LPS-encoding genes and thus induces antibodies which are only detectable in an in-house whole-cell ELISA and not in the commercial LPS-based ELISAs. It was evaluated as a stand-alone measure in one group of piglets and combined with in-feed coated calcium-butyrate in another group.

No significant differences in the *Salmonella*-specific antibody titres were observed between the intervention groups and the positive control group. In the combination group DIVA vaccine + coated calcium-butyrate, significantly less *Salmonella*-positive samples were present in the faeces of the contact animals and in the tissue samples of all pigs, when compared to the positive control group. Both vaccinated groups demonstrated only a non-significantly lower transmission ratio, but confirmed the DIVA capacity of the ‘Salmoporcs*-\text{ΔrfaJ}’ vaccine candidate in the in-house whole-cell ELISA. The
vaccine thus merits further research with larger sample sizes to optimize its use for *Salmonella* programmes.

The effect of one control measure from the two transmission studies was investigated on a larger scale under field conditions (Chapter 3.3). The commercial *Salmonella* Typhimurium live vaccine was selected in this field trial for several reasons. Firstly, the oral vaccination at three and 28 days of age with this vaccine (*i.e.* the most recent vaccination protocol) was considered an easier and more practical intervention candidate to investigate than the administration of organic acids to water or feed. Secondly, certain results of our first transmission experiment needed further investigation in the field, namely the promising reduction of *Salmonella* excretion on the one hand, and the possible persistence of vaccine-induced antibodies and the vaccine strain itself on the other hand. In addition, although currently not registered in Belgium, this vaccine is readily available for use in primary production, whereas the evaluated DIVA vaccine candidate is not. The field trial was conducted in three *Salmonella* Typhimurium-positive farrow-to-finish pig herds. Vaccinated pigs were compared with non-vaccinated ones from suckling till slaughter age, by collecting data on the weight gain, and serological and bacteriological parameters.

At the end of the fattening period, a significantly lower *Salmonella*-specific antibody titre and significantly less *Salmonella*-excreting pigs were observed in the vaccinated group over all herds, compared to the control group. The vaccinated animals of one herd showed additionally significantly less *Salmonella*-positive samples in the lymph nodes, while this was the case for caecal contents in another herd. In the vaccinated group of each herd, the daily weight gain between three days and 29 weeks of age was significantly higher than in the non-vaccinated control group. These results indicate that the current vaccination strategy decreased the overall *Salmonella* infection pressure. However, the serological and bacteriological results differed largely between the three herds throughout the trial. This points out that different measures targeting all components of the farm system are indispensable, and that all pigs in a compartment or herd should be vaccinated to improve the overall vaccination effect. Another important finding was the isolation in all three herds of a *Salmonella* Typhimurium strain with the same auxotrophic characteristics and genotype as the vaccine strain, from faecal, lymph node and caecal content samples. Further research on this isolated strain is warranted.

Based on the results of both transmission studies, the field trial and literature findings, several conclusions regarding control measures on-farm are discussed in Chapter 4. The beneficial effects of the organic acid administration on *Salmonella* (sero)prevalence may be influenced by different factors, such as the formulation, the dosage, the application route (feed or drinking water), the treatment duration, and the age of the treated pigs. Further, it was concluded that vaccination with the commercial
Salmonella Typhimurium live vaccine decreases the Salmonella infection pressure on-farm and improves the daily weight gain. However, the observed herd variability in the serological and bacteriological effect makes it difficult to predict to what extent this vaccination would decrease human salmonellosis in the EU. Moreover, clarifications regarding the possible vaccine strain persistence are indispensable, since this might have important implications for bacteriological monitoring programmes, and might be a possible public health concern. The experimental ‘Salmoporc-ΔrfaJ’ vaccine appeared to give little protection against Salmonella transmission in piglets after weaning. Its DIVA capacity merits further research however, to improve its efficacy.

The feasibility in practice of these intervention strategies were described in Chapter 4 as well. The costs of control measures like vaccination or the administration of organic acids, may be considered too high by pig producers compared to the benefits of a better pig performance. Therefore, without governmental support, too few pig producers might voluntarily implement these control measures.

Not only Salmonella control, but also the monitoring procedures in Belgium need adaptations in the near future. In Chapter 4, the (dis)advantages of the possible scenarios for optimizing the current SAP are discussed, as are the perspectives for further research, based on our experiments.

Several possible interventions are presented in this thesis, but the future for Salmonella control will involve a continuous exploration of new control measures, together with the maintenance of high biosecurity levels. In order to tackle the Salmonella problem in swine and humans effectively, this approach must be applied not only in primary production, but in all links of the pork production chain.
2. Samenvatting

*Salmonella enterica* subspecies *enterica* is één van de voornaamste oorzaken van bacteriële voedselgerelateerde ziekten ter wereld. Jaarlijks worden er wereldwijd, in de EU en in België respectievelijk ongeveer 93,8 miljoen, 95500 en 3200 humane salmonellose-gevallen gerapporteerd. Het echte voorkomen van salmonellose bij mensen is naar alle waarschijnlijkheid zelfs 10 tot 100 keer hoger, aangezien slechts een fractie van alle infecties officieel gemeld wordt. Tien jaar geleden werd aan de EU lidstaten gevraagd om targets te stellen voor een *Salmonella* reductie, via het implementeren van nationale controleprogramma’s in de veestapel. Het *Salmonella* controleprogramma voor pluimvee (voornamelijk via vaccinatie van de leghennen) is succesvol gebleken in Europa, na de waargenomen spectaculaire daling in het aantal humane *Salmonella* Enteritidis gevallen in de EU sinds de implementatie ervan. Recent werd 57% en 74% van de gerapporteerde humane salmonellose-gevallen in respectievelijk de EU en België toegewezen aan varkens. Dit wijst er op dat de belangrijkste *Salmonella*-bron voor humane ziekte zich verplaatst heeft van de pluimvee- naar de varkensindustrie. Maar liefst 8,3% en 18,8% van de varkenskarkassen in respectievelijk de EU en België werden inderdaad *Salmonella*-positief bevonden. **Hoofdstuk 1** beschrijft het voorkomen, de epidemiologie en symptomen geassocieerd met *Salmonella* infecties in mensen en varkens.

Ondanks het vele onderzoek naar *Salmonella* en de implementatie van het *Salmonella* Actie Plan (SAP) tijdens de voorbije jaren werd er weinig verbetering gezien in de *Salmonella* seroprevalentie in de Belgische varkensbedrijven. Aangezien België één van de grootste varkensvlees-exporterende EU-landen is, is de druk groot om te voldoen aan de eisen van de importerende landen omtrent *Salmonella*-reductie. *Salmonella*-infecties hebben bovendien een belangrijke negatieve impact op de gewichtsaanzet van varkens. Daarom dient de *Salmonella*-monitoring en -controle in de Belgische varkensproductie dringend verbeterd te worden. In **Hoofdstuk 1** is een overzicht gegeven van de huidige diagnostische methoden voor *Salmonella* in varkens, van mogelijke controlemaatregelen op het bedrijf, en van de huidige *Salmonella* controle programma’s in België en andere EU landen.

Het algemene doel van deze thesis (**Hoofdstuk 2**) was het onderzoeken en optimaliseren van controlemaatregelen tegen *Salmonella*-infecties in varkens op het bedrijf.

In **Hoofdstuk 3.1** werd het effect geëvalueerd van drie interventiestrategieën op de transmissie van *Salmonella* Typhimurium in drie- tot 14-weken oude biggen. De eerste interventie in dit transmissie experiment omvatte het gebruik van 0,3% m/m gecoot calcium-butyraat als voedersupplement. De tweede interventiestrategie bestond uit een orale vaccinatie met een commerciële, verzwakte histidine-adenine-auxotroof *Salmonella* Typhimurium stam op drie en zes weken leeftijd. De derde interventie was een drinkwateraanzuring tot een pH van 3,6-3,8 d.m.v. een 0,09% vol organische-zuren-
mengeling. Op acht weken leeftijd werden enkele biggen van elke interventiegroep oraal geïnfecteerd met een *Salmonella Typhimurium* stam. De transmissie van deze infectiestam naar de naïeve contactdieren werd opgevolgd in de zes weken nadien d.m.v. een LPS-ELISA van individuele bloedstalen en d.m.v. de ISO 6579:2002/Amd1:2007 Annex D analyse van stalen van faeces en weefsel bij alle varkens. Transmissie werd gekwantificeerd via een ‘aangepaste’ reproductie-Ratio ‘$R_a$’ voor de bacteriologische resultaten.

De gemiddelde serum-antistoffentiter van de gevaccineerde groep was significant hoger doorheen het experiment, in vergelijking met de geïnfecteerde/onbehandelde positieve-controlegroep. De laagste proporties van infectiestam-positieve faecale en weefselstalen werden gevonden in de vaccinatiegroep en in de groep met gecoat calcium-butyraat in het voeder, in vergelijking met de positieve-controlegroep en de groep met aangezuurd drinkwater. De vaccinatiegroep telde echter evenveel vaccinstam-positieve als infectiestam-positieve weefselstalen, wanneer een differentiërende biochemische kit van de vaccinfabrikant gebruikt werd. De laagste $R_a$ over alle bacteriologische matrices heen werd genoteerd in de voedersupplementgroep, kort gevolgd door de vaccinatiegroep, terwijl de positieve-controlegroep en de groep met aangezuurd drinkwater een veel hogere $R_a$ vertoonden. De resultaten van deze studie suggereerden dat de vaccinatie met dit commerciële, levende *Salmonella Typhimurium* vaccin en de voedersupplementatie met de gebruikte dosis gecoat calcium-butyraat de *Salmonella*-transmissie in de biggen limiteerden en nuttige controlemaatregelen zouden kunnen zijn. De aanzuring van het drinkwater met deze zuurcomponenten aan de toegepaste dosis leek de biggen echter geen bescherming te bieden gedurende de behandelde periode.

De antistoffen geïnduceerd door het commerciële vaccin, dat in de eerste transmissiestudie gebruikt werd, zijn in LPS-ELISA’s niet te onderscheiden van de antistoffen geïnduceerd door natuurlijke *Salmonella*-infecies. Daardoor kunnen deze eerste interfereren met de meeste huidige Europese serosurveillance-programma’s. Een tweede, gelijkaardig transmissie-experiment werd uitgevoerd (Hoofdstuk 3.2) om de beschermende capaciteiten te evalueren van een orale ‘DIVA’-vaccinkandidaat tegen *Salmonella Typhimurium*-infecies. Dit levende ‘Salmoporc-ΔrfaJ’ vaccin differentieert geïnfecteerde van gevacineerde dieren, aangezien het LPS-coderende genen mist en dus antistoffen induceert die niet detecteerbaar zijn in de commerciële LPS-ELISA’s, maar wel in een in-house vollekiem-ELISA. Het vaccin werd geëvalueerd als alleenstaande maatregel in één groep biggen en gecombineerd met gecoat calcium-butyraat gesupplementeerd voeder in een andere groep.

Er werden geen significante verschillen gevonden in de *Salmonella*-specifieke antistoffentiters van de interventiegroepen en de positieve-controlegroep. In de combinatiegroep DIVA + gecoat calcium-butyraat waren significant minder *Salmonella*-positieve stalen aanwezig in de faeces van de contactdieren en in de weefselstalen van alle dieren in vergelijking met de positieve-controlegroep.
Beide gevaccineerde groepen vertoonden slechts een niet-significant lagere transmissieratio, maar bevestigden wel de DIVA-capaciteit van de ‘Salmoporc-ΔrfaJ’ vaccinkandidaat in de in-house vollekiem-ELISA. Verder onderzoek met stataalnames op grotere schaal zijn dus vereist om het gebruik van het vaccin in Salmonella programma’s te optimaliseren.

Het effect van één controlemaatregel uit de twee transmissiestudies werd onderzocht op grotere schaal onder voldemortstandigheden (Hoofdstuk 3.3). Het commerciële, levende Salmonella Typhimurium-vaccin werd geselecteerd voor deze veldstudie omwille van verschillende redenen. Ten eerste werd de orale vaccinatie op drie en 28 dagen leeftijd met dit vaccin (i.e. het meest recente vaccinatieprotocol) beschouwd als een gemakkelijkere en praktischere interventie om te evalueren dan de toediening van organische zuren via drinkwater of voeder. Ten tweede vereisten bepaalde resultaten van het eerste transmissie-experiment verder onderzoek in het veld. Dit betreft zowel de veelbelovende reductie van de Salmonella-excretie als de mogelijke persistentie van vaccin-geïnduceerde antistoffen en de vaccinstam zelf. Dit vaccin is bovendien, hoewel nog niet geregistreerd in België, wel onmiddellijk beschikbaar voor gebruik in de primaire productie, hetgeen niet geldt voor de geëvalueerde DIVA-vaccinkandidaat. De veldstudie werd uitgevoerd op drie Salmonella Typhimurium-positieve gesloten varkensbedrijven. Gevaccineerde varkens werden van zoogetot slachtleeftijd vergeleken met niet-gevaccineerde varkens via het verzamelen van gegevens over de gewichtsaanzet en over serologische alsook bacteriologische parameters.

Aan het einde van de mestperiode werd er een significant lagere Salmonella-specifieke antistoffentiter waargenomen in de gevaccineerde groep over alle bedrijven heen, alsook significant minder excreterende varkens in vergelijking met de controlegroep. De gevaccineerde dieren van één bedrijf vertoonden daarbij ook significant minder Salmonella-positieve stalen in de lymfeknopen, terwijl dit het geval was voor de caecuminhoud van een ander bedrijf. In de vaccinatiegroep van elk bedrijf was de dagelijkse gewichtsaanzet tussen drie dagen en 29 weken leeftijd significant hoger dan in de controlegroep. Deze resultaten indiceren dat de gebruikte vaccinatiestrategie de algemene Salmonella-infectiedruk verlaagde. De serologische en bacteriologische resultaten varieerden echter sterk tussen de drie bedrijven doorheen de studie. Dit wijst er op dat verschillende maatregelen, die alle bedrijfscponenten aanpakken, onmisbaar zijn, en dat alle varkens in een compartiment of bedrijf gevaccineerd zouden moeten worden om het algemene vaccinatie-effect te verbeteren. Een bijkomende belangrijke bevinding was de isolatie van een Salmonella Typhimurium stam uit stalen van faeces, lymfeknopen en caecuminhoud in de drie bedrijven met dezelfde auxotrofe eigenschappen en hetzelfde genotype als de vaccinstam. Verder onderzoek naar deze geïsoleerde stam is aan te raden.
Gebaseerd op de resultaten van beide transmissiestudies, de veldproef en literatuurbevindingen, werden er in Hoofdstuk 4 meerdere conclusies besproken betreffende controlemaatregelen op het bedrijf. De gunstige effecten van de toediening van organische zuren op Salmonella (sero)prevalentie kunnen beïnvloed worden door verschillende factoren, zoals de formulatie, de dosering, de toedieningsweg (voeder of drinkwater), de behandelingsduur, en de leeftijd van de varkens. Voorts werd er geconcludeerd dat vaccinatie met het commerciële, levende Salmonella Typhimurium vaccin de Salmonella-infectiedruk op het bedrijf verlaagt en de dagelijkse gewichtsaanzet verbetert. De waargenomen bedrijfsvariabiliteit in de serologische en bacteriologische resultaten maakt het echter moeilijk om te voorspellen in hoeverre deze vaccinatie de humane salmonellose zou kunnen verlagen in de EU. Verklaringen omtrent de mogelijke persistentie van de vaccinstam zijn bovendien noodzakelijk, aangezien dit belangrijke implicaties kan hebben voor bacteriële monitoringprogramma’s, en ook een mogelijke probleem vormt voor de volksgezondheid. Het experimentele ‘Salmonoporc-ArfαJ’ vaccin leek weinig bescherming te bieden tegen Salmonella-transmissie in gespeende biggen. Zijn DIVA-capaciteiten moedigen verder onderzoek echter aan om de efficaciteit te verbeteren.

Ook de haalbaarheid in de praktijk van deze interventiestrategieën werd beschreven in Hoofdstuk 4. De kosten van controlemaatregelen zoals vaccinatie of de toediening van organische zuren zouden te hoog beschouwd kunnen worden door de varkensproducenten, in vergelijking met de baten van betere varkensprestaties. Zonder overheidssteun zullen wellicht te weinig varkensproducenten deze controlemaatregelen vrijwillig implementeren.

Niet alleen de Salmonella-controle, maar ook de Salmonella-monitoringsmethodes in België dienen aangepast te worden in de nabije toekomst. In Hoofdstuk 4 werden de voor- en nadelen besproken van de mogelijke scenario’s om het huidige SAP te optimaliseren, alsook de perspectieven voor verder onderzoek, gebaseerd op onze experimenten.

Verschillende mogelijke interventies werden voorgesteld in deze thesis, maar de toekomstige Salmonella-controle zal een voortdurende exploratie van nieuwe controlemaatregelen vragen, samen met de handhaving van hoge bioveiligheidsnormen. Om het Salmonella-probleem in varkens en mensen efficiënt aan te pakken, dient deze benadering bovendien niet alleen in de primaire productie toegepast worden, maar in alle schakels van de varkensvleesproductieketen.
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1. Curriculum Vitae


In december 2009 vatte ze haar doctoraatsonderzoek aan op de afdeling Bacteriologie van het Centrum voor Onderzoek in de Diergeneeskunde en Agrochemie (CODA) te Ukkel, getiteld ‘Optimalisatie van de bestrijding van Salmonella-infecties bij varkens’ en met als acroniem ‘SalmoSu’. Dit project werd gefinancierd door de Federale OverheidsDienst Volksgezondheid, Veiligheid van de Voedselketen en Leefmilieu (FOD VVVL), en verliep in samenwerking met de Dierengezondheidszorg Vlaanderen (DGZ) en het Instituut voor Landbouw- en Visserij-Onderzoek (ILVO). Na twee jaar van experimenteel onderzoek in zowel het laboratorium te Ukkel als het dierproefcentrum te Machelen, voerde ze de tweede helft van dit project voor het CODA uit op de faculteit Diergeneeskunde, in de Vakgroep Voorplanting, Verloskunde en Bedrijfsbegeleiding. Daar was ze ook betrokken bij opleiding van de studenten Diergeneeskunde in hun laatste Masterjaar.

Lotte De Ridder is (mede)auteur van meerdere wetenschappelijke publicaties en ontving in 2013 de Junior Scientist Prize van The Veterinary Journal voor beste paper van het jaar van recent-afgestudeerde wetenschappers. Ze nam deel aan verschillende (inter)nationale congressen waar zij haar onderzoeksresultaten toelichtte aan de hand van posters en presentaties.
2. Bibliography

Scientific publications


De Ridder L., Maes D., Dewulf J., Butaye P., Pasmans F., Boyen F., Haesebrouck F. and Van der Stede Y. (2013) Usefulness of a live Salmonella Typhimurium vaccine to control Salmonella infections on farrow-to-finish pig herds. Vaccine. (Submitted)

Oral presentations at (inter)national conferences


Poster presentations at (inter)national conferences


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Dankwoord

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