“Knowledge is like a baobab tree – one person’s arms are not enough to encompass it.”

(African proverb)

Vakgroep Veterinaire Volksgezondheid en Voedselveiligheid
Vakgroep Voortplanting, Verloskunde en Bedrijfsdiergeneeskunde

CONTROL OF *SALMONELLA* IN THE PORK PRODUCTION CHAIN

BEHEERSING VAN *SALMONELLA* IN DE PRODUCTIEKETEN VAN VARKENSVLEES

Proefschrift voorgedragen tot het behalen van de graad van Doctor in de Diergeneeskundige Wetenschappen aan de Faculteit Diergeneeskunde, Universiteit Gent

door

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19 december 2012

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ISBN: 9789058643223

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LIST OF ABBREVIATIONS

BPW	Buffered Pepton Water
DFD	Dark, Firm and Dry
DIASSALM	Diagnostic Semi-Solid *Salmonella* agar
DIVA	Differentiation of Infected and Vaccinated Animals
EFSA	European Food Safety Authority
ELISA	Enzyme Linked Immunosorbent Assay
ERIC	Enterobacterial Repetitive Intergenic Consensus
EU	European Union
FASFC	Federal Agency for the Safety of the Food Chain
HACCP	Hazard Analysis and Critical Control Point
IFC	Information of the Food Chain
ISO	International Organization for Standardization
LPS	Lipopolysaccharide
MKTTn	Muller Kauffmann Tetrathionate novobiocin broth
MS	Member States
MSRV	Modified Semisolid Rappaport Vassiliadis agar
NRSS	National Reference Centre for *Salmonella* and *Shigella*
OD	Optical Density
OIE	Office International des Epizooties
OR	Odds Ratio
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
RV	Rappaport Vassilisadis broth
RVS	Rappaport Vassiliadis broth with Soya
SAP	*Salmonella* Action Plan
SMS	Simple Method *Salmonella*
S/P	Sample to Positive ratio
SSAP	*Salmonella* Specific Action Plan
TSB	Tryptone Soya Broth
TSI	Triple Sugar Iron agar
XLD	Xylose Lysine Desoxycholate agar
YOPI	Young, Old, Pregnant and Immunocompromised

PREFACE

On average, 28% of all human cases of salmonellosis in the EU can be linked to the consumption of pork. This makes pork, besides eggs, the second most important contamination source for human salmonellosis in the European Union (European Food Safety Authority, 2011). In Belgium, however, 73.7% of the foodborne human salmonellosis cases can be attributed to the consumption of pork (Pires et al., 2011). The most common *Salmonella* serotypes found in humans are Typhimurium (62.8%) and Enteritidis (14.9%) (NRSS, 2011).

Pigs infected with *Salmonella* Typhimurium usually do not show clinical symptoms, but they may shed the bacteria in the feces. Shedding occurs continuously or intermittently and infected pigs can become carriers. Carriers can start shedding *Salmonella* bacteria following many different stress factors such as commingling, food deprivation, transport, etc. In this way, they contribute to contamination of the environment. At slaughter, pigs carrying or shedding *Salmonella* pose directly or indirectly a threat to human health through contamination of the carcass.

To lower the risk of foodborne infections in humans, the European Commission has set deadlines for its Member States (MS) to initiate *Salmonella* surveillance programs in different livestock species, including pigs (Anonymous, 2003). Before July 2009, surveillance and control programs had to be established in every MS to control *Salmonella* in pigs at the pre-harvest stage.

As there is currently no easy, practical and economical control measure to eradicate *Salmonella* from all types of pig herds, several possible control options have been proposed. These include acidifying feed and drinking water, feeding meal instead of pellets, adapting the purchase policy as well as improving hygiene and biosecurity. The actual benefit of these control measures to control *Salmonella* is difficult to measure.

Not only the primary production has to be targeted. Also at the slaughterhouse, several critical steps occur that might increase the risk for *Salmonella* contamination.

An integrated approach from “farm-to- fork” is necessary to achieve the ultimate goal of reducing contaminated pork and the number of human salmonellosis.

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CHAPTER 1

GENERAL INTRODUCTION

Modified from: *Salmonella* control in live pigs and at slaughter: a review. Conditionally accepted in The Veterinary Journal (2012)

GENERAL INTRODUCTION

1. Taxonomy and characteristics of *Salmonella*

The genus *Salmonella* encompasses Gram-negative, motile, non-spore forming, facultative anaerobic bacilli with peritrichous flagella belonging to the family of the Enterobacteriaceae. Currently the genus *Salmonella* is divided into two species: *Salmonella enterica* and *Salmonella bongori* (Guibourdenche, et al., 2010).

The species *Salmonella enterica* consists of six subspecies: *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae* and *indica* whereas no subspecies has been assigned to *Salmonella bongori* (Su and Chiu, 2007). Based on the combination of bacterial surface-antigens, the genus *Salmonella* is subdivided into 2,541 serovars (also called serotypes). For convenience, the serovars are denominated by genus and serovar only (e.g. *Salmonella enterica* subspecies *enterica* serovar Typhimurium is called *Salmonella* Typhimurium). According to Popoff *et al.* (2004), 1,504 serovars belong to *Salmonella enterica* subspecies *enterica*. Though all *Salmonella* serovars are considered potentially pathogenic for humans, the degree of host adaptation varies, which affects the pathogenicity.

Salmonellae are hardy and ubiquitous bacteria multiplying at 7-45°C with a pH range from 4.0 to 9.5 (Ekperigin et al., 1998). The organism has all the necessary tools to ensure a wide distribution: they have many reservoir hosts, are efficiently shed from carrier animals, persist easily in the environment and take effective use of transmission vectors (feed, fomites, vehicles,…).

1.1 *Salmonella* in humans

Salmonellosis is with 99,020 confirmed cases in the European Union (EU) in 2010, the second most common reported zoonosis (following campylobacteriosis) (European Food Safety Authority (EFSA), 2012). The global human health impact of non-typhoidal *Salmonella* is estimated at 93,8 million illnesses, of which an estimated 80,3 million are foodborne, and 155,000 deaths each year (Majowicz, 2010). In Belgium, 3,231 human *Salmonella* cases were reported in 2011 to the National Reference Centre for *Salmonella* and *Shigella* (NRSS, 2011). This number however, only represents a fraction of the total community cases as some criteria has to be fulfilled before official registration takes place. An ill person must seek medical care, submit a specimen, the laboratory must test for

Salmonella and report a positive finding. Further, the laboratory confirmed infection must be ascertained by public health authorities.

The reservoir for salmonellae is the intestinal tract of endo- and ectothermic animals leading to a whole variety of foodstuffs of both animal and plant origin that might directly or indirectly be contaminated. Transmission occurs when salmonellae are introduced in food preparation areas and are allowed to multiply in the produced food, *e.g.* due to inadequate storage temperatures, inadequate cooking or cross contamination of food. The organism can also be transmitted through direct contact with fecal contaminated environments, infected animals or humans.

Figure 1 shows the evolution of *Salmonella* Typhimurium and *Salmonella* Enteritidis isolated from human cases (number of cases/year) in Belgium during the period 1980-2011 (NRSS, 2011). Human *Salmonella* Enteritidis cases are most commonly associated with the consumption of contaminated eggs and poultry, while *Salmonella* Typhimurium cases are most commonly associated with the consumption of contaminated pig, poultry and bovine meat. In 2005, a commercial combination vaccine against *Salmonella* Typhimurium and *Salmonella* Enteritidis was registered for poultry. Since 2007 vaccination of laying hens became mandatory (Anonymous, 2007a) and resulted in a major decrease of the number of *Salmonella* Enteritidis cases in humans. In 2011, *Salmonella* Typhimurium was isolated most frequently (62.8%), followed by the serotype Enteritidis (14.9%).

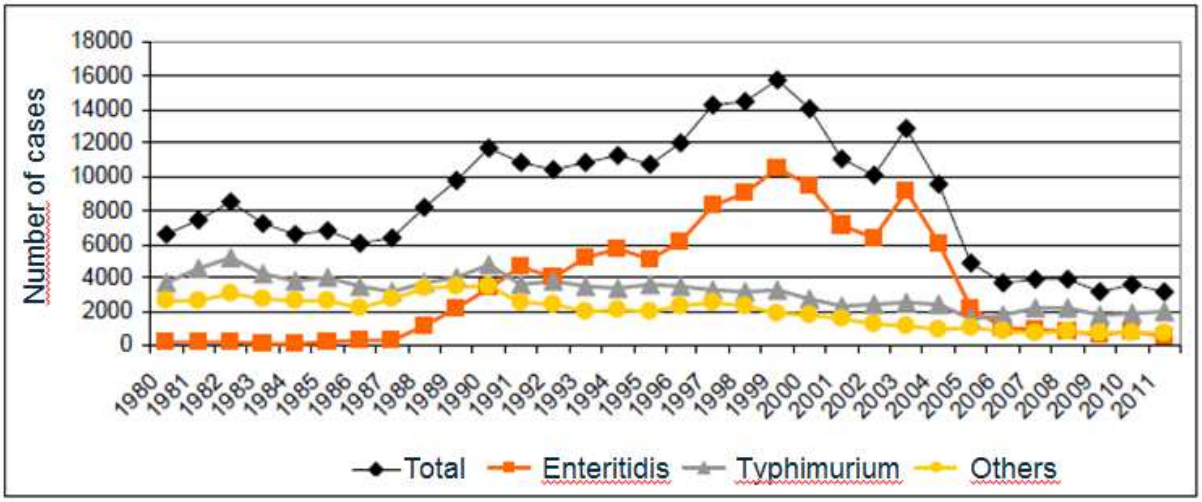

Figure 1: The number of identified human salmonellosis cases (number of cases/year) in Belgium from 1980-2011 (NRSS, 2011).

Human salmonellosis is usually characterized by fever, abdominal pain, diarrhea, nausea and sometimes vomiting after an incubation period of 12-36 hours. The illness usually lasts 4–7 days, and most persons recover without treatment. However, in some patients as the very young and the elderly, the infection can be more severe and the associated dehydration can be life-threatening. When the bacteria enter the bloodstream and cause systemic infection, effective antimicrobials are essential for treatment.

The most susceptible population group for a *Salmonella* infection is the YOPI group (Young, Old, Pregnant and Immunocompromised). In Belgium, 38% of all registered salmonellosis cases occurred in children younger than 5 years (NRSS, 2011). During July-September a seasonal increase of human cases is noticed.

Further, in 2011, an increased antimicrobial resistance was noticed in the serotypes Typhimurium and Hadar. Multi-drug resistance (≥ 4 of the 14 tested antimicrobials) was shown in 46.9% and 50% of the human isolates, respectively. In contrast, 93.1% of the tested isolates of the serotype Enteritidis were susceptible for all antimicrobials tested (NRSS, 2011).

1.2 *Salmonella* in pigs

Pathogenesis of Salmonella *infection*

The transmission of *Salmonella* mainly occurs via the fecal-oral route, but nose-to-nose transmission by contaminated oral-pharyngeal secretions is also possible (Oliveira et al., 2007). Transmission over short distances may be possible through contaminated aerosols, feces and dust particles (Griffith et al., 2006).

During ingestion, *Salmonella* enters the tonsils and persists in the tonsillar crypts and on the tonsillar epithelium (Horter et al., 2003). The mode of tonsil colonization and persistence seems to be very different from intestinal colonization and persistence and is mediated by tissue-specific gene expression (Boyen et al., 2006; Van Parys et al., 2011).

Following ingestion, *Salmonella* enters the stomach where an acidic environment exists. Due to the production of acid shock proteins, *Salmonella* is able to resist to a pH as low as 2.3 (Viala et al., 2011). The presence of feed can further act as a buffer, increasing the chance that bacteria reach the gut (Mikkelsen et al., 2004).

After reaching the distal part of the intestines, *Salmonella* Typhimurium attaches to the intestinal mucosa by using adhesins, such as type 1 fimbriae (Althouse et al., 2003).

Following adhesion, *Salmonella* penetrates the mucosa and invades the intestinal epithelium. In the lamina propria, *Salmonella* is phagocytized by macrophages in which the bacteria can survive, multiply and spread into organs such as Peyer's patches and gastric, hepatic, jejunal and ileocecal lymph nodes (Tam et al., 2008). It was demonstrated that two hours after oral inoculation, *Salmonella* Typhimurium was already found within porcine enterocytes and mesenteric lymph nodes (Reed et al., 1986).

After infection, the pathogen can remain in the tonsils, gastrointestinal tract and gut-associated lymphoid tissue (Wood et al., 1989; Vieira-Pinto et al., 2005; Boyen et al., 2008a; Scherer et al., 2008). These so-called carrier animals do not show clinical signs of infection, but intermittently shed *Salmonella* in the feces. In live animals, this carrier state is difficult to detect and might therefore bias monitoring programs. During periods of stress, re-excretion can occur, leading to contamination of the environment. Especially in the pre-salughter environment, this may lead to *Salmonella* transmission to non-infected pigs and increase the risk of carcass contamination.

Both the intestinal and the systemic phase of infection are regulated by genes on pathogenicity islands, which are clusters of genes that encode virulence factors involved in different stages of *Salmonella* pathogenicity (Blondel et al., 2009). Especially *Salmonella* Pathogenicity Island 1 (SPI-1) and 2 (SPI-2) are important for bacterial penetration of the epithelial cells of the intestine (Boyen *et al.*, 2006; Lara-Tejero *et al.*, 2009) and survival and proliferation in porcine macrophages (Boyen et al., 2008a). Van Parys et al. (2012) recently showed that *Salmonella* Typhimurium downregulates the expression of major histocompatibility complex class II molecules (MHC II) on porcine macrophages. The extent of downregulation differed among *Salmonella* strains, indicating that besides SPI-1 and SPI-2 also other factors are involved in this *Salmonella* induced downregulation of MHC II expression (Van Parys et al., 2012). Verbrugghe et al. (2011) demonstrated that stress-induced excretion of *Salmonella* is correlated with increased serum cortisol. Cortisol further promotes intracellular proliferation of *Salmonella* Typhimurium in porcine macrophages which is caused by an indirect effect through the cell (Verbrugghe et al., 2011).

Atlhough recent studies carried out in pigs improved the knowledge on the pathogenesis of *Salmonella* infections in pigs, further research on this topic remains necessary.

Salmonella *epidemiology and monitoring at primary production*

In 2010, 6,4 million pigs were present in Belgium. Of these 94% are situated in Flanders. Although the number of pig herds has decreased over the past ten years, the number of pigs per herd has increased from 789 pigs/herd in 2000 towards 1174 pigs/herd in 2010 (Anonymous, 2011).

As a result of the EU Regulation No 2160/2003, the Belgian Federal Agency for the Safety of the Food Chain (FASFC) implemented in January 2005 a national *Salmonella* sero-surveillance and control program in fattening pigs (farms with ≥ 31 fattening pigs) called *Salmonella* Action Plan (SAP). The aim was to designate the top ten percent of herds with a high risk for *Salmonella* based on the mean level of *Salmonella* specific antibodies detected in blood samples taken in the context of the eradication and monitoring program for Aujeszky's disease (Anonymous, 1999). A maximum of 12 pigs of different weight categories (≤ 40kg, 40-59kg, 60-79kg and ≥ 80kg) are sampled randomly by the herd veterinarian every 4 months. If the mean Sample to Positive (S/P) ratio of the herd is above 0.6 during three consecutive sampling rounds, the herd is designated as a high risk herd. Since July 2007, these risk herds are required to take part in a *Salmonella* Specific Action Plan (SSAP) consisting of bacteriological examination in pens of different age groups, filling in a checklist to detect risk factors and implementing herd specific control measures designated to reduce the risk of *Salmonella* infection (Anonymous, 2007b). When the mean S/P ratio drops below 0.6 the herd is withdrawn from the high risk status. Table 1 shows the number of pig herds designated as a risk herd from 2007 onwards as well as the number of pig herds that were not able to be withdrawn from the program.

Table 1: Number of high risk herds and herds relapsed in Belgium in the period 2007-2012 (Brossé, Diergezondheidszorg Vlaanderen, personal communication, 2012)

Year	Total risk herds	Risk herds for the first time	Herds relapsed
2007	269	269	-
2008	426	385	41
2009	314	228	85
2010	156	107	49
2011	114	83	31
2012 (until June)	56	37	19

Since January 2008, the farmer has to fill in the document 'Information of the Food Chain" (IFC) and send it to the slaughterhouse 24h before the delivery of the pigs. This document contains information about the health status and the use of veterinary medication of the delivered slaughter batch (Anonymous, 2004a). Farmers also have to mention whether the herd is a *Salmonella* risk herd and report the latest mean S/P ratio. In this way, the operator of the slaughterhouse can decide to take preventive measures to avoid or minimize contamination and cross contamination.

A critical evaluation of the Belgian SSAP showed that herds recovered more slowly from their high risk status before the SSAP was implemented (period 2005-2007) than afterwards. However, results also demonstrated that 29% of the herds were withdrawn from the program due to sampling error (Méroc et al., 2012).. The importance of implementing control measures at the level of the sows was further addressed by Méroc et al. (2012) as closed pig herds were at higher risk compared to herds with fattening pigs and mixed herds Vangroenweghe et al. (2010) investigated the serological status of sows in herds designated as risk herds as well as in "non-risk" herds. The results of that study (Table 2) showed that sows at risk herds had a significantly higher mean S/P ratio compared to sows at non-risk herds. In general, *Salmonella* antibodies were detected in 98.7% of the sows and using a cut-off S/P value of 0.6, 63.6% of the sows were *Salmonella* positive.

Table 2: Number of sampled sows (blood) per herd category and their mean S/P value (Vangroenweghe et al., 2010)

	Number of sows	Mean S/P ratio ± S.E.M.
Risk farms	583	$1.14^{a} \pm 0.03$
Non-risk farms	555	$0.70^{b} \pm 0.02$
Total	1138	0.92 ± 0.02

The importance of the sow in maintenance and spread of *Salmonella* in the herd has also been demonstrated by other authors (Nollet et al., 2005a; EFSA, 2010; Hill et al., 2011).

A baseline survey on the prevalence of *Salmonella* in breeding and production holdings in the EU was carried out (EFSA, 2009) by sampling selected holdings through the use of fresh voided pooled fecal samples. Samples were tested by the National Reference Laboratory (or an authorized laboratory) using the latest ISO 6579 Annex D method

(International Organization for Standardization (ISO), 2007). At EU level, 28.7% of the breeding holdings were *Salmonella* positive. Among the Member States (MS) a wide variation (0-64.0%) was noticed. The EU prevalence of the production holdings was 33.3%, with a variation of 0-55.7% among the MS (EFSA, 2009). At country level, there was a strong and significant positive association between the *Salmonella* prevalence at breeding and production holdings (EFSA, 2011). In Belgium, 18.8% of the sampled breeding holdings (n=16) (Fig. 2a) and 36.4% of the sampled production holdings (n=209) (Fig. 2b) were *Salmonella* positive (EFSA, 2009).

Hill et al. (2011) concluded that MS with high breeding herd prevalence (i.e. > 10-15% of breeding herds are infected with *Salmonella*) must tackle the breeding herd as part of any national control program in order to achieve a significant reduction in national slaughter pig prevalence.

Although a weak agreement between serology (serum and meat juice) and bacteriology has been shown by several authors (Nollet et al., 2005b; Korsak et al., 2006; Methner et al. 2011; Visscher et al., 2011), most European *Salmonella* surveillance programs are based on the detection of antibodies against *Salmonella* (Cortinas Abrahantes et al., 2009). Nollet et al. (2005b) found a weak agreement between serology (blood) and bacteriological examination of the lymph nodes of the same pigs. In the group of pigs that were *Salmonella* positive on culture, only 34.5% (cut-off 40%) or 82.8% (cut-off 10%) were seropositive. Having serologically negative animals is thus no guarantee that *Salmonella* is not present. Increasing the sample size however can influence this agreement. To classify all culture positive herds as serological positive, Nollet et al. (2005b) stated that minimum 20 samples per herd have to be taken. Other authors (Laevens and Mintiens, 2005; Snary, 2010) reported that at least 40 pigs of 16 weeks or older are needed to improve the correlation between serology and bacteriology at herd level.

Korsak et al. (2006) and Visscher et al. (2011) also demonstrated a weak concordance between serologic results obtained from meat juice and bacteriological results. Results of Methner et al. (2011) showed that only 3.4% of all *Salmonella* positive pigs, identified after bacteriological examination of tonsils, ileocaecal lymph nodes, caecal and rectum content at slaughter, also showed a positive meat juice result. Detection of antibodies is therefore only useful to verify whether pig herds were previously exposed to *Salmonella*, but the *Salmonella* status of individual pigs at slaughter and the associated risk of dissemination can only be assessed by bacteriological examination.

Although blood serum and meat juice were thought to be equally suitable for diagnosis of *Salmonella* in pigs (Nielsen et al., 1998), a large field study showed an important disagreement between ELISA performed in serum and meat juice (Vico and Mainar-Jaime, 2011). The OD% values from the serum were consistently higher than those from the meat juice ELISA. This would be related to a higher concentration of immunoglobulins in the serum (Steinbach et al., 2003), suggesting a lower chance for detecting an infection and an underestimation of the actual seroprevalence when ELISA is performed on meat juice. The serum antibody concentration is further influenced by the kind of serotype present in the animal. In blood, the probability of serological detection of *Salmonella* Typhimurium or *Salmonella* Derby was higher than that of *Salmonella* Goldcoast, *Salmonella* Panama or *Salmonella* Livingstone (Van Winsen et al, 2001, Nollet et al., 2005b). Not all pigs do seroconvert and if seroconversion takes place, the probability of detecting antibodies will also depend on the infection level and the onset of infection (Nielsen et al., 1995).

Considering the results obtained by the above mentioned authors, it is clear that the sampling method used in the Belgian SAP so far is insufficient. The Scientific Committee of the Federal Agency for the Safety of the Food Chain (FASFC) has proposed to shift from a serological monitoring towards a bacteriological monitoring (Anonymous, 2012b). Possible monitoring scenarios include using pooled fecal samples at herd level, gut content or ileo-ceacal lymph nodes at slaughterhouse level and both ileo-ceacal lymph nodes and carcass swabs of the same animal at population level at the slaughterhouse. Bacteriology has the additional advantage that the isolates can be stored for further sero- and genotyping or antimicrobial resistance analysis. At this stage, it is unclear when and how the current control strategy will be changed and which of the suggested control strategies will be finally selected by the Belgian Food Agency.

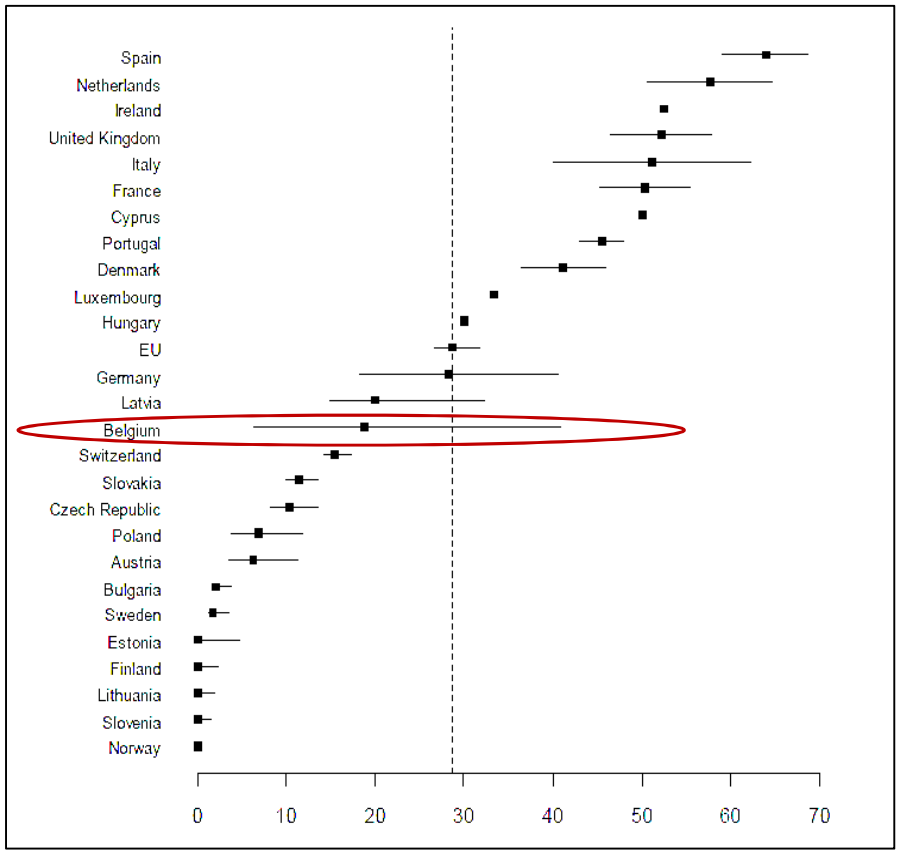

Figure 2a) Prevalence of *Salmonella*-positive breeding holdings in the EU (2008), with 95% Confidence Interval (horizontal bars), (EFSA, 2009)

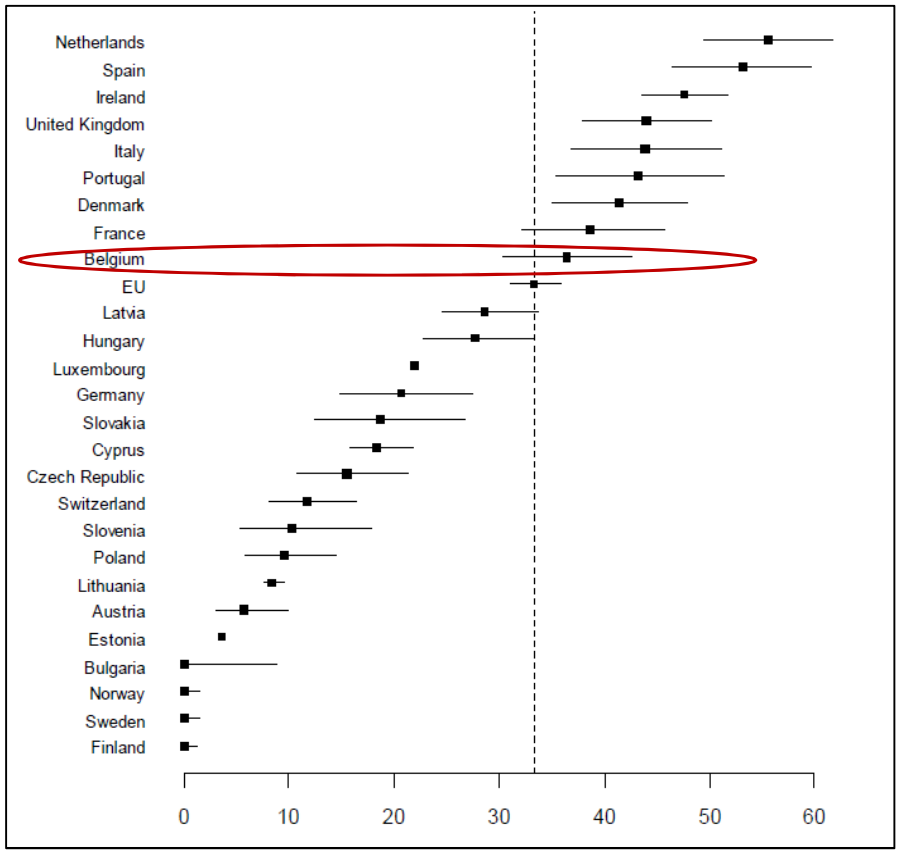

Figure 2b) Prevalence of *Salmonella*-positive production holdings in the EU (2008), with 95% Confidence Interval (horizontal bars), (EFSA, 2009)

Salmonella *epidemiology and monitoring at slaughterhouse level*

In Belgium, pigs are slaughtered at 6 to 7 months of age when the live weight is 105-120 kg. In 2011, 11.8 million pigs were slaughtered and 1.1 billion kg of pork was produced. Pork production mainly takes place in Flanders. The self-sufficiency rate of pork in Belgium is 239% (2009). Meat is mainly exported to Germany (38%), Poland (11%), the Netherlands (7%), Italy (6%), the UK (5%), Russia and France (both 4%) (Anonymous, 2012a).Before transport to the slaughterhouse, pigs are usually fastened for 12-18 hours. Feed withdrawal increases the well-being of the animals during transport (Bradshaw et al., 1996; Guàrdia et al., 1996), reduces carcass contamination due to the lower risk of accidental cutting into the intestines (Berends et al., 1996; Saucier et al., 2007), and improves pork quality (Guàrdia et al., 2004, 2005). The average transport time for slaughter pigs in Belgium is on average 74 minutes (De Sadeleer et al., 2008).

After arrival at the slaughterhouse pigs are unloaded and kept in lairage pens to recover from fatigue and stress. Pigs spend on average 126 minutes (5-720 minutes) in the lairage area (De Sadeleer at al., 2008) during which the health inspection can be performed. In Figure 3 a graphical representation of the slaughter process is made indicating the main activities.

Before actual slaughtering, pigs undergo electrical or carbon dioxide stunning resulting in unconsciousness after which they are bled (exsanguination).

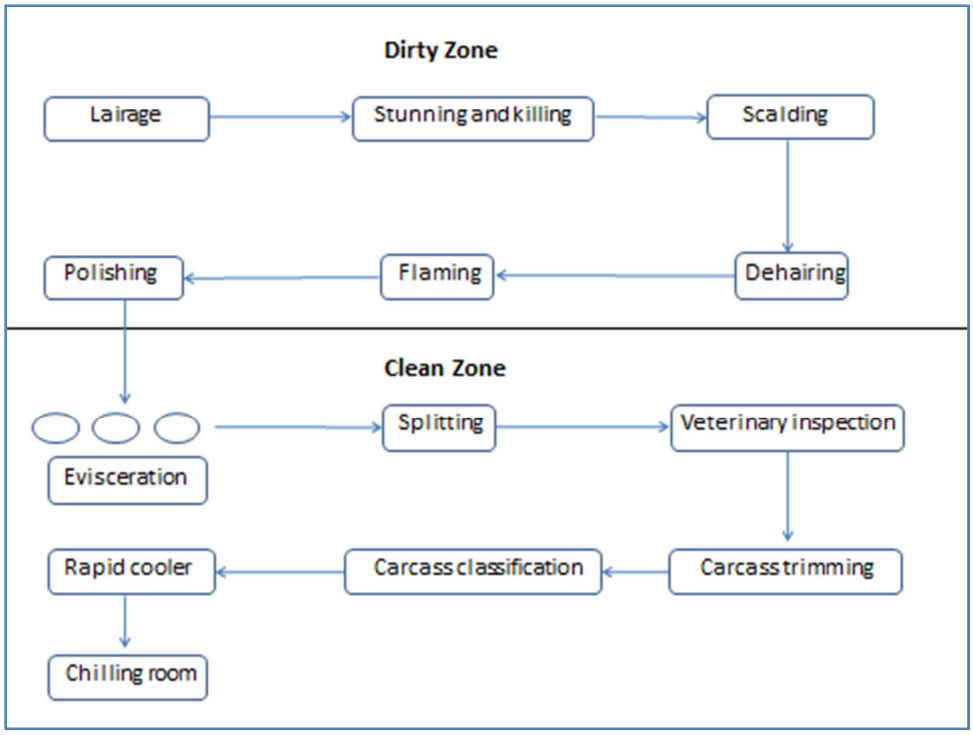

Fig. 3: Description of the pig slaughter process

To facilitate the dehairing process, pig carcasses are passed through a scalding tank or steam tunnel before they enter the dehairing machine. To remove the remaining hairs, pig carcasses are singed. After singeing, the carcasses are scraped and washed a final time in the polishing machine. From then onwards, they are allowed to enter the clean part of the slaughterhouse.

The first step in the clean part of the slaughterhouse is the evisceration. The rectum is made loose, manually or automatically by using a bung cutter, and the belly is opened down the entire length of the carcass. Abdominal organs are removed and placed in a moving gut pan. Lungs, liver, heart, oesophagus and tongue are hanged up next to the gut pan and move forward along with the carcass to make inspection of the whole pig possible further down the slaughter line. Carcasses are then cut into two halves by the splitting machine. Afterwards a veterinarian inspects the carcass (Anonymous, 2004b) and the carcass is further cleaned up or trimmed (removal of the kidneys, diaphragm, fat,…). Carcasses are weighted and classified according to the SEUROP system that is based on the percentage of lean meat of the carcass. Chilling is necessary to decrease the temperature of the carcass until 7°C within 12-16 hours. Most slaughterhouses have a rapid cooler (blast chilling) after which the carcasses are placed in the chilling room.

As mentioned above, the document “Information of the Food Chain” has to arrive at the slaughterhouse 24h before the slaughter batch. In this way, the operator can choose to slaughter batches originating from high *Salmonella* risk farms together at the end of the slaughter day or slaughter week. This so called logistic slaughter should decrease the risk of transmission from pigs bearing *Salmonella* to susceptible (negative) pigs.

In respect to the auto-control programs and Hazard Analysis and Critical Control Point (HACCP), the food business operators of slaughterhouses have to take samples for microbiological analysis from five randomly selected carcasses once a week (Anonymous, 2007c). The day of sampling has to be changed each week to ensure that each day of the week is covered. Carcasses must be sampled after dressing, but before chilling by using the abrasive sponge sampling method. Due to the technical difficulties associated with carcass sampling along the slaughter line, sampling has to be performed between 2 and 4 hours after slaughter (Anonymous, 2005a). The total sampling area has to cover a minimum of 400 cm^2. Of the 50 samples derived from 10 consecutive sampling sessions, not more than 5 may be *Salmonella* positive. If this limit is exceeded, improvements in slaughter hygiene and review of process control has to be done. Further, the origin of the slaughtered animals and the biosecurity measures in the farms of origins should be evaluated. In Belgium, the FASFC has implemented a so called action limit. If more than 3/50 carcasses reveal the presence of *Salmonella*, action has to be taken by evaluating and improving the auto-control program thoroughly (Directive CONT/2010/98/580647).

The FASFC also sends out inspectors to control the efficiency and accuracy of the operators own-check programs. Therefore, carcass samples are taken and *Salmonella* isolation has been performed. Table 3 shows the percentage *Salmonella* positive carcasses detected by the FASFC and by the auto-control program of the slaughterhouse during the period 2008-2010. The results show that samples taken by the FASFC reveal systematically more positive carcasses than the once taken by the operator himself. The correlation coefficient between both percentages of positive carcasses is moderate to low (0.55) (Anonymous, 2012b).

Table 3: Percentage *Salmonella* positive carcasses taken in the period 2008-2010 by the FASFC monitoring and auto-control program of the slaughterhouse (Anonymous, 2012b)

Year	% positive by FASFC	% positive by auto-control
2008	14.6% (n=281)	-
2009	13.4% (n=828)	5.96% (n=5135)
2010	8.93% (n=750)	6.83% (n=4700)

During the period 2006-2007, a baseline study was performed in the EU to assess the prevalence of *Salmonella* positive carcasses and lymph nodes at slaughter in the different MS (EFSA, 2008a). In the EU, 10.3% of the slaughter pigs carried *Salmonella* in the lymph nodes and 8.3% of the carcasses were contaminated with *Salmonella.* The results of the carcass sampling showed that Belgium has a high (18.8%) percentage of positive carcasses in comparison with the other MS (Table 4).

Table 4: Prevalence of *Salmonella* on carcasses and in lymph nodes of different Member States (MS) as sampled by the EFSA protocol (EFSA, 2008a) during the period 2006-2007

MS	*Salmonella* prevalence carcasses			*Salmonella* prevalence lymph nodes		
	n	%	CI	n	%	CI
Austria	617	1.2	0.4-3.7	617	2.0	1.1-3.6
Belgium	381	18.8	14.1-24.6	601	13.9	9.8-19.3
Cyprus	359	3.3	3.2-3.4	359	12.4	10.1-15.2
Czech Republic	417	3.7	2.2-6.3	654	5.8	3.8-8.9
Denmark	344	3.3	1.3-8.5	998	7.7	5.5-10.7
France	413	17.6	11.8-25.4	1163	18.1	16-20.5
Ireland	422	20.0	10.8-34	422	16.1	15.6-16.7
Latvia	391	3.3	1.2-8.9	392	5.6	3.3-9.1
Lithuania	461	1.6	0.6-4	461	1.8	0.8-3.9
Poland	447	1.3	0.5-3.2	1176	5.1	3.7-6.9
Slovenia	441	0	-	431	6.2	4.2-9.1
Sweden	402	0	-	394	1.3	1.2-1.5
UK	641	13.5	9.9-18.1	639	21.2	17.8-25

Belgium as well as Ireland are the only two MS where the *Salmonella* prevalence of the carcasses is higher than the one of the lymph nodes, indicating that cross contamination in the slaughterhouse is an important feature. The latter is in accordance with results obtained by Wonderling et al. (2003) showing that most *Salmonella* genotypes on the carcasses of slaughtered pigs were different from those in the feces of the corresponding pigs, indicating that the contamination of the pigs intestines was not the primary cause of carcass contamination. Other authors already suggested that the slaughter practices have the highest impact on the number of contaminated carcasses (Swanenburg et al., 2001a; van der Gaag et al., 2004; Alban and Stärk, 2005).

Although MS have to consider whether on farm intervention, slaughterhouse intervention or a combination of both offer the optimum control strategy (EFSA, 2008a), quantitative microbiological risk assessment (QMRA) showed that specific slaughterhouse interventions are, at present, more likely to produce larger reductions of human illness than interventions in the primary production (Baptista et al., 2010a; Bollaerts et al., 2010; EFSA, 2010). Interventions at harvest level should be based on prevention of direct/indirect fecal contamination during transport, lairage, slaughter and dressing processes and/or by effective carcass decontamination. Control at herd level remains important to prevent further spread in the pig sector and potential zoonotic infections due to contact with infected pigs and manure (Lo Fo Wong et al., 2002; Hendriksen et al., 2004). The overall input of *Salmonella* to the slaughterhouse is particularly important for small slaughterhouses where implementation of a decontamination step might not be cost-effective (Lawson et al., 2009).

Diagnostic tools

Bacteriological examination

Microbiological culture is the "gold standard" diagnostic test for *Salmonella* serovars. If a positive test result is obtained, a *Salmonella* isolate is available for further identification (serotype, genotype, sensitivity testing). As the bacterial isolate can be definitively identified, microbiological culture has a perfect specificity (no false positive results). However, microbiological culture has also some weaknesses namely it is costly, time-consuming and the sensitivity is poor. Fecal culture has the advantage of being performed ante mortem and samples are easy to collect, but it is also susceptible to sampling error due to intermittent shedding of the pathogen.

Salmonella can be isolated using a variety of techniques. Most of them include pre-enrichment to resuscitate damaged salmonellae, enrichment with media containing inhibitory substances to suppress competing organisms and selective plating to differentiate *Salmonella* from other Enterobacteriaceae (Office International des Epizooties (OIE), 2010).

Pre-enrichment is usually carried out in buffered peptone water (BPW), stimulating growth of *Salmonella*, but also from accompanying flora. In the second step, selective enrichment is performed. Different selective enrichment media ara available that can be divided in two groups: the semi-solid agars developed for the detection of motile *Salmonella* and the enrichment broths. Modified semi-solid Rappaport-Vassiliadis (MRSV) and Diagnostic Semi-Solid *Salmonella* agar (DIASSALM) are both semi-solid media whereas Rappaport Vassilisadis broth (RV), Rappaport Vassiliadis broth with Soya (RVS) and Muller Kauffmann Tetrathionate novobiocin broth (MKTTn) are enrichment broths. Xylose Lysine Desoxycholate (XLD), Xylose-Lysine-Tergitol 4 (XLT-4) or Brilliant Green Agar (BGA) can be used as a selective isolation step.

As the outcome of the analyses depends on the method, international standard methods have been developed to allow proper comparisons between results of different countries or laboratories (Mooijman, 2010). The International (ISO) and European Standardisation (EN) Organisations developed different EN/ISO methods for the detection of *Salmonella* in different matrices (Table 5).

Table 5: EN/ISO methods for the detection of *Salmonella* in different matrices (Mooijman, 2010)

	Matrix	Selective enrichment	Selective plating
ISO 6579:2002	Food, animal feeding stuffs	RVS and MKTTn	XLD and second
ISO 6579:2002/Amd1, Annex D, 2007	Animal feces, environment primary production	MSRV	XLD and second
ISO 6785/IDF 93, 2001	Milk and milk products	RVS and SC	BGA and second
ISO/DIS 19250, 2007	Water	RVS, optional MKTTn	XLD and second
EN 15215-3 (not yet published)	Soils and sludges	2x RV, at 36°C and 41°C	BPLS

SC: Selenite Cystine broth / BPLS: Brilliant Green Phenol Lactose Sucrose agar

As shown in Table 5, the detection of *Salmonella* spp. in animal feces and in samples from the primary production is described in Amendment 1, Annex D of the ISO 6579 (ISO, 2007). The first step in the *Salmonella* isolation protocol is pre-enrichment in which the sample is diluted 1:9 with BPW, homogenized in a stomacher blender and incubated at 37°C. After 16-20h incubation, 0.1 ml of the BPW broth is dispersed in three drops on MRSV. After incubation of the selective enrichment media for 24h at 42°C, MSRV plates are examined for the presence of typical migration zones. A loopful of the migration zone with the inoculum taken from the furthest edge of the visual growth zone is streaked on XLD. After incubation for 24h at 37°C, all XLD plates are examined for the presence of typical colonies. Biochemical confirmation is performed using triple sugar iron agar (TSI), indole and lysine broth. This isolation method requires 5 days. Instead of biochemical confirmation, a multiplex PCR can be performed to confirm the *Salmonella* genus (Aabo et al., 1993) and identify the *Salmonella* Typhimurium serotype (Lin and Tsen, 1999).

To rapidly detect *Salmonella* in food matrices, the VIDAS *Salmonella* (SLM) Easy *Salmonella* method can be used (Crowley et al., 2011). This method is a specific enzyme-linked fluorescent immunoassay that is performed in an automated VIDAS instrument. As it is based on a simple two-step enrichment procedure, results are obtained within two days.

Real-time PCR is another rapid method that can be used for the detection of

Salmonella in food (Krascsenicsová et al., 2008). It is recommended to prolong the enrichment step or to include an additional short selective enrichment/concentration step before PCR is applied to avoid false negative results when low levels of sub-lethally injured cells are present (Jasson et al., 2011). For positive results a confirmation step is additionally needed.

All methods have their specific advantages and disadvantages. The challenge is to select a method that fulfills most of the preferred characterisitcs for the user's practical context, including the technical aspects of the method, its operational requirements and sustainability. Overall, the classical culture methods remain the basis but also evolve by using more differential media and broths which enhance resuscitation and growth (Jasson et al., 2010).

Serology

To evaluate if an animal has been infected with *Salmonella* at some stage of the production cycle, body fluids such as blood serum or meat juice (from a slaughtered animal) can be analyzed to detect antibodies to *Salmonella***.** In the SAP, blood samples taken in the eradication and monitoring program for Aujeszky's disease (Anonymous, 1999) are also used to detect *Salmonella* antibodies. This is done by the use of an indirect ELISA test (HerdCheck Swine *Salmonella* Antibody Test Kit, Idexx Laboratories, Inc., Maine, USA). The presence of *Salmonella* specific antibodies is determined by calculating the S/P (Sample to Positive) ratio by using following formula, with OD meaning optical density values:

$$\text{S/P ratio} = (OD_{\text{sample}} - OD_{\text{negative kit control}}) / (OD_{\text{positive kit control}} - OD_{\text{negative kit control}})$$

A farm is categorized as a high risk farm if the mean S/P ratio is higher than 0.6 during three consecutive sampling rounds.

Correlation bacteriology-serology

In Belgium, only 60% of the designated risk farms were also positive after bacteriological examination of feces taken at the herd (Laevens and Mintiens, 2005). To improve the correlation between serology and bacteriology, more samples have to be taken

(sample size of minimal 40 pigs) and the sampled animals should be old enough (>60kg) (Laevens and Minitens, 2005; Snary, 2010). Also at the slaughterhouse, a weak agreement was found between serology (blood) and bacteriological examination of the lymph nodes of the same pigs (Nollet et al., 2005b). Depending on the cut-off level used, only 34.5% (cut-off 40%) or 82.8% (cut-off 10%) of the culture positive (lymph nodes) animals were seropositive (Nollet et al., 2005b). To classify herds correctly the sample size as well as the used cut-off level are of major importance.

Characterization of the isolates

Phenotypic typing techniques

Serotyping is the most common way to differentiate *Salmonella* isolates. Standard serotyping methods rely on the detection of somatic (O) and flagellar (H) antigens present on the cell surface of *Salmonella*. The isolate is classified according to the Kauffman-White classification scheme. Full serotyping is usually performed in only one or a few numbers of official laboratories in each country.

Antimicrobial susceptibility testing and the generation of antimicrobial resistance profiles are also phenotypic techniques. (Foley et al., 2007). Phage typing is a method that is often used to discriminate between closely related *Salmonella* (*i.e.,* within the same serotype). It assesses the lytic patterns of test isolates that have been exposed to a defined set of bacteriophages. A major drawback of phage typing is the production and continuous quality control of phages as well as the limited number of available phages, rendering many strains untypable (Amavisit et al., 2001).

Molecular or genotypic techniques

Genotyping methods have been developed for genetic discrimination of *Salmonella* isolates in outbreaks.

Pulsed field gel electrophoresis (PFGE) is a restriction enzyme digestion-based method that made it possible to separate large DNA fragments in agarose gels by periodic alternation of the angle of the electric field's direction. It has remarkable discriminatory power and reproducibility (van Belkum et al., 2007) and is often considered as the "gold standard" for comparative typing of many bacteria (Tenover et al., 1995; Olive and Bean, 1999). However, PFGE is time-consuming, labor-intensive and relatively expensive PFGE

equipment is required. Gels need to be analyzed closely and carefully, even after digitalization and computerized processing (van Belkum et al., 2007). Further, PFGE does not display equal discriminatory power within different serovars (Liebana et al., 2001; Kerouanton, et al., 2007).

Multilocus variable number of tandem repeat analysis (MLVA) is an amplification-based method and the ease of performance and interpretation makes it a valuable technique (Kruy et al., 2011). A challenge for the future is to set up an MLVA technique using a panel of markers that allows the genotyping of a large variety of *Salmonella* serovars (Kruy et al., 2011).

Multilocus sequence typing (MLST) is a DNA sequence-based method. This technique relies on the use of housekeeping genes, which are stable, defined markers for typing. Due to their genetic stability, they may not provide enough variability to distinguish among strains with more recent genetic divergence (Foley et al., 2007). As virulence genes are under greater selective pressure, they may be used in a MLST scheme redefined as multivirulence-loci sequence typing or MVLST (Chen et al., 2007; Zhang et al., 2004).

Control measures

Pre-harvest

Purchase policy

Salmonella can be introduced in the herd through infected purchased pigs (Fedorka-Cray et al., 1997) and in this way increase the *Salmonella* prevalence at slaughter (van der Heijden et al., 2005). Davies et al. (2000) showed that gilts after introduction in a new herd showed increased *Salmonella* excretion. Purchasing replacement stock from more than three and finishers from more than one supplier herd, increased the odds to test seropositive (Lo Fo Wong et al., 2004; Quessy et al., 2005). If necessary, pigs should be purchased from herds with a known *Salmonella* status, ideally from *Salmonella*-free herds. However, it is not clear which criteria should be fulfilled before the *Salmonella*-free status can be claimed. 1) should the assignment be based on serological (blood) results obtained from sows and/or bacteriological examination of feces from the different age groups present at the herd? 2) which sample size should be used? 3) how many sampling rounds per year should be foreseen?, are to be answered.

According to EFSA (200a, 2009) and Hill et al. (2011), breeding herd prevalence is correlated with the slaughter pig prevalence. If only *Salmonella* negative breeding stock would be allowed, it has been estimated that a *Salmonella* reduction of 70-80% in high prevalence countries, and a reduction of 10-20% in low prevalence countries could be obtained (EFSA, 2010). Nollet et al. (2005b) demonstrated that in farrow-to-finish herds sows are an important source for indirect transmission of *Salmonella*. Monitoring of sows by serological analysis is recommended, although sample size has to be calculated and adjusted to the expected *Salmonella* prevalence, the desired precision and herd size. As an increase in *Salmonella* shedding was seen in sows seven days after weaning (Nollet et al., 2005b), additional pooled fecal samples (EFSA, 2007) should be taken preferably at this time-point. As sows produce approximately 2.3 litters a year, two-to-three sampling rounds per year should be performed in order to establish the *Salmonella* status. Once a free status has been assigned, the sampling strategy might be adapted, and become less intensive in time.

Salmonella-*free feed*

As mentioned by Sauli et al. (2005) safe feed is the first step for ensuring safe food, especially in a “Farm to Fork” food safety concept. *Salmonella* can be introduced into the feed by contaminated ingredients, but also during processing, transport, storage at the farm, distribution and administration (Davies and Hinton, 2000; Jones and Richardson, 2004). *Salmonella* could be isolated from feedstuff in 17.6% of pig herds amongst five EU countries and 6.9% of all feed samples (Lo Fo Wong and Hald, 2000). Results of pig feed monitoring by the FASFC (Belgium) showed an average prevalence of 2.84% in the years 2008-2010 in compounded pig feed. The recovered serotypes were Anatum, Livingstone, Senftenberg, Oranienburg and Lexington (Anonymous, 2012). The EU *Salmonella* prevalence in pig feed as established by EFSA (2006) showed contamination rates up to 1.7%. The dominant serotypes found in samples of compounded feed were Livingstone, Senftenberg and Montevideo. In Belgium, five *Salmonella* serotypes are classified as critical in pig feed, namely: Anatum, Derby, Enteritidis, Infantis and Typhimurium (Anonymous, 2010). The link between animal feed and both animal and human salmonellosis has been regularly established as demonstrated by EFSA (2008b) and Jones (2011).

Salmonella in feed normally involves small numbers of organisms distributed in a non-uniform way within very large consignments of materials, making correct sampling difficult (EFSA, 2008b; Jones, 2011). Mitchell and McChesney (1991) even suggested that 30

samples should be analyzed to determine if a batch of feed is *Salmonella* negative. More recent studies (EFSA, 2008b; Davies and Wales, 2010) reported that analysis of dust, spilled feed and debris around processing equipment is a more sensitive strategy than direct sampling of feed. Sampling of pig feed should be continued and intensified as it is believed that feeding *Salmonella* free feed results in a significant decrease of *Salmonella* prevalence, especially in countries with a low *Salmonella* prevalence (EFSA 2010, Hill et al., 2011). In such countries, *Salmonella* is mostly introduced in the herd through contaminated feed (Häggblom, 2009).

As stated by Jones (2011), *Salmonella* control in animal feed should be performed at three levels: 1) prevention of *Salmonella* entering the facility: purchasing *Salmonella*-free ingredients, controlling dust, optimizing internal biosecurity, controlling rodents and wild birds and ensuring the sanitation of transport vehicles, 2) reducing *Salmonella* multiplication inside the facility and 3) killing the pathogen by thermal processing or chemical additions as organic acids, formaldehyde or a combination of both.

Heat treatment is able to eliminate *Salmonella* when conditioning and pelleting is performed at 93°C for 90 seconds with a 15% moisture (Himathongkham et al., 1996). These conditions are however, rarely obtained in practice due to the higher energy cost involved, the heat damage to vitamins and other nutrients and the adverse effect on the integrity of the pellets (Peisker, 2006). As the number of *Salmonella* in feed are usually less than one cfu/g (Taylor and McCoy, 1969), temperatures of at least 80°C during conditioning followed by pelleting are mostly successful in eliminating *Salmonella* (Veldman et al., 1995). Heat treatment has no residual effect so feed can be readily recontaminated after treatment. Chemical treatment can be done using *e.g.* organic acids and their salts, formaldehyde and bacterial membrane disruptors such as terpenes and essential oils. Organic acid treatment reduces recontamination after feed preparation (Iba and Berchieri, 1995; Ricke, 2005), but the use might mask the presence of *Salmonella*, when assessed by standard culture methods (Carrique-Mas et al., 2006). The use of formaldehyde is of concern as it is potentially harmful for humans (Arts et al., 2006). Many products use blends of agents from the same or different chemical groups to achieve synergistic effects. However failure of protection is noticed along with problems in usage such as corrosion and reduced palatability (Wales et al., 2010).

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Feed composition and drinking water

Lo Fo Wong et al. (2004) showed that feeding non-pelleted feed to finishers decreased the level of seropositivity in comparison to pigs fed pelleted feed. Feeding coarsely ground meal decreased the survival of *Salmonella* during stomach passage (Mikkelsen et al., 2004; Canibe et al., 2005), possibly by a slower gastric passage and a lower gastric pH. However, this type of feed is associated with a lower growth rate in pigs. A strong reducing effect of fermented liquid feed, including whey on *Salmonella* shedding and seroprevalence has also been reported (van der Wolf et al., 2001a; Lo Fo Wong et al., 2004; Farzan et al., 2006; Poljak et al., 2008).

The addition of organic acids to feed or drinking water is in general beneficial to reduce *Salmonella* shedding, but the effects largely depend on the type of product and the treatment strategy *e.g.* dosage, duration, age groups, *Salmonella* infection levels. The theory behind the use of acidification to reduce *Salmonella* prevalence is that the supplemental organic acids enter the bacterial cell and dissociate due to the higher pH within the cell. This in turn lowers cellular pH and prevents DNA synthesis and hence replication of the bacteria (Rubin et al., 1982; Kirchgessner et al., 1992). However, large differences are noticed in the antibacterial effect of one acid versus another, indicating that factors such as chain length, side chain composition, pKa-values and hydrophobicity could affect the antimicrobial activity (Van Immerseel et al., 2006).

When organic acids are administered in the drinking water, the pH of the water can be lowered to 4, at which Enterobacteriaceae cannot multiply (Ostling and Lindgren, 1993). Practical problems associated with water supplementation are clogging of drinking nipples and corrosion. Van der Heijden *et al.* (2005), van der Wolf *et al.* (2001b), Hansen *et al.* (1999) and Letellier *et al.* (1999) all supplemented the drinking water of finishing pigs with organic acids. Van der Wolf *et al.* (2001b) and van der Heijden *et al.* (2005) observed a significant reduction of the *Salmonella* seroprevalence. Hansen *et al.* (1999) and Lettellier *et al.* (1999) however, did not.

There is similar variability on the effect of organic acid supplementation of feed with Papenbrock *et al.* (2005) reporting a 30% reduction in *Salmonella* prevalence in feces and Walsh *et al.* (2003) reporting no beneficial effect. Further, McLaren *et al.* (2001) found that feed acidification increased the prevalence of *Salmonella* in both weaning and finishing pigs. A combination of lactic and formic acid added to pelleted feed was beneficial (Creus et al.,

2007), but reduction in *Salmonella* prevalence and number of carriers was only obvious after treatment during the whole fattening period.

Virulence gene expression and invasion can however, be decreased and inhibited by certain short and medium chain fatty acids *in vitro* (Boyen et al., 2008b). *In vivo* coated butyric acid was effective to decrease fecal shedding, intestinal colonization (Boyen et al., 2008b) and transmission (De Ridder et al., 2011).

The inconsistent results can further (partially) be explained by the so called "acid tolerance response" in which adaptation to mild (pH 5.8) or moderate (pH 4.4) acid conditions enables the organism to endure periods of severe acid stress (pH 3) (Bearson et al., 1998). However, further research should be carried out to gain more knowledge in this topic.

Cleaning and disinfection strategies

To remove *Salmonella* contamination in a pig herd, currently applied cleaning and disinfection procedures of the accommodation are often insufficient (Berends et al., 1996; Funk et al., 2001; Wales et al., 2009). The environmental robustness of the organism (Guan and Holley, 2003), the poor efficacy of many cleaning and disinfection regimens (Mannion et al., 2007; Wales et al., 2009), the limitations of many disinfectants in farm environments (McLaren et al., 2011) and the ubiquity of rodent vectors make it difficult to obtain a *Salmonella*-free accommodation. Most disinfectants (based on sodium hypochlorite or quaternary ammonium compounds) are able to eliminate *Salmonella* bacteria, although their effectiveness can be decreased by inadequate cleaning (through remaining organic material), incorrect dosage or contact time. A sanitary transition period should be included to obtain a dry environment, if necessary with extra heating and ventilation. In the cleaning and disinfection procedure any tools such as brooms, tools for scratching feces, boards for moving pigs and transport vehicles as well as areas to which the animals have no direct contact such as ante-rooms for changing clothes and boots and alleys for pig movements have to be included (Bode et al., 2007). As shown in poultry, biofilms can be developed by *Salmonella* strains, leading to resistance against several disinfectants (Marin et al., 2009). In addition, several *Salmonella* serovars showed survival in (King et al., 1988; Tezcan-Merdol, 2004) and subsequent release from protozoa (Brandl et al., 2005). The bacterium-protozoan association further allows increased resistance to free chlorine residuals, which can lead to persistence of bacteria in chlorine treated water (King et al., 1988). More research needs to be done on this

last topic to gain more insight in the interaction pathogen-protozoa and its effect on regular cleaning and disinfection procedures.

Biosecurity and management

A high level of biosecurity with accurate cleaning and disinfection procedures should be achieved, especially in herds aiming to obtain the free-status. All possible stress factors should be minimized as stress enhances *Salmonella* shedding and depresses immunity (Hurd et al., 2001b). Pig flow and housing conditions, stable climate, health, feeding strategy, pig density, handling and moving of pigs as well as optimal fastening (12-18h) and loading (with a minimum of stress) are points of attention. Farmers should select well skilled drivers for the transport of their animals to the slaughterhouses as stress occurring during transport enhances fecal shedding and increases contamination and cross-contamination.

Vaccination against Salmonella

The immune system uses different strategies to protect a host against infectious agents. The innate immunity recognizes invading micro-organisms, produces cytokines and soluble factors and activates phagocytic cells (Dougan et al., 2011). The innate immune response lacks any form of memory and is, although successful in controlling the initial growth of *Salmonella*, insufficient to ensure resistance to a *Salmonella* infection (Dougan et al., 2011). The acquired immune system includes the humoral (mostly against extracellular invaders) and cell-mediated (mainly against intracellular invaders) immune response, allowing the clearance of *Salmonella* and the establishment of a long-lasting immunity against re-infection (Mastroeni et al., 2000). Where killed *Salmonella* whole-cell vaccines lack the ability to induce proper cell-mediated immunity (Xu et al., 1993; Yamane et al., 2000; Davies and Breslin, 2003), attenuated live vaccines provide better protection due to the cellular immune response and the induction of mucosal IgA production (Haesebrouck et al., 2004).

Maes et al. (2001) demonstrated that vaccination with a live *Salmonella* Choleraesuis vaccine at 3 and 16 weeks was able to significantly lower the lymph node contamination of slaughter pigs. Recently, Schwarz et al. (2011) demonstrated that in herds with a high *Salmonella* prevalence, the administration of a *Salmonella* Choleraesuis attenuated vaccine on the first day of life decreased the *Salmonella* isolation from lymph nodes and the seroprevalence in pigs at slaughter. However, antibodies induced by different *Salmonella* serovars show only a low level of cross-protection (Wallis, 2001).

In Europe, only one *Salmonella* Typhimurium live vaccine is commercially available (Salmoporc*ΔompD*, IDT Biologika GmbH) (Lindner et al., 2007) and can be administered subcutaneously in sows (primo-vaccination: 6 and 3 weeks ante partum, revaccination: 3 weeks ante partum) and orally in piglets (at day 3 of life and 3 weeks later). It has shown to reduce both shedding and colonization of host tissues (Selke et al., 2007) and to induce a substantial antibody response (Eddicks et al., 2009). The protein OmpD is one of the most abundant proteins in the outer membrane of *Salmonella enterica* and is not found in other gram-negative bacteria, which makes it a suitable marker protein (Santiviago *et al.*, 2003). This negative-marker vaccine allows differentiation of infected from vaccinated animals (DIVA) using an OmpD-specific peptide-based ELISA (Selke et al., 2007). Most European *Salmonella* surveillance programs are however based on the detection of antibodies against the lipopolysaccharides (LPS) of *Salmonella* (Cortinas Abrahantes et al., 2009) by using an O-antigen based ELISA which does not allow distinction between infected and Salmoporc®-vaccinated pigs. Moreover, Gil-Cruz *et al.* (2009) showed that the *Salmonella* Typhimurium protein, OmpD, is important in mediating a protective B-cell antibody response.

The principle of DIVA is based on the absence of at least one immunogenic protein in the vaccine strain which is present in the wild-type strain (Selke, 2006). A vaccine based on deletion of LPS biosynthesis encoding genes could circumvent this limitation (Nagy et al., 2008a, 2008b; Kong et al., 2011; Leyman et al., 2011). Such a vaccine could be used within the current serology-based monitoring programs.

However, following important questions remain: which animals should be vaccinated, should vaccination become obligatory, which sector of the pork production chain should bear the costs. As a first step, vaccination could be limited to breeding herds, combined with appropriate biosecurity and management strategies (Hotes et al., 2011; Schwarz et al., 2011). Future research should be conducted to assess the cost-effectiveness of vaccination (Baptista 2010b).

Harvest

Transport

Hurd et al. (2001b) and Rostagno et al. (2011) showed that the proportion of pigs shedding *Salmonella* significantly increased during transport from the farm to the slaughterhouse. This increase depends on the amount of animals shedding *Salmonella* (infected and carrier animals) and on the duration of the transport. Due to stress, carriers can start re-shedding and are in this way an important *Salmonella* source for their travelling mates.

Stress occurs by rough handling at the time of loading and unloading, but it may also be influenced by stocking density during transport (Anonymous, 1995), transport duration, drivers skills, weather conditions and general health status of the pigs. Stress induced by feed withdrawal should also not be neglected as in practice the total feed withdrawal time (at farm-transport-lairage) can increase up to 24 h and longer. Warriss et al. (1996) noted that fasting periods >18h caused hunger and aggressiveness in pigs. Furthermore, increased feed withdrawal times may be associated with changes in the gut microbial ecosystem with increasing levels of Enterobacteriaceae in the cecum and *Salmonella* in the feces (Martín-Peláez et al., 2009).

The transport vehicle itself should be cleaned and disinfected after the delivery at the slaughter plant (Anonymous, 2005b). Although it can be questioned if the cleaning and disinfection is always performed properly as mentioned above (section Cleaning and disinfection).

Lairage

Holding pigs in lairage for 2 to 3 h is necessary for the pigs to recover from transport stress (Warriss et al., 1992). Longer lairage times increase the level of dark, firm and dry (DFD) pork and skin blemishes (Warriss et al., 1998; Nanni Costa et al., 2002). Stress should further be avoided as it does not only harm animal welfare and meat quality, but it also leads to a higher amount of *Salmonella* shedders and increases the susceptibility to *Salmonella* infection (Hurd et al., 2001b). The movement of pigs in the lairage area can be facilitated by well-designed infrastructure *e.g.* long and narrow pens with entry and exit at opposite ends, well lighted corridors with few bends, and careful handling. Minimal human intervention obtained by the use of automatic gates improves the ease of handling and leads to a minimum

of stress (Barton Gade et al., 1993). If human intervention is necessary, prudent behavior of the slaughterhouse staff is recommended to avoid excitement, pain or suffering of the animals (Lammens et al., 2007; Anonymous, 2009). Fighting of the pigs can be prevented by keeping the pigs in small (*n*=15) groups (Barton Gade et al., 1993). When the ambient temperature rises above 10°C, pigs should be showered (Schütte et al., 1996).

All these measures can only be meaningful in preventing *Salmonella* infection if pigs are housed in a clean and *Salmonella*-free environment. The latter however, is still a major problem in lairages all over the world. Rossel et al. (2009) demonstrated that carcass contamination is directly related to skin contamination of live pigs before stunning. This skin contamination was connected with the contamination of the lairage area. Effective cleaning and disinfection is necessary, but difficult to achieve as shown by Swanenburg et al. (2001b). The use of roughened slatted floors in the lairage area may be a good contribution as it keeps the animals clean, minimizes contact with feces, and prevents animals from falling or slipping as recommended in the animal welfare legislation of slaughter animals (Anonymous, 2009).

Slaughter line

Along the slaughter line, several steps are critical for *Salmonella* contamination (Figure 1) such as dehairing, scalding, polishing, removal of the intestines, removal of the pluck set and meat inspection procedures (Borch et al., 1996). During these steps, the carcasses can be contaminated with feces and bacteria can spread over the same and subsequent carcasses and contaminate the slaughter equipment and environment.

Hald et al. (2003) showed that if scalding water was *Salmonella* positive, pluck removal was associated with a higher risk of carcass contamination. Survival of *Salmonella* in the scalding tank increases if the water temperature drops below the recommended 62°C (Hald et al., 2003) and/or if the amount of organic material is sufficient to protect the organism against the heat (Sörqvist et al., 1990). Continuous monitoring of the temperature of the scalding water is necessary, although scalding with steam is a good alternative (Delhalle et al., 2008).

Da Silva et al. (2012) demonstrated that a significantly higher number of *Salmonella* positive carcasses was observed after the dehairing process compared to carcass contamination after singeing and evisceration. The contamination level before singeing could be associated with dirty pigs entering the slaughterhouse (Letellier et al., 2009),

contamination from the scalding water (Hald et al., 2003) or the dehairing process itself (Pearce et al., 2004).

Berends et al. (1997) suggested based on a literature review that 5-15% of all carcass contamination occurred during polishing. Contamination and cross-contamination can occur easily at this step as carcasses are "wringed out" and the equipment is difficult to clean, allowing bacteria to establish on the surface of the brushes and scrapes (Borch et al., 1996).

Although singeing is performed before polishing, adding a second flaming device after polishing is helpful to avoid that contaminated carcasses enter the clean part of the slaughterhouse (Delhalle et al., 2008; De Sadeleer et al., 2008).

During evisceration, 55-90% of all carcass contamination occurs (Berends et al., 1997). Good fasting of the delivered pigs, a correct evisceration technique and proper training of the slaughterhouse personnel are helpful to diminish the risk of accidental cutting in the intestines. Further application of a bung cutter in connection with enclosing the anus and rectum in a plastic bag (Alban and Stärk, 2005) should prevent leaking of feces from the rectum.

Already in 1977 (Childers and Keahey), it was shown that carcass contamination could be reduced by 50% when the eviscerating operative wore plastic gloves and sanitized the knife in 82°C water between carcasses. However, De Sadeleer et al. (2008) showed that the temperature of the water used for cleaning the evisceration knives and the bung cutter varied over 10 slaughterhouses from 47°C to 81°C, whereas this should be at least 82°C. The knives were not cleaned between every carcass, but were held for one second in hot water every third or fourth carcass. In the worst case situation, this was done only when the intestines were accidentally opened. The limited cleaning frequency for the knives is probably due to the high slaughter line speed in the slaughterhouses (between 285 to 550 pigs per h). The use of plastic gloves is further not easy to implement, as safety gloves are mandatory at different places along the slaughter line (Anonymoys, 2005c).

The carcass splitter (Swanenburg et al., 2001c; Bertrand et al., 2010, Smid et al., 2012) as well as the hands of slaughterhouse personnel (Bertrand et al., 2010; Duggan et al., 2010) and meat inspectors (Vieira-Pinto et al., 2006) can further also cause cross-contamination of carcasses. Cleaning and disinfection of the splitter machine several times a day was shown to be beneficial in reducing *Salmonella* contamination (Delhalle et al., 2008).

The chilling process can result in slight increases (Bolton et al., 2002), decreases (Arguello et al., 2012) or no changes (Nesbakken et al., 2008) of bacterial contamination. Factors such as air speed, air flow, relative humidity, temperature, duration and carcass spacing modulate the impact of chilling (Bolton et al., 2002; Loretz et al., 2011). As the proliferation of bacteria is prevented, chilling should be used as an important critical control point (Bolton et al., 2002).

Decontamination of the carcass

Since January 2006, the use of substances other than potable or clean water to remove microbial surface contamination from food of animal origin is allowed in the EU (Anonymous, 2004a), with the restriction that product approval must be obtained from the EFSA BIOHAZ Panel and that the decontamination treatment is an added tool to good hygiene practices (Bertrand et al., 2010).

It has been shown that hot-water decontamination and the use of acidified sodium chlorite reduce the *Salmonella* prevalence on the pork carcass (Jensen and Christensen, 2000; Hamilton et al., 2010). Hot-water decontaminated carcasses are showered with water at 80°C for 14-16 s. This temporary increase in meat surface temperature might lead to minor changes of the meat color immediately after decontamination, but they disappear after chilling (Goldbach and Alban, 2006). As hot-water decontamination implies major investment costs, alternatives as hand-held decontamination by use of steam suction and the combination of steam and ultra-sound are further investigated (Goldbach and Alban, 2006). In the study of Hamilton et al. (2010), acidified sodium chlorite was applied for approximately 15 seconds per carcass side and resulted in a “whitening”of the skin and the fat. Particularly for Asian export markets this was judged as a positive esthetic improvement. The product is approved for use on edible products in the USA, while citric acid and sodium chlorite as distinct components are approved processing aids in Australia (Hamilton et al., 2010).

In Europe, chemical decontamination of carcasses is at present not be performed, although a proposal has been made concerning the use of lactic acid (2-5% at a maximum temperature of 55°C) in reducing the microbial surface contamination of bovine carcasses (Anonymous, 2012c). As the process of dehiding constitutes a major source of carcass contamination (Biss and Hathaway, 1995; Sheridan, 1998; Antic et al., 2010), decontamination treatments are of special interest in the slaughter of cattle and sheep. Lactic acid spraying (2%) on naturally contaminated pig carcasses was shown to reduce the

prevalence of *Campylobacter* and *Salmonella* (Epling et al., 1993). Lactic acid spraying on inoculated pork carcass surface parts and on pork variety meats (liver, intestines, stomach, heart) yielded reductions of *Salmonella* between 0.5 log cfu/sample (King et al., 2012) and 1.8 log cfu/cm^2 (Fabrizio and Cutter, 2004).

Post-harvest

Once a contaminated carcass leaves the slaughter line, there is no decontamination step available until the product has reached the consumer. Although, the contamination level might be decreased due to the removal of the skin for a large part of the pork meat and due to dilution effects for example in the preparation of minced meat originating from different pig carcasses.

Freezing is a common practice to preserve meat and prevent microbial growth, although it does not eliminate bacterial pathogens in food (Farkas, 1997). Although a clear trend to a decrease of *Salmonella* counts was seen during frozen storage, the survival of *Salmonella* in minced beef (Barrell, 1988) and pork (Escartín et al., 2000) during 10 to 78 weeks of frozen storage was demonstrated. Differences among *Salmonella* serovars were noticed, with *Salmonella* Agona surviving frozen storage better (Escartín et al., 2000).

Good hygiene practices, awareness of possible contamination and education in avoiding cross contamination during food handling are important issues at transformation, distribution and consumer level.

In Belgium, the FASFC stimulates all operators producing or working with food to do this according to a validated auto-control program. Further, some campaigns are running to sensitize the consumers in handling food properly as cross contamination in the kitchen and inadequate cooking often occurs.

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CHAPTER 2

AIMS OF THE THESIS

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Salmonella is an important cause of foodborne illness in humans. The relative contribution of contaminated pork as a source of salmonellosis has increased during the last years. Further EU Member States are obliged to take appropriate measures to reduce the number of salmonellosis in their country by targeting the primary production stage. Each Member State has to consider whether on farm intervention, slaughterhouse intervention or a combination of both offer the optimum control strategy. The general aim of this thesis was to obtain more knowledge on the epidemiology, diagnostics and control of *Salmonella* in the primary production as well as at the slaughterhouse.

Efficient control measures are not possible without proper diagnostic tools. As multiple serotypes can be present in samples from naturally infected animals, the isolation method as well as the origin and the amount of samples taken or colonies tested are very important. Therefore the first specific aim was to investigate the impact of different enrichment media used in the *Salmonella* isolation protocol on the recovery and diversity of *Salmonella* found in the intestinal content of slaughter pigs.

Reducing *Salmonella* infections at herd-level might be very challenging. Several measures are described to lower the *Salmonella* spread and shedding of pigs, although no efficient, practical and economical measure applicable to all kind of herds has yet been described. The second specific aim was to study the effect of the administration of organic acids in the drinking water of slaughter pigs during the last 14 days prior to slaughter on the *Salmonella* prevalence obtained from slaughterhouse samples.

As recent quantitative microbiological risk assessment (QMRA) results suggest that slaughterhouse intervention may lead to a more rapid and greater decrease in salmonellosis than interventions taken at the primary production level, the third specific aim was to study the *Salmonella* prevalence at different stages of the slaughter process and to search for clonal relationships and associations between positive samples.

CHAPTER 3

EFFECT OF THE ENRICHMENT MEDIUM ON THE DETECTION AND DIVERSITY OF *SALMONELLA* FROM PORCINE DUODENAL CONTENT

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Modified from Foodborne Pathogens and Disease: accepted (2012)

Abstract

This study assesses the effect of the enrichment medium used on the isolation of *Salmonella* from the duodenal content of naturally infected slaughter pigs. From 6 slaughterhouses, the duodenum was collected from 458 randomly chosen pigs and examined in the laboratory. Three semi-solid enrichment media (modified semi-solid Rappaport-Vassiliadis medium (MSRV), diagnostic semi-solid *Salmonella* medium (DIASALM), and Simple Method *Salmonella* agar) and three enrichment broths (Rappaport-Vassiliadis, Rappaport Vassiliadis broth with Soya (RVS), and Muller Kauffmann Tetrathionate novobiocin broth (MKTTn)) were evaluated. If a migration zone was present on the semi-solid media, a loopful was taken both near the inoculation drop and at the edge of the migration zone and streaked on a Xylose Lysine Desoxycholate (XLD) agar plate. Each enrichment broth was streaked on XLD and three presumptive colonies were further examined. Detection rate was calculated and isolates were, after serotyping, genotyped by performing pulsed-field gel electrophoresis (PFGE). The overall frequency of *Salmonella* isolated in at least one of the six different media was 15.5% (71/458). No significant differences in relative sensitivity were obtained within semi-solid media and within liquid media. Semi-solid media showed a significant higher relative sensitivity than the one obtained with liquid media. A relative sensitivity higher than 83.1%, namely of 94.4%, could only be obtained by combining three different enrichment media (MSRV+DIASALM+RVS or MKTTn). In 13.4% of the positive pigs more than one serotype was found within the duodenum of one pig. In 12.9% of the duodenal contents different genotypes were found within the same serotype. Differences in serotypes and genotypes were found predominantly within the same enrichment medium. In conclusion, to obtain the highest *Salmonella* detection rate in naturally contaminated pig samples, MSRV should be used as enrichment medium. However, to obtain a realistic picture of the sero- and genotypes present, different samples per enrichment medium and different enrichment media should be tested.

Introduction

EU directives foresee reduction targets for *Salmonella* in food and animal populations as part of the overall EU strategy to reduce food-borne diseases in humans (EC No 2160/2003). In this context, several surveys obtaining reliable and comparable data on the *Salmonella* prevalence in pigs in EU Member States have already been carried out (Hald et al., 2003; EFSA, 2008; EFSA, 2010a). Further, national monitoring programs have been established in which bacteriological isolation forms an important part. To obtain comparable results from different countries, it is essential that *Salmonella* isolation procedures are performed accurately and in a standardized manner.

Different media and culture methods are available for the isolation of *Salmonella.* The detection of *Salmonella* spp. in animal feces and in samples from primary production is described in ISO 6579:2002/Amd 1 2007, in which the selective enrichment medium used is the modified semi-solid Rappaport-Vassiliadis (MSRV), developed for the detection of motile *Salmonella* (EFSA, 2010b) and in which subculture is carried out from the migrated culture, with the inoculum taken from the edge of the visual growth zone. Non-motile *Salmonella* are therefore not isolated. As only *Salmonella* Gallinarum and *Salmonella* Pullorum are not motile (May and Goodner, 1927) and are host specific (poultry), the use of MSRV should not lead to false negative results in *Salmonella* isolation from porcine samples. However, not all Salmonellae have the same capacity to migrate on the medium and some may have evolved into non-motile variants (Grimont et al., 2000).

Previous research has shown that the semi-solid media diagnostic semisolid *Salmonella* medium (DIASALM) and MSRV are not suitable for all *Salmonella* serotypes (O'Donoghue and Winn, 1993; Read et al., 1994). A combination of one of these with the liquid medium Rappaport-Vassiliadis (RV) leads to a higher detection rate of *Salmonella* (Voogt et al., 2001). A further complication is that multiple serotypes can be present in samples from naturally infected animals (Funk et al., 2000; O'Carroll et al., 1999), raising the question whether all of these have an equal chance of being detected (Singer et al., 2009). Probably a particular serotype grows over the others during incubation. This selection pressure has already been described by Harvey and Price's (1967) demonstrating that different *Salmonella* serotypes had different growth characteristics in the same selective enrichment broths. However, Singer et al. (2009) suggest that the factors influencing this *in vitro* variability are not solely due to growth competition among *Salmonella* serotypes, as

inconsistent results were also found in a fecal experiment tube containing a single *Salmonella* strain.

Further questions involve the number of samples which should be taken and how many suspected colonies derived from the enrichment medium should be tested. These often depend on the objective of the study (*e.g.* to indicate the samples as *Salmonella* positive or negative, to study the epidemiology of *Salmonella* or to investigate the origin of contaminated food involved in an outbreak). As in the latter two, identifying the sero- and genotypes present is of major importance.

In this study the impact of six different enrichment media on the detection rate and diversity of *Salmonella* from duodenal content of slaughter pigs is examined.

Materials and Methods

Samples

The study was conducted from December 2006 to August 2007. A total of 458 pigs were randomly selected from six different slaughterhouses (A to F). In total, 22 slaughterhouse visits were performed (2-10 visits per slaughterhouse; 10-28 pigs per visit) and 56 different slaughter batches were included. (A slaughter batch contains pigs originating from the same herd.) During evisceration, stomach-gut packages were collected, the duodenum was ligated, taken out, transferred into a sterile bag and transported to the laboratory.

Salmonella *isolation*

Upon arrival in the laboratory, the samples were immediately processed for *Salmonella* isolation. Each duodenum was immersed in 95% ethanol and dried in air before being cut open with sterile utensils. Ten grams of duodenal content was diluted 1/10 with buffered peptone water (BPW, Bio-Rad, Marnes-La-Coquette, France), homogenized in a stomacher blender and incubated at 37°C. After 16-20h, 0.1 ml of the BPW broth was added to 10 ml RV (Oxoid Ltd, Hampshire, UK) and 10 ml of Rappaport Vassiliadis broth with Soya (RVS, Bio-Rad), spotted on DIASALM (Lab M Ltd. Topley House, Lancashire, UK) and Simple Method *Salmonella* (SMS, AES Chemunex, BruzCedex, France) agar, and dispersed in three drops on MRSV (Lab M Ltd. Topley House). Subsequently, 1 ml of the

BPW culture was also added to 10 ml Muller Kauffmann Tetrathionate novobiocin broth (MKTTn, Oxoid Ltd.). After incubation of the enrichment media for 24h at 42°C (but 37°C for MKTTn), the DIASALM, SMS and MSRV plates were examined for the presence of typical migration zones and a loopful of the migration zone near the inoculation drop and also from the edge of the migration zone was streaked on a Xylose Lysine Desoxycholate (XLD, Bio-Rad) agar plate. A loopful from each RV, RVS and MKTTn enrichment broth was also streaked on a XLD agar plate. After incubation for 24h at 37°C, all XLD plates were examined for the presence of typical colonies, ranging from pink colonies with large, glossy black centers to almost completely black ones. From the semi-solid agars and the enrichment broths, one and three typical colonies respectively per XLD plate were selected for identification. In this way, a maximum of 15 colonies per duodenal sample was obtained. Collected isolates were biochemically tested using triple sugar iron, indol and lysine.

Evaluation of strain motility

In nine pigs, *Salmonella* could only be isolated after enrichment in one or more broths. The isolates (n=18) obtained from these pigs were cultured in Trypto-Casein-Soy Broth (TSB) (Bio-Rad) for 24h in 37°C. After overnight incubation, two dilutions (10^6 and 10^3 cfu/ml) were made in 0.1% Peptone Water. Of these dilutions 0.1ml was spotted on MSRV in three drops and incubated for 24h at 42°C. The migration capacity of the strains was evaluated by checking the presence of a migration zone on the MSRV agar after incubation (Fig. 1). As a reference, 31 isolates obtained from the enrichment broths, but originating from a duodenal sample that tested positive on all enrichment media were included and submitted to the above mentioned culture method.

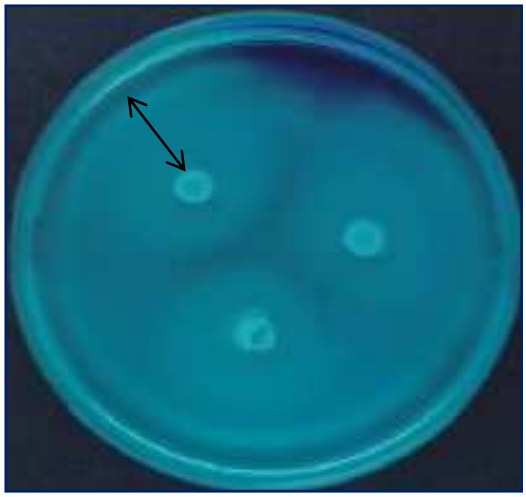

Figure 1: A *Salmonella*-positive MSRV agar plate with the migration zone indicated by the black arrow

Salmonella *serotyping*

To limit the number of strains that had to be serotyped, all *Salmonella* isolates were subjected to an enterobacterial repetitive intergenic consensus (ERIC) PCR (Rasschaert et al., 2005). At least two isolates per cluster were then selected and serotyped by the Belgian reference laboratory for *Salmonella* (Veterinary and Agrochemical Research Centre, Ukkel, Belgium) using the Kaufmann-White scheme (Popoff and Le Minor, 1992).

Salmonella *genotyping*

When more than one isolate with the same serotype was present in a duodenal sample, characterization on strain level was performed by pulsed-field gel electrophoresis (PFGE). The PulseNet protocol (Ribot et al., 2006) was used with the following modification. The running condition was 6V/cm at 14°C in 0.5 x Tris-Borate-EDTA buffer for 20h with a ramping time from 2.2 to 54.2. Profiles were obtained by GelCompar II (3.5) (Applied Maths, Sint-Martems-Latem, Belgium) using the Dice coefficient with 2.1% position tolerance and clusters were generated through the unweighted pair group method using arithmetic averages algorithm (UPGMA). A PFGE genotype was assigned on the basis of a difference in at least one band in the *XbaI* fingerprint. Genotypes within serotypes are indicated below by the capital of the name of the serotype followed by a number (e.g. *Salmonella* Typhimurium genotype 1 is indicated as T_1).

Statistical analysis

The relative sensitivity of each culture method was calculated as the number of positive duodenal samples detected by that method divided by the total number of samples that tested positive by at least one of the six methods (gold standard).

Possible differences between the proportions of positive pigs obtained with each culture method were analyzed using McNemar tests (SPSS version 19). The statistical level of significance used was $P<0.05$.

To detect the agreement between the results of the culture methods used, kappa values were calculated (SPSS 19). Interpretation of the kappa values was made according to Landis and Koch (1977): ≤ 0: poor agreement, 0.01 to 0.20: slight agreement, 0.21 to 0.40: fair agreement, 0.41 to 0.60: moderate agreement, 0.61 to 0.80: substantial agreement, 0.81 to 1.00: almost perfect agreement.

Results

In 15.5% (71/458) of the samples, *Salmonella* was isolated by at least one of the media. The relative sensitivity of each is shown in Table 1.

Table 1: Relative sensitivities of enrichment media used for *Salmonella* isolation from duodenal content of slaughter pigs (71 items positive in at least one method)

Enrichment medium	Number of positive pigs	Relative sensitivity (%)
MSRV	59	83.1
DIASALM	58	81.7
SMS	58	81.7
RV	40	56.3
RVS	39	54.9
MKTTn	34	47.9
All media	71	100

No significant differences were found between the proportion of positive samples obtained by the different semi-solid media or by the different liquid media. However, the proportion of positive samples obtained by the semi-solid media significantly ($P<0.05$) differ from those obtained by the liquid media (Table 2).

Calculation of the kappa value reveals an almost perfect agreement between MSRV and DIASALM ($\kappa=0.95$), between MSRV and SMS ($\kappa=0.95$), and between DIASALM and SMS ($\kappa=0.94$). Between all the other media, a substantial ($\kappa=0.61$-0.80) agreement was found (Table 2).

Table 2: Results of McNemar test and level of agreement (kappa values) for different enrichment media

		Kappa-values					
		MSRV	DIASALM	SMS	RV	RVS	MKTTn
p-value	MSRV		0.95	0.95	0.74	0.68	0.61
McNemar	DIASALM	1.00		0.94	0.75	0.67	0.62
test	SMS	1.00	1.00		0.73	0.69	0.62
	RV	< 0.01	< 0.01	< 0.01		0.74	0.65
	RVS	< 0.01	< 0.01	< 0.01	1.00		0.63
	MKTTn	< 0.01	< 0.01	< 0.01	0.31	0.42	

The combined use of MSRV with RVS, MSRV with MKTTn or DIASALM with RVS increased the detection rate to 63 (88.7%) positive samples. Combining MSRV or DIASALM with RVS and MKTTn led to a rate of 94.4% (67/71 positive duodenal samples).

In total, 113 isolates were collected from the MSRV media, of which 57 were picked close to the inoculation drop and 56 at the edge of the migration zone. On DIASALM and SMS, 51 and 50 isolates respectively were picked close to the drop and 55 and 49 respectively at the edge of the migration zone.

From each slaughterhouse, 4-29 pigs tested positive and *Salmonella* was isolated in the duodenal content of pigs originating from 28 (50%) slaughter batches. For each batch, an average of 6.5 pigs were sampled with 2.5 of these (on average) being *Salmonella* positive.

Evaluation of strain motility

In 4 of the 9 pigs were *Salmonella* was only isolated after enrichment in one of the broths, serotyping of the isolates (n=9) was not possible (Table 3). Six of the 9 isolates were able to migrate in MSRV when inoculated at the concentration level of 10^6/ml. When inoculated at the lower concentration level (10^3) only 2 did migrate on MSRV. In 5 pigs, *Salmonella* could only be isolated using RV and MKTTn (n=9) and different serotypes were identified (Typhimurium (n=3), Arizonae (n=3), Derby (n=2) and O4:i:- (n=1)). Eight of the nine isolates were able to migrate on MSRV when inoculated at the concentration level of 10^6/ml. When inoculated at the lower concentration level (10^3), this number was declined to three. One of the three *Salmonella* Typhimurium isolates showed no migration capacity when inoculated at both concentration levels.

The 31 isolates selected from duodenal samples where *Salmonella* could be isolated from all the enrichment media, showed full migration capacity on MSRV when inoculated at both concentration levels.

Salmonella *serotyping*

In total, 595 isolates from the duodenal content of 71 pigs were further serotyped. Table 3 shows the number of pigs for which a particular serotype had been isolated by the different enrichment media used. In general, *Salmonella* Typhimurium was less isolated after enrichment in RVS and *Salmonella* Derby after enrichment in RV. *Salmonella* Anatum was equally detected in all types of enrichment media used, but was in terms of percentage more identified after enrichment in the different broths. *Salmonella* Brandenburg and *Salmonella* Rissen were not isolated after enrichment in MKTTn, while *Salmonella* O4:i:- was not detected after enrichment in DIASALM and SMS. Further, it was not possible to isolate *Salmonella* Ohio from MSRV, SMS and MKTTn. *Salmonella* Arizonae could only be detected in isolates of MKTTn (Table 3).

Table 3: Number of pigs (with percentages) positive for specific *Salmonella* serotypes using different enrichment media

	MSRV	DIASALM	SMS	RV	RVS	MKTTn	Total positive pigs
Total positive pigs	59	58	58	40	39	34	71
S. Typhimurium	41 (69.5%)	41 (70.7%)	38 (65.5%)	25 (62.5%)	19 (48.7%)	21 (61.8%)	45
S. Derby	12 (20.3%)	12 (20.7%)	13 (22.4%)	7 (17.5%)	10 (25.6%)	7 (20.6%)	15
S. Anatum	3 (5.1%)	3 (5.2%)	3 (5.2%)	3 (7.5%)	3 (7.7%)	3 (8.8%)	3
S. Infantis	1 (1.7%)	1 (1.7%)	1 (1.7%)	1 (2.5%)	1 (2.6%)	1 (2.9%)	1
S. Brandenburg	2 (3.4%)	1 (1.7%)	1 (1.7%)	1 (2.5%)	1 (2.6%)	0	2
S. Rissen	3 (5.1%)	1 (1.7%)	2 (3.4%)	1 (2.5%)	1 (2.6%)	0	4
S. O4:i:-	1 (1.7%)	0	0	1 (2.5%)	1 (2.6%)	2 (5.9%)	3
S. Ohio	0	1 (1.7%)	0	1 (2.5%)	1 (2.6%)	0	1
S. Arizonae	0	0	0	0	0	1 (2.9%)	1
Auto-agglutinated	0	0	0	1 (2.5%)	4 (10.2%)	0	4
Total number of identified serotypes/medium	7	7	6	8	8	6	

S.: *Salmonella*

No *Salmonella* could be detected from the edge of the migration zone on SMS (n=9), MSRV (n=3) and DIASALM (n=3) in nine animals, while *Salmonella* (Typhimurium, Derby and Brandenburg) was isolated from cultures taken close to the inoculation drop. In 15 pigs, *Salmonella* (Typhimurium, Derby and Rissen) could be obtained from the edge of the migration zone, but not close to the inoculation drop (SMS (n=8), DIASALM (n=7) and MSRV (n=2)).

The number of serotypes found in the isolates of a single slaughter batch varied from one to three. In the majority of the positive pigs (86.6%), only one serotype was present, in the remaining pigs two serotypes (Table 4). Differences in serotypes amongst all the isolates were found between and within the media used.

Salmonella *genotyping*

Genotyping was performed on 578 isolates, resulting in 34 different genotypes. For *Salmonella* Typhimurium, 19 genotypes were obtained, for *Salmonella* Derby five, for *Salmonella* O4:i:- three, for *Salmonella* Rissen and *Salmonella* Brandenburg two and for the remaining serotypes one. In MSRV 82.3% of the genotypes were identified, in DIASALM 73.5%, in SMS 64.7% and in the liquid media RV, RVS and MKTTn, 58.8%, 44.1% and 50.0% respectively. Different genotypes (151 isolates) within and/or between the media were found in the duodenal content of 15 (24.2%) pigs. In eight pigs, different genotypes were found within the same serotype. The different genotypes were detected between the different media and within the same medium (Table 5).

The number of genotypes found in the duodenal content of pigs per slaughter batch varied from one to six. The number of different genotypes in each pig varied from one to four, with one genotype found in 77.6% of the pigs, two genotypes in 16.4%, three in 4.5% and four in 1.5%.

Table 4: Breakdown of 9 *Salmonella*-positive pigs with more than one serotype in their duodenal content according to the type of enrichment medium used. From the enrichment broths (RV, RVS and MKTTN) three (1-3) presumptive *Salmonella* colonies were further analyzed.

Pig	MSRV		DIASALM		SMS		RV			RVS			MKTTn		
	close	edge	close	edge	close	edge	1	2	3	1	2	3	1	2	3
7	Derby	Derby	Ohio	Derby	Derby		Ohio			Ohio					
15	Brand		Derby		Derby		Derby			Derby			Derby		
57	Derby	Derby	Derby	Derby	Derby	Derby	Rissen			Derby	Derby	Derby	Derby	Derby	Derby
278	Typh	Typh	Typh	Typh	Typh	Typh	Typh	Typh	Typh	Typh	Typh	Typh	O4:i:-	O4:i:-	Typh
280	O4:i:-	Typh	Typh	Typh	Typh	Typh	O4:i:-	Typh	Typh	O4:i:-	Typh	Typh	Typh	Typh	Typh
282	Typh	Typh	Typh	Typh	Typh	Typh				Typh	Typh	Derby			
323	Rissen	Typh	Typh	Typh		Rissen	Typh	Typh	Typh						
325	Typh	Rissen	Typh	Rissen		Rissen									
327	Typh	Rissen	Typh	Typh		Typh				Rissen	Rissen				

close: at start of migration zone / edge: at edge of migration zone / Typh.: Typhimurium / Brand.: Brandenburg

Table 5: Breakdown of 8 *Salmonella*-positive pigs with more than one genotype within the same serotype in their duodenal content according to the type of enrichment medium used. From the enrichment broths (RV, RVS and MKTTN) three (1-3) presumptive *Salmonella* colonies were further analyzed.

SH	Batch	Pig	MSRV		DIASALM		SMS		RV			RVS			MKTTn		
			close	edge	close	edge	close	edge	1	2	3	1	2	3	1	2	3
B	9	139	T_{19}	T_{19}	T_{19}	T_{9}	T_{19}	T_{19}	T_{19}	T_{19}	T_{9}	T_{19}	T_{19}	T_{19}	T_{19}	T_{19}	
		142	T_{9}	T_{13}	T_{15}	T_{15}	T_{15}	T_{15}	T_{15}	T_{15}		T_{15}	T_{15}		T_{15}	T_{15}	
	15	273	T_{13}	T_{13}	T_{13}	T_{13}	T_{16}	T_{13}	T_{13}	T_{13}	T_{13}	T_{13}	T_{13}	T_{13}	T_{13}	T_{13}	T_{13}
		280	O4:i:-$_{1}$	T_{13}	T_{13}	T_{13}	T_{13}	T_{13}	O4:i:-$_{1}$	T_{13}	T_{13}	O4:i:-$_{3}$	T_{13}	T_{13}	T_{13}	T_{13}	T_{13}
	16	314	T_{14}	T_{12}	T_{12}	T_{12}	T_{14}								T_{12}		
D	22	325	T_{6}	R_{2}	T_{7}	R_{1}		R_{1}									
	24	353	T_{4}	T_{4}		T_{4}			T_{4}	T_{2}	T_{4}						
F	28	456	T_{14}	T_{11}	T_{11}	T_{14}	T_{1}	T_{1}	T_{14}	T_{1}	T_{1}				T_{1}	T_{1}	T_{1}

SH: slaughterhouse / Batch: slaughter batch / T: Typhimurium / R: Rissen / close: at start of migration zone / edge: on edge of migration zone

Discussion

The detection rate of *Salmonella* isolated from naturally contaminated pig samples depends on the enrichment medium used. This study showed that although semi-solid media are most suitable as enrichment media, a combination of different media is necessary to increase the relative sensitivity. Migration capacity can be influenced by the *Salmonella* serotype (auto-agglutinated or not) and depends on the concentration level of the organism in the sample. Isolates originating from liquid media only were in 77.8% of cases able to migrate on MSRV when inoculated from a high concentration. At a lower concentration (10^3 cfu/ml), migration capacity decreased to 27.8%. If *Salmonella* is present in the pre-enrichment broth (BPW) at low concentrations, it can easily be missed when semi-solid media are used in the isolation protocol (ISO, 2007). In our procedure, the combination of MSRV or DIASALM with either RVS or MKTTn increased relative sensitivity from 83.1% (MSRV) or 81.7% (DIASALM) up to 88.7%. These results are in accordance with those obtained by Voogt et al. (2001), Dam-Deisz et al. (2003), and Botteldoorn et al. (2003), indicating that combining media (MSRV or DIASALM with RV) yields a higher number of *Salmonella* positive samples. The highest relative sensitivity (94.4%) in this study was achieved only by combining three different enrichment media (one semi-solid and two liquid media), increasing the labor intensity and therefore the costs involved.

The distribution of the serotypes showed that some serotypes were less or not recovered from certain media. This finding is also reported by Dam-Deisz et al. (2003), although the patterns of serotype detection in the different media used by these authors (MSRV, DIASALM and RV) are not similar to those found in the present study. Future experimental studies with different serotypes in porcine fecal samples will allow us to gain more insight into the behavior of *Salmonella* serotypes in the standard MSRV medium, and also in other enrichment media. Singer et al. (2009) showed already that the probability of detecting a specific *Salmonella* serotype in a sample depends on its ability to compete in the cultivation media and on the specific mixture of *Salmonella* bacteria present in the sample. However, explanations for this variability within serotypes need to be further examined.

More than one serotype was identified in the duodenal content of 13.4% of the pigs. The isolation of multiple serotypes from individual pigs has previously been reported (Kampelmacher et al., 1962; O'Carroll et al., 1999; Funk et al., 2000) and is important regarding epidemiological studies. Rostagno et al. (2005) demonstrated that asynchronous

growth curves among serotypes were due to the selective enrichment media used in the *Salmonella* isolation protocol. In our study differences in serotypes were predominantly found within the same media, demonstrating that it is useful to select more than one colony per sample. For semi-solid media, samples should be taken both close to the inoculation drop and also on the edge of the migration zone. In this way, less motile *Salmonella* serotypes can also be detected. An explanation for the decreased motility of some strains on MSRV cannot be given by the results of this study, as all genotypes (except one) which were found at the start of the migration zone but not on the edge migrated in samples belonging to other pigs. It is possible that the salmonellae were overgrown by competitive bacteria present in the duodenal content of the pig, hindering isolation. In total, 34 genotypes were found, with the largest variation within the serotype Typhimurium. In 13% of the duodenal content, different strains were found within the same serotype. These differences were predominantly seen within the same medium. This finding again emphasizes the fact that examining more parts of the migration zone of semi-solid media or more colonies derived from the enrichment broths increases the probability of detecting multiple genotypes.

Conclusions

This study shows that even by sampling a rather low number of pigs multiple sero- and genotypes can be detected within a slaughter batch as well as within one pig. Taking into account the variation resulting from the type of enrichment medium used and the number of colonies taken, it is clear that obtaining realistic and valid data is a real challenge. Although increasing the number of colonies analyzed and using multiple enrichment media inherently involve higher costs and more labor, on occasions when detecting the *Salmonella* source is of primary importance, such as in outbreak investigations, it should be seriously considered.

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CHAPTER 4

EFFECT OF ORGANIC ACIDS IN DRINKING WATER DURING THE LAST TWO WEEKS PRIOR TO SLAUGHTER ON *SALMONELLA* SHEDDING AND CARCASS CONTAMINATION

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Modified from Zoonoses and Public Health (2009) 56, 129-136

Abstract

In this study, we investigated the effect of adding organic acids to the drinking water of finishing pigs 2 weeks prior to slaughter on the shedding and prevalence rate of *Salmonella* at slaughter. Approximately 600 animals from four Belgian pig herds infected with *Salmonella* were included. At two herds, the study was conducted twice. Before the start of the study, overshoes were taken at the different herds. Two weeks prior to the expected slaughter date, the pigs were randomly divided into two groups (treatment and control group) each containing on average 50 animals within each herd. The treatment group received from this day onwards acidified drinking water (pH = 3.6–4.0), the control group received non-treated water (pH = 7.8–8.5). All other housing, feeding and management factors were identical in both groups. At the slaughterhouse, 10 pigs of each group (20 pigs for each group of study group 6) were randomly selected and sampled (blood, contents of ileum and rectum, mesenteric lymph nodes and carcass swabs). All samples were immediately transported to the laboratory and submitted to *Salmonella* isolation. *Salmonella* was isolated out of 11.9% (66/554) of the samples taken at the slaughterhouse, with the highest frequency found in the content of the ileum (18.7%), followed by 17.8% in the lymph nodes, 7.2% in the content of the rectum and 3.6% in the carcass swabs. The results did not reveal a significant difference between the treatment and control groups for the different slaughterhouse samples. The study documented that the investigated control strategy namely, the strategic application of organic acids during the last 2 weeks prior to slaughter was insufficient to decrease *Salmonella* shedding and contamination shortly before and during slaughter.

Introduction

Salmonella is one of the most important zoonotic pathogens and the consumption of pork meat is a major source of human infection (Van Loock et al., 2000). In 2011, 3231 infections in human had been registered in Belgium, of which *Salmonella* Typhimurium (62.8%) was the most occurring serotype (NRRS – National Reference Centre for *Salmonella* and *Shigella* 2011). In 2010, the reported number of cases of human salmonellosis in the EU were 99 020 cases (European Food Safety Authority, Community Summary Report on Zoonoses, 2012).

Salmonella is known to survive well in the environment (Sandvang et al., 2000) and pigs become mostly orally infected with the organism by a contaminated environment or through direct contact with *Salmonella* shedding pen-mates (Schwartz, 1999). Nollet et al. (2005) demonstrated that the number of animals shedding *Salmonella* clearly increased at the time of stress such as transfer to another unit. Transport to the slaughterhouse and waiting in the lairage area are also stressful moments for the pigs (Isaacson et al., 1999; Seidler et al., 2001) resulting in a higher risk of carcass contamination and a potential danger for food safety.

Different strategies have been investigated to control *Salmonella* in the pig industry including the use of organic acids in the feed. Several studies revealed the benefit of acidifying feed and/or drinking water during a long period in the reduction of the *Salmonella* prevalence (van der Wolf et al., 1999, 2001; Creus et al., 2007). The effectiveness of fermented liquid feed, containing a large amount of organic acids and large numbers of lactic acid producing bacteria or organic acids administered in drinking water is largely based on the fact that the pH is lowered to a level of 4.2 or lower at which Enterobacteriacea cannot multiply (Ostling and Lindgren, 1993). In addition, organic acids have the ability to enter bacteria in their non-dissociated form and affect the protein and DNA synthesis of this bacteria after dissociating in the cell (Rubin et al., 1982; Kirchgessner et al., 1992).

While the effectiveness of the long-time application of organic acids is well established, no information is available on the strategic use of acidified drinking water prior to slaughter. If contamination could be reduced, this would entail major economic benefits to the farmer.

The aim of this study was to evaluate whether the strategic use of acidified drinking water during only 14 days prior to slaughter was able to affect the shedding of *Salmonella* and to influence the *Salmonella* prevalence at the slaughterhouse.

Materials and Methods

Study population and experimental design

This study took place from April 2006 until February 2007 and was conducted on four different Belgian farrow-to-finish herds (A, B, C and D). In herds A and D, the study was performed twice, resulting in six study groups (Table 1). All the herds were chosen from a list containing the herds with the highest *Salmonella* infection status as determined by serology in the Belgian *Salmonella* Surveillance Program (Animal Health Care Flanders, 2006, personal communication).

Two weeks prior to the expected slaughter date, the pigs in each herd were randomly divided into two groups (treatment and control group) each containing on average 50 animals. The treatment group received from this day onwards acidified drinking water. A mixture of different organic acids (Nutri-Ad) was added by means of a dose-measuring pump (Medikator unit Mini, Schippers, Belgium) until a pH of maximum 4 was achieved. This mixture of organic acids contained formic acid (50%), propionic acid (10%), acetic acid (16%), sorbic acid and a liquid carrier. During the treatment period, the pig farmer or the person in charge of the pig herd was asked to write down and to adjust the pH of the drinking water daily and to control the drinking nipples regularly to avoid an insufficient water supply due to clogging.

The concentration of the product in the drinking water varied among the different herds and ranged between 0.25% and 0.40%. The control group received the normal, untreated drinking water (deep pit water) from which the pH and the quality were measured before the start of the study.

All other factors including housing and feeding were identical in both groups. All finishing units of the different herds were equipped with a fully slatted floor. At herd A, a wet feed system (drinking nipple in the feeding trough) was used during the whole fattening period; at the other herds, dry feed (meal) was provided. The number of animals per pen varied between 10 and 14 pigs. Animals were fasted 4–24 h before transport to the slaughterhouse, but the drinking water was not closed off.

The animals in each group were individually identified at the time of loading and held separately during transport and in the lairage area. The time between loading and slaughtering was registered. Further, it was also registered which group (treatment or control) was slaughtered first and whether the pigs were slaughtered first in the morning.

Five different slaughterhouses, located in Flanders, took part in this study. Only study group 1 and 5 were slaughtered in the same slaughterhouse (Table 1).

Sample collection

Overshoes were taken at the different herds (except for herd A where the study started) one week before the start of the treatment period. Samples were taken from every pen containing pigs expected to be slaughtered at the end of the treatment period (8–12 overshoes per study group). On top of disposable plastic overshoes, woven overshoes with a high absorbing capacity were added. The latter were changed after sampling a pen by moving through the pen along the side walls in a lingering way. A sample of the drinking water was taken one week before the start of the study at the source of the drinking water. This sample was immediately transported to the laboratory for bacteriological, chemical and macroscopic examination.

Ten randomly selected pigs of each group were sampled in the slaughterhouse (20 per group in study group 6) (Table 1). The total number of sampled pigs allowed detecting a reduction in prevalence of 20% to 5% with a confidence level of 95% and a power of 80%. Blood samples were taken at exsanguination. After evisceration, contents of ileum and rectum as well as mesenteric lymph nodes were collected. Carcass swabs were taken after chilling. One side of the carcasses was swabbed, according to the procedure described by the EU Regulation EC No 2073/2005 of 15 November 2005. The outside of the ham (100 cm^2) and the shoulder (100 cm^2) were swabbed together with the pelvic canal (100 cm^2) and the sternum along the incision line (300 cm^2). All swabs from one carcass were pooled into a sterile stomacher bag. All samples were immediately transported to the laboratory for further processing.

Sample analyses

Overshoes and samples taken at the slaughterhouse were submitted to *Salmonella* isolation. Two hundred and twenty five milliliter buffered peptone water (BPW) was added to the overshoes samples and thereafter they were manually shaken. Ten grams of content of

ileum and rectum was collected. To avoid cross-contamination, the outer walls of the ileum and rectum were sprinkled with alcohol and the intestines were opened with sterile utensils. From each mesenteric lymph node, the fat tissue was aseptically removed. Ten grams of mesenteric lymph nodes was collected using sterile utensils. The samples of the lymph nodes were passed through a flame to decontaminate the surface. Afterwards, they were transferred to sterile stomacher bags. At all weighed samples, 90 ml BPW was added. After homogenization during 1 min with a stomacher blender, the homogenates were incubated at 37°C for 16–20 h. Following incubation, 100 µl was spotted onto a Modified Semisolid Rappaport-Vassiliadis agar (MSRV) plate and incubated for 24 h at 42°C. If migration zones were present on the MSRV plates, a loopful of the edge of the migration zones was streaked on a xylose lysine desoxycholate (CM 469; Oxoid, Hampshire, UK) agar plate. After incubation for 24 h at 37°C, plates were examined for the presence of typical colonies. These colonies were biochemically confirmed.

The blood samples were analyzed using a commercial indirect mix-ELISA (HerdCheck Swine *Salmonella* Antibody Test Kit; Idexx Laboratories, Inc., Westbrook, ME, USA).

Salmonella *serotyping*

All *Salmonella* isolates were sent to the Belgian reference laboratory (Veterinary and Agrochemical Research Centre, Ukkel, Belgium) for serotyping following the Kaufmann–White scheme (Popoff and Le Minor, 1992).

Statistical analyses

Possible differences in the number of positive animals (concerning content of ileum and rectum, lymph nodes and carcass swabs) in both groups (treatment and control) were analyzed using logistic regression analyses (SPSS 15.0; STATCON, Witzenhause, Germany). The percentage of positive animals was the dependent variable and the treatment group, the independent variable. The effect of the herd (as a fixed factor) was also included in the statistical models. The serological results of the different study groups were submitted to an univariate analysis of variance (SPSS 15.0). Differences between treatment and control groups were considered significant if *P*-values were lower than 0.05 (two-sided test).

Results

Herd and transport information

The pH of the drinking water of the control group, measured once before the start of the study at the source of the normally used drinking water, varied between 7.8 and 8.5. The pH of the treatment group was measured daily during the study and the average pH of the drinking water in this group varied between 3.6 and 4.0. While testing the quality (chemical and bacteriological) of the provided drinking water, no abnormalities were found.

The initial average S/P ratios of the different herds indicated a high (S/P ratio > 1) antibody level.

The transport time from the herd to the slaughterhouse ranged from 1 to 3.5 with an average of 2.38 h ± 0.85.

Only pigs from herd B and C were slaughtered as first batch in the morning.

Prevalence of Salmonella *in the different samples*

At herds B and C, all overshoes (12 and 8, respectively) tested negative for *Salmonella*. Isolation of *Salmonella* from overshoes taken in herd D revealed one positive sample in each study group (in study group 4, eight overshoes were taken, in study group 6, 11 overshoes were taken) (Table 2). In both cases, *Salmonella* Typhimurium O5+ was isolated out of pens belonging to the control group (Table 2).

The serological results of the blood taken at the slaughterhouse are shown in Table 1. No serological results for study group 1 and 3 are available because the samples were lost in the laboratory. The average S/P ratio was calculated for the control and treatment group of the different study groups. The average S/P ratios of herds B and D indicated a low- (S/P ratio 0.25–0.50) to-moderate (S/P ratio 0.5–1) antibody level. However, study group 5 (herd A second time) had a high antibody level (S/P ratio 1–2). In all study groups, the differences in S/P ratio between treatment and control group were small and statistically not significant (P = 0.57).

Table 1: Serological and bacteriological results of the samples taken in the slaughterhouse of the pigs of the treatment and control group from four different herds

SG	Herd	SH	Group	Blood (SD)	IL	R	LN	CS	Total*
1	A	1	Treatm.	ND	2/10	0/10	3/10	3/10	6/10
			Control	ND	4/10	1/10	5/10	0/10	6/10
2	B	2	Treatm.	0.46 (0.112)	0/10	0/10	0/10	0/10	0/10
			Control	0.55 (0.305)	0/10	0/10	0/10	0/10	0/10
3	C	3	Treatm.	ND	1/10	2/10	1/10	1/10	3/10
			Control	ND	2/9	0/8	3/10	0/10	4/10
4	D	4	Treatm.	0.91 (0.391)	3/10	1/10	1/10	0/10	3/10
			Control	0.81 (0.264)	2/10	0/9	1/10	0/10	3/10
5	A	1	Treatm.	1.47 (0.525)	2/10	4/10	6/10	0/10	8/10
			Control	1.75 (0.325)	3/10	2/10	5/10	0/8	8/10
6	D	5	Treatm.	0.48 (0.265)	6/20	0/20	0/20	0/20	6/20
			Control	0.35 (0.206)	1/20	0/20	0/20	1/20	2/20
		Total	Treatm.		14/70 (20.0%)	7/70 (10.0%)	11/70 (15.7%)	4/70 (5.7%)	26/70 (37.1%)
			Control		12/69 (17.4%)	3/67 (4.5%)	14/70 (20.0%)	1/68 (1.5%)	23/70 (32.8%)

SG: study group / SH: slaughterhouse / IL: content of ileum (10g) / R: content of rectum (10g) / LN: mesenteric lymph nodes (10g) / CS: carcass swabs / Total*: number of animals tested positive in at least one sample / number of animals tested / Blood: average S/P ratio per group / SD: standard deviation / ND: not determined

The results of the contents of the intestines, the lymph nodes and the carcass swabs are summarized in Table 1. *Salmonella* could be isolated from 66 out of 554 (11.9%) samples taken at the slaughterhouse. For some pigs, not enough content of ileum or rectum was observed because of fasting. *Salmonella* was mostly isolated out of samples belonging to herd A. In herd B, no *Salmonella* organisms were detected.

In general, the highest frequency of *Salmonella* was found in the content of the ileum (treatment group 20.0%, control group 17.4%) and in the lymph nodes (treatment group 15.7%, control group 20.0%). Contents of ileum and rectum, together with the carcass swabs, are slightly more positive (20.0%, 10.0% and 5.7% respectively) in the treatment group than in the control group (17.4%, 4.5% and 1.5% respectively). However, in the lymph nodes, an opposite result was observed.

The difference between treatment and control groups concerning the number of animals tested positive in at least one sample was variable and small (37.1% and 32.8% respectively). None of the observed differences in *Salmonella* prevalence between treatment and control group was statistically significant ($P > 0.05$).

There was always a significant influence of the herd on the *Salmonella* prevalence ($P < 0.01$). A description of the *Salmonella* serotypes isolated from the overshoes taken at the herd and from the different slaughterhouse samples is given in Table 2. The three most prevalent serotypes were *Salmonella* Typhimurium O5+ (67.6% of the isolates), *Salmonella* Typhimurium O5- (11.8%) and *Salmonella* O4:i- (7.3%). In the treatment group of study group 1, *Salmonella* Enteritidis was isolated from the content of the ileum and from the lymph nodes of the same animal. One animal in the control group of this study group was positive in all slaughterhouse samples except for the carcass swabs.

Table 2: *Salmonella* serotypes isolated from the overshoes and slaughterhouse samples. Number of isolates (between brackets)

SG	Herd	Group	Herd	Slaughterhouse samples			
			Overshoes	Ileum	Rectum	Lymph nodes	Carcass swabs
1	A	Treatm.	ND	Typh O5- (1) Enteritidis (1)	negative	Typh O5+ (2) Enteritidis (1)	Derby (2) Agona (1)
		Control	ND	Typh O5+ (4)	Typh O5+ (1)	Typh O5+ (5)	negative
2	B	Treatm.	negative	negative	negative	negative	negative
		Control	negative	negative	negative	negative	negative
3	C	Treatm.	negative	Typh O5+ (1)	Typh O5+ (1) Braenderup (1)	Typh O5+ (1)	Typh O5+ (1)
		Control	negative	Typh O5+ (2)	negative	Typh O5+ (2) Typh O5- (1)	negative
4	D	Treatm.	negative	Typh O5- (1) Typh O5+ (2)	Typh O5+ (1)	Typh O5- (1)	negative
		Control	Typh O5+ (1)	Typh O5- (2)	negative	Enteritidis (1)	negative
5	A	Treatm.	ND	Typh O5+ (2)	Typh O5+ (4)	Typh O5+ (6)	negative
		Control	ND	Typh O5+ (3)	Typh O5+ (1) Rissen (1)	Typh O5+ (5)	negative
6	D	Treatm.	negative	Typh O5- (1) O4:i- (5)	negative	negative	negative
		Control	Typh O5+ (1)	Typh O5- (1)	negative	negative	ND

SG: Study group / Typh: Typhimurium / ND: not determined

The positive *Salmonella* Typhimurium O5+ samples in the treatment group of study group 3 belonged to the same animal except for the positive carcass swabs. In the control group, one animal carried *Salmonella* Typhimurium O5+ in the content of the ileum and in the lymph nodes. In study group 4, *Salmonella* Typhimurium O5- was isolated from the content of ileum and in the lymph nodes of the same animal. *Salmonella* Typhimurium O5+ was isolated from the content of ileum and rectum of animals belonging to the treatment group. One animal tested *Salmonella* Typhimurium O5+ positive in both contents. In study group 5, in both treatment and control group *Salmonella* Typhimurium O5+ was found in the content of ileum and rectum and in the lymph nodes. Two animals tested positive in all these samples (one animal belonging to the treatment group and one to the control group). Three different serotypes (Derby, Agona and Typhimurium) were isolated from the carcasses. These contaminated carcasses belonged to pigs slaughtered at two different slaughterhouses.

Discussion

Different studies have demonstrated the benefit of acidifying feed and/or drinking water during a long period (from approximately 25 kg live weight to slaughter age) in the reduction in the *Salmonella* prevalence (van der Wolf et al., 1999, 2001). However, it was not yet known whether strategic administration of acidified drinking water during a limited period of time could also reduce the *Salmonella* prevalence. Therefore, we investigated the effect of acidifying the drinking water during the last 14 days before slaughter.

The participating herds had a high mean *Salmonella* antibody level before the start of the study as determined by serology in the Belgian *Salmonella* Surveillance Program (Animal Health Care Flanders, personal communication, 2006). Studies have shown that there is a moderate correlation between serology and positive bacteriology in the feces at the herd level (Lo Fo Wong et al., 2003). Therefore, we expected that herds with a high mean antibody level were at higher risk for shedding *Salmonella* at the end of the fattening period. However, in herd B, all samples taken at the herd and in the slaughterhouse remained negative for *Salmonella*. The drinking water used in these herds was examined in the laboratory and was declared fit for consumption by pigs. The pH values of the drinking water for the different herds before the start of the study were rather high. Nevertheless, it was possible in all farms to decrease sufficiently the pH of the drinking water of the treatment groups (3.6–4.0) and to maintain these values by adding the appropriate amount of acids.

Because of the intermittent shedding of *Salmonella* in pigs, overshoes were taken before the start of the study to investigate if there was an active shedding at the herd. The overshoes taken at herd D contained the same serotype as found in the content of ileum and rectum of three pigs belonging to the treatment group of study group 4 suggesting that these pigs were infected at the herd or were re-shedding *Salmonella* because of stress during transport and/or lairage.

The results of the samples taken at the slaughterhouse revealed a high *Salmonella* contamination in herd A. *Salmonella* could be isolated in 34.95% of the pigs sampled at the different slaughterhouses. In the study of Botteldoorn et al. (2003), 28% of the pigs carried *Salmonella* in their feces and/or mesenteric lymph nodes. In general, *Salmonella* was mostly detected in the content of ileum (18.7%) and in the lymph nodes (17.6%). In the control group, *Salmonella* was mostly isolated out of the lymph nodes (20.0%) instead of the content of the ileum (17.4%). Botteldoorn et al. (2003) and Vieira-Pinto et al. (2005) also found a higher contamination level of the mesenteric lymph nodes in comparison with the contents of the colon. During slaughter, lymph nodes and intestines containing *Salmonella* can be a primary source of carcass contamination.

The predominant serotype found in the overshoes at the herd and in the slaughterhouse samples was *Salmonella* Typhimurium O5+ (67.6%). *Salmonella* Derby and *Salmonella* Agona were detected on three different carcasses of animals belonging to the treatment group of study group one. These serotypes were only detected on these carcasses and are probably caused by contamination from the slaughterhouse environment.

In general, the *Salmonella* prevalence in the treatment and control groups were not different from each other (37.1% and 32.8%, respectively; $P = 0.92$), hence, the present study could not demonstrate a significant reduction in *Salmonella* positive samples in finishing pigs receiving acidified drinking water for 14 days prior to slaughter. This can be explained by the fact that the period of acidifying was too short, the administered dose was too low and/or the possibility of cross-contamination and infection during transport and lairage.

Acidifying the drinking water is preferably done at any time of stress during the pig's life. However, this implies a higher cost. The administered dose used in this study was sufficient to lower the pH of the drinking water to a $pH = 4$. Further lowering this pH is not recommended because of the decrease in water intake by the pigs (De Busser et al., 2008).

Cross-contamination and infection can occur during transport and in the lairage area (Boes et al., 2001; Hurd et al., 2001). During transport, pigs belonging to a different group (treatment or control) were loaded separately (in different compartments) in a clean and disinfected truck. The use of a separate truck for each group would have been better, but given the fact that this study was conducted under practical field conditions, this was practical, but not economically feasible. Not all pigs were slaughtered as the first batch in the morning. This implies that although held separately in the lairage area, pigs could have been put in already contaminated pens and slaughtered in an already contaminated environment.

Furthermore, cleaning and disinfection may not always be performed adequately and so the presence of *Salmonella* in the truck, lairage area and along the slaughter line is still possible (Swanenburg et al., 2001; Boughton et al., 2007a). Logistic slaughter was not performed in the participating slaughterhouses.

An additional problem in the control of *Salmonella* is the existence of carriers, hiding the pathogen in the internal organs such as the mesenteric lymph nodes (Schwartz, 1999). When *Salmonella* carriers are transported to the slaughterhouse, shedding can be reactivated because of stress. The average transport time in the study was 2.38 h. This corresponds to the average transport time of pigs in Belgium (approximately 2 h). This period is sufficient to enhance shedding that may proceed in the lairage area resulting in cross-contamination of the slaughter pigs (Hurd et al., 2001; Boughton et al., 2007b). In this study, 40% of the serotypes found in the lymph nodes were also detected in the content of the intestines. This percentage is most probably because of carrier animals' reshedding during transport and in the lairage area.

The occurrence of new infections cannot be ruled out (Erdman et al., 2002; Hurd et al., 2002). Stress reduces the immune response and influences the gastrointestinal motility resulting in more susceptible animals and a higher defecation rate (Williams and Newelln, 1967). Therefore, an optimal fasting period of 12–16 h before transport is important. Such a fasting period will also lead to a lower risk for contamination of the carcasses because it reduces the possibility of accidental laceration of the intestines during evisceration. New infections could have occurred because only *Salmonella* Typhimurium O5+ was found in the overshoes of herd D, whereas in the slaughterhouse samples, both *Salmonella* Typhimurium O5- and *Salmonella* Enteritidis were detected. To prevent new infections in the lairage area, it may be an option to acidify the drinking water in the lairage area to try to reduce these

infections after a period of stress. However, this measure would not be completely able to prevent *Salmonella* shedding by carrier animals (when animals carry the organism in the mesenteric lymph nodes).

Despite the fact that our study did not reveal a significant benefit of the used organic acids during a short period, acidifying feed and drinking water at strategic time points earlier in life remains an option to consider in the control of *Salmonella* on pig herds. Yet, the control of *Salmonella* remains a joint responsibility in which all stakeholders along the production line need to take their responsibility.

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CHAPTER 5

DETECTION AND CHARACTERIZATION OF *SALMONELLA* IN LAIRAGE, ON PIG CARCASSES AND INTESTINES IN FIVE SLAUGHTERHOUSES

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Modified from The International Journal of Food Microbiology (2011) 145, 279-286

Abstract

In this study, conducted at five slaughterhouses, individual pigs were sampled and followed up from stunning to cooling down of the carcasses. In this way, *Salmonella* prevalence and possible risk points were described. At the lairage area, pens were sampled using overshoes. At stunning and bleeding, pigs were individually identified and subsequently swabs were taken of the oral cavity, the carcass after polishing, splitting and forced chilling. Additionally, duodenum, ileum, rectum and mesenteric lymph nodes were extracted and samples were taken of the scalding water. All samples were submitted to *Salmonella* isolation and *Salmonella* isolates were serotyped and genotyped by pulsed-field gel electrophoresis (PFGE). Of all samples taken (n=1953), 14.1% was *Salmonella* positive. The prevalence of *Salmonella* in the lairage area varied widely (from 0 to 100%) between the slaughterhouses. Of the sampled pigs (n=226), 48.2% was positive in at least one sample. Statistical analysis revealed that the contamination of the lairage area was related to a higher amount of positive carcasses after polishing. Furthermore, the contamination of the carcasses after splitting and forced chilling was related to the contamination level of the carcass after polishing. A relation between the outer (carcass) contamination and the inner (gut content and lymph nodes) contamination of a pig could not be established. The predominant serotypes were *Salmonella* Typhimurium (58.7%) and *Salmonella* Rissen (17.4%). Genotyping revealed 46 different PFGE profiles amongst the 276 *Salmonella* isolates. The same genotype at the lairage area as in the oral cavity of the pigs was found in 95%. The results indicate that the lairage area is a primary source of *Salmonella* in slaughter pigs and that carcass contamination originates from the environment rather than from the pig (inner contamination) itself. It further shows that slaughterhouses vary in their capability of dealing with *Salmonella* positive pigs. A slaughterhouse specific approach is needed, however, general guidelines should be provided to decrease the contamination level of the lairage area and the slaughter environment.

Introduction

Salmonella is one of the most important zoonotic pathogens in Europe and the consumption of pork meat is a major source for human infection (Van Loock et al., 2000). In Belgium, 3944 *Salmonella* infections in humans have been registered in 2008, of which *Salmonella* Typhimurium (57%) was the most prevalent serotype (NRSS, 2008). However, due to underreporting, the actual incidence of human salmonellosis in Belgium as well as in the EU is much higher (European Food Safety Authority (EFSA), 2008b). In England, it has been determined that for every laboratory-confirmed case, 3.8 cases occurred in the community (Wheeler et al., 1999), for the Netherlands, the multiplier is approximately 13.4 (Kreijl et al., 2006).

In pigs, the most common serotypes are *Salmonella* Typhimurium and *Salmonella* Derby (Letellier et al., 1999; Davies et al., 2004; Gebreyes et al., 2004; Nollet et al., 2004; Valdezate et al., 2005; EFSA, 2006 and Rostagno et al., 2007). Pigs usually get infected through oral intake of the organism. After infection, animals can become carriers in the tonsils, the intestines and the gut-associated lymphoid tissue (Wood et al., 1989; Fedorka-Cray et al., 2000). Most of the time, carriers are not excreting the bacteria but under stressful conditions, re-shedding may occur. In this way, carriers are a permanent potential source of infection for other animals, including humans. Stress factors can occur during the fattening period, but also prior to slaughter, for instance during transport to the slaughterhouse or during the stay in the lairage (Isaacson et al., 1999; Seidler et al., 2001; Rostagno et al., 2010).

Along the slaughter line, several steps can be critical for *Salmonella* contamination: dehairing, polishing, removal of the intestines, removal of the pluck set and meat inspection procedures (Borch et al., 1996). During these steps, the carcass can be contaminated with feces and bacteria can be spread all over the carcass and to subsequent carcasses. Based on a recent EFSA report (EFSA, 2008a), 10.3% of the slaughter pigs in the EU were found to be infected with *Salmonella* in the lymph nodes and 8.3% of the carcasses was contaminated with *Salmonella*. Belgium (13.9%) as well as eleven other EU member states had an observed prevalence above the average EU-level (24 member states) prevalence of *Salmonella* in the lymph nodes. Ireland, Belgium (18.8%), France and the United Kingdom had an observed prevalence of *Salmonella* on the carcasses above the average EU-level (13 member states). Other studies performed in Belgian slaughterhouses showed a carcass contamination of 27% (Korsak et al., 1998) and 37% (Botteldoorn et al., 2003). Interestingly, the study performed by

EFSA (EFSA, 2008a) indicates that only Ireland and Belgium showed a higher observed *Salmonella* prevalence on the carcass than in the lymph nodes. Although not all participating member states took carcass swabs of slaughtered pigs, this finding indicates that cross-contamination in the slaughterhouse is an important feature.

To overcome carcass contamination, it is crucial to identify the sources of contamination throughout the slaughter process. Therefore, the aim of this study was to investigate the prevalence of *Salmonella* contamination along the slaughter line and to identify possible contamination sources. This was achieved by following individual pigs from resting in the lairage until carcasses hanging in the chilling room. In the lairage and along the slaughter line, different samples of the same pig were taken, making it possible to look for accurate associations between positive samples. Furthermore, sero- and genotyping was performed to define clonal relationships between *Salmonella* strains and to assess the distribution of the recovered strains.

Materials and methods

Slaughterhouses

Five pig slaughterhouses (A to E) were selected from the 10 largest Belgian slaughterhouses. They represented 30% of the annual number of pigs slaughtered in Belgium. The five slaughterhouses were visited twice with one to two weeks interval. A description of the slaughterhouses is shown in Table 1. The study was performed from March until August 2007.

Each visit was performed on Tuesday, and sampling started with the first batch of pigs slaughtered that day. One out of 4 to one out of 10 successive pigs (depending on the speed of slaughtering), with a total of 20-28 pigs per slaughterhouse visit, were individually identified at exsanguination and followed-up along the different slaughter steps. The number of herds and the number of pigs originating from these herds during the slaughterhouse visits are shown in Table 1.

Table 1: Description of the five slaughterhouses (A-E) included in the study with the number of sampled herds and number of sampled pigs per herd

SH	SR (pigs/h)	Floor type*	Cleaning*	Disinfection*	Additional information	SH visit	Number of herds	Number of pigs/herd
A	300	Fully slatted	Never	Never	Scalding tank	1	2	10
						2	2	9-10
B	550	Solid	Daily: dry	Twice a year	Scalding tank	1	7	2-4
			Weekly: water under low pressure	(external)	After polishing:second flaming	2	6	1-5
C	580	Solid	Daily: water under high pressure	Daily	Scalding tank	1	4	5-9
						2	3	6-8
D	420	Solid	Daily: water under high pressure	Once a month	Steam tunnel	1	4	1-8
						2	3	5-10
E	170	Solid	Daily: with water	Once a week	Scalding tank	1	3	5-12
						2	3	7-9

SH: slaughterhouse / SR: slaughter rate / *: lairage / (external): by an external company

Slaughterhouse samples

In each slaughterhouse, samples were taken at seven different stages throughout the slaughter process (Figure 1).

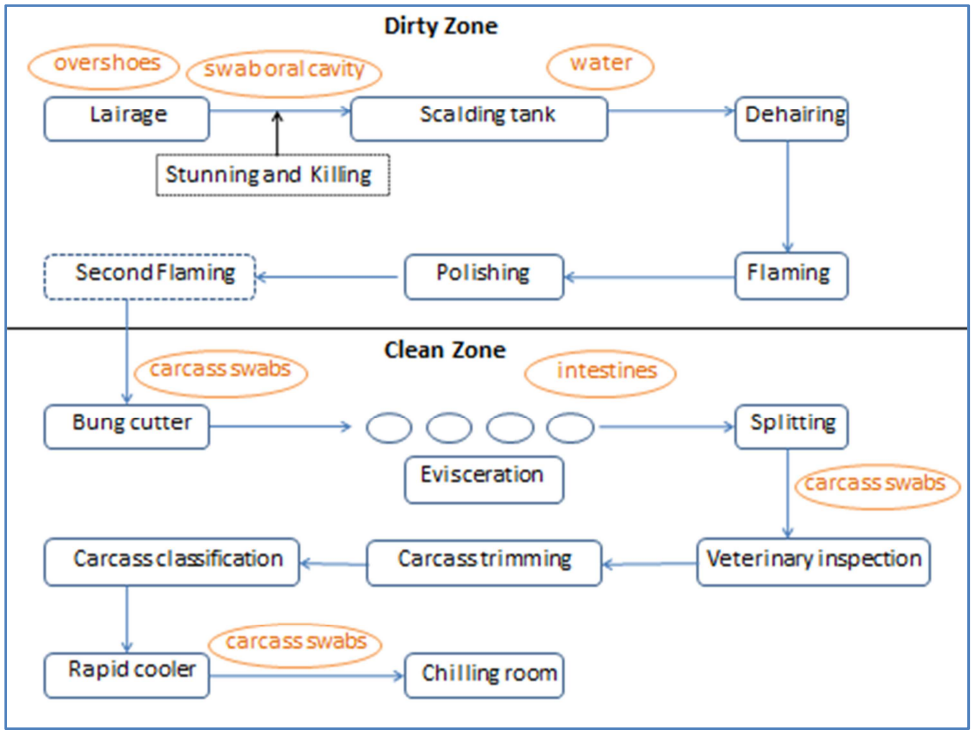

Figure 1: Slaughterhouse production path, with location of the slaughterhouse samples taken.

a) Overshoes

At arrival of the researchers, pens in the lairage were already filled with pigs to be slaughtered that day. As much pens as possible (harboring pigs that were further sampled along the slaughter line as well as pigs that were not followed up) were sampled using the overshoe method.

b) Swabs oral cavity

At exsanguination, an individual swab sample of the oral cavity (on the inside of the cheek) of each identified pig was taken, using a sterile moistened (0.1% peptone water) cotton cosmetic pad. After sampling, the swab was put into a sterile stomacher bag.

c) Scalding water

Before and during slaughter activities, water samples were taken at the in- and outlet of the scalding tank. In slaughterhouse D, these samples were not taken due to the use of a steam tunnel instead of a scalding tank.

d) Carcass swabs

Carcass swabs were taken at three different places: after polishing, splitting and forced chilling. Sterile cotton cosmetic pads were first moistened with peptone and then used for swabbing, according to the procedure described by Ghafir and Daube (2008). The swabs taken after polishing were taken from the shoulder, breast and inner side of the ham on the right side of the carcass, representing in total a swabbing area of 500 cm^2. After splitting, swabs were taken from the shoulder, the sternum along the incision line, the inner side of the ham and pelvic canal of the left side of the carcass representing in total 600 cm^2. After forced chilling, the inner side of the ham and the shoulder were swabbed together with the pelvic canal and the sternum along the incision line of the right side of the carcass representing in total 600 cm^2. All swabs of one carcass and according to one sampling place were pooled in one sterile stomacher bag.

e) Intestines

During evisceration, the stomach-gut package of each identified pig was collected in a sterile plastic bag. In a separate room within the slaughterhouse, duodenum, ileum, rectum and mesenteric lymph nodes were dissected.

Sample analyses

Bacteriological isolation

After collection, all samples were immediately transported to the laboratory and submitted to *Salmonella* isolation within 2 hours using the following procedure. To the overshoes 225 milliliter buffered peptone water (BPW) was added and to the swabs 100 ml BPW. Of the content of duodenum, ileum, rectum and mesenteric lymph node tissue, ten grams of each were aseptically collected. Ninety ml BPW was added to all weighed samples. After homogenization, all homogenates were incubated at 37°C for 16–20 h. Following incubation, 100 µl was spotted onto a Modified Semisolid Rappaport-Vassiliadis agar (MSRV) plate and incubated for 24 h at 42°C. If migration zones were present on the MSRV

plates, a loopful of the edge of the migration zones was streaked on a Xylose Lysine Desoxycholate (CM 469; Oxoid, Hampshire, UK) agar plate. After incubation for 24 h at 37°C, plates were examined for the presence of typical colonies. One suspected colony per plate was biochemically confirmed.

Salmonella serotyping and genotyping

An enterobacterial repetitive intergenic consensus (ERIC) PCR was used to limit the number of strains that had to be serotyped as different strains belonging to the same serotype are, with this method, clustered together with a delineation of 95% (Rasschaert et al., 2005). After performing ERIC-PCR, representative *Salmonella* isolates were selected (at least two isolates per cluster) and sent to the Belgian reference laboratory (Veterinary and Agrochemical Research Centre, Ukkel, Belgium) for serotyping following the Kaufmann-White scheme (Popoff and Le Minor, 1992).

All isolates were further genotyped by using pulsed-field gel electrophoresis (PFGE) after digestion with *XbaI* (modificated PulseNet protocol according to Ribot et al., 2006). Samples of the *Salmonella* Rissen serotype were additionally analyzed using 25 U of the restriction endonuclease *BlnI*. The running conditions were 6V/cm at 14°C in 0.5 x Tris-Borate-EDTA buffer for 20h with a ramping time from 2.2 to 54.2 s. PFGE profiles were clustered with GelCompar II (3.5) (Applied Maths, Sint-Martems-Latem, Belgium) using the Dice coefficient (1% position tolerance) and the unweighted pair group method using arithmetic averages algorithm (UPGMA). A PFGE genotype was assigned on the basis of a difference in the presence of at least one band in the *XbaI* and *BlnI* fingerprint. Genotypes within serotypes were indicated by the capital of the name of the serotype followed by a number (e.g. *Salmonella* Typhimurium genotype 1 is indicated as T1).

Statistical analysis

In the analysis, samples were grouped as those representing external (carcass swabs taken after polishing, spitting and after forced chilling) and internal (content of duodenum, ileum and rectum and lymph nodes) contamination. The gut content represents the content of duodenum, ileum and rectum. Logistic mixed regression models using 1st order penalized quasi-likelihood algorithms were fit in MLwiN 2.02 (Centre for Multilevel Modeling, Bristol, UK). Herd and slaughterhouse were included as random effects to correct for clustering of herds within slaughterhouse and pigs within herd as herds slaughtered in the same

slaughterhouse are more related and as pigs belonging to the same herd are more related to each other.

Univariable associations were tested between the binary (0= *Salmonella* negative; 1= *Salmonella* positive) outcome variables (1) carcass swabs after polishing at pig level, (2) carcass swabs after splitting at pig level, (3) carcass swabs after forced chilling at pig level, and all independent variables at the slaughterhouse, herd and pig level. Subsequently all variables with a *P*-value < 0.2 were tested in a multivariable model again with herd and slaughterhouse as random variables. The model was built in a stepwise backward manner excluding at each step the least significant variable until only significant variables remained. In this analysis, *P*-values lower or equal to 0.05 were considered as statistically significant (two-sided test). Finally, all 2-way interactions were evaluated.

Results

Salmonella *isolation*

Overall, *Salmonella* was isolated from 14.1% (276/1953) of all the samples taken. In total, 226 pigs were followed up during slaughter. Of these 226 pigs, 109 (48.2%) were positive for *Salmonella* in at least one sample. An overview of the results according to slaughterhouse and slaughterhouse visit is shown in Table 2. Overshoes (n=61) taken in the lairage area were highly contaminated with a large variation (ranging from 0 to 100%) between slaughterhouses. Contents of ileum and the mesenteric lymph nodes had the highest *Salmonella* prevalence of all the pig samples taken, with 23.0% and 17.7% positives, respectively. Of the 226 stomach-gut packages taken at evisceration, 4.4% (10/226) were only positive in the lymph nodes, 20.3% (46/226) only in the gut content (duodenum and/or ileum and/or rectum content) and 12.8% (29/226) in the lymph nodes as well as in the gut content (duodenum and/or ileum and/or rectum content). No *Salmonella* was isolated from the scalding tank water.

Table 2: Number of *Salmonella* positive samples at different points in five slaughterhouses (A-E) classified per slaughterhouse visit

	A		B		C		D		E		Total x/y (%)
	A1	A2	B1	B2	C1	C2	D1	D2	E1	E2	
Lairage	0/10	0/9	1/4	3/7	5/5	6/6	5/5	3/5	1/5	4/5	28/61 (46%)
Swabs oral cavity	1/20	0/20	0/30	3/30	21/30	9/30	3/28	0/30	0/30	2/30	39/278 (14%)
Carcass after polishing	0/20	0/19	0/22	1/21	21/28	0/23	0/21	3/23	0/24	0/25	25/226 (11%)
Carcass after splitting	0/20	0/19	1/22	1/21	22/28	1/23	4/21	1/23	1/24	0/25	31/226 (14%)
Duodenum content	0/20	3/19	0/22	3/21	5/28	9/23	3/21	0/23	1/24	2/25	26/226 (11%)
Ileum content	3/20	5/19	2/22	5/21	4/28	10/23	7/21	4/23	4/24	8/25	52/226 (23%)
Rectum content	0/20	2/19	2/22	2/21	7/28	8/23	7/21	1/23	1/24	0/25	30/226 (13%)
Mesenteric lymph nodes	4/20	1/19	3/22	2/21	4/28	11/23	7/21	4/23	2/24	2/25	40/226 (18%)
Carcass after chilling	0/20	0/19	0/22	0/21	4/28	0/23	0/21	1/23	0/24	0/25	5/226 (2%)

x/y (%): positive/total samples examined (percentage of positive samples)

Samples of the scalding tank water not included, as they were all *Salmonella* negative

The results of the univariable and multivariable analysis are shown in Table 3. Detection of *Salmonella* in the lairage area increased the odds of a contaminated carcass after polishing (OR=13.5) and of *Salmonella* positive mesenteric lymph nodes (OR=4.0) significantly. Isolation of *Salmonella* from the oral cavity and from a carcass after polishing increased the odds (OR=10.2 and OR= 10.5, respectively) of a positive carcass after splitting (P<0.01). Detecting *Salmonella* on a carcass after polishing increased the odds (OR=36.7) of a positive carcass after forced chilling (P<0.01). Further, isolation of *Salmonella* from the content of the ileum (P<0.01) and of the content of rectum (P<0.01) increased the odds (OR=6.4 and OR=4.6, respectively) of a positive content of the duodenum. Additionally, detection of *Salmonella* in the content of the duodenum (P=0.01) and in the mesenteric lymph nodes (P<0.01) increased the odds (OR=4.0 and OR=5.9, respectively) of a positive content of the ileum. Isolation of *Salmonella* from the duodenum content increased the odds (OR=3.6) of a *Salmonella* positive rectum content (P=0.02). The model further showed that isolation of *Salmonella* from the ileum content increased the odds (OR=3.1) of positive mesenteric lymph nodes (P=0.05). The evaluation of the two-way interactions showed no significant differences (P>0.05).

Table 3: Results of the univariable and multivariable analysis on 226 pigs slaughtered at 5 different slaughterhouses, with *Salmonella* status of carcass swabs after polishing, carcass swabs after splitting and carcass swabs after forced chilling, lairage area, swabs of the oral cavity, gut (duodenum, ileum, rectum) content and mesenteric lymph nodes as dependent variables.

	UNIVARIABLE			**MULTIVARIABLE**		
	Odds ratio	**Confidence interval**	**Significance level: *P*-value**	**Odds ratio**	**Confidence interval**	**Significance level: *P*-value**
Carcass swabs after polishing						
Lairage area	13.50	0.94 – 194.52	0.05	13.50	0.94 – 194.52	0.05
Swabs oral cavity	0.86	0.17 – 4.32	0.85			
Duodenum content	0.86	0.18 – 4.02	0.85			
Ileum content	0.68	0.20 – 2.38	0.55			
Rectum content	0.52	0.10 -2.77	0.81			
Gut content	0.96	0.22 – 2.04	0.48			
Mesenteric lymph nodes	0.69	0.16 – 2.89	0.61			
Inner	0.85	0.20 – 1.98	0.77			
Carcass swabs after splitting						
Lairage area	6.53	0.88 – 48.52	0.07			

Swabs oral cavity	3.38	0.84 – 13.55	0.08	10.24	3.32 – 31.59	<0.01
Carcass swabs after polishing	3.38	0.71 – 15.99	0.12	10.46	2.90 – 29.84	<0.01
Content of duodenum	1.33	0.35 – 5.03	0.67			
Content of ileum	1.70	0.61 – 4.74	0.31			
Content of rectum	1.55	0.44 – 5.42	0.49			
Gut content	1.66	0.62 – 4.42	0.31			
Mesenterial lymph nodes	0.85	0.23 – 3.09	0.80			
Inner	1.33	0.51 – 3.46	0.56			
Carcass swabs after forced chilling						
Lairage area	Nc	Nc	Nc			
Swabs oral cavity	1.29	0.07 – 20.00	0.86			
Carcass swabs after polishing	36.71	3.79 – 355.90	<0.01	36.71	3.79 – 355.90	<0.01
Carcass swabs after splitting	7.07	0.89 – 56.35	0.06			
Content of duodenum	Nc	Nc	Nc			
Content of ileum	0.75	0.06 – 9.07	0.82			
Content of rectum	Nc	Nc	Nc			

Gut content	0.21	0.01 – 3.97	0.30			
Mesenteric lymph nodes	3.31	0.57 – 29.18	0.28			
Inner	0.55	0.06 – 4.89	0.60			
Lairage area						
Content of duodenum	1.01	0.34 – 3.01	1.00			
Content of ileum	1.03	0.47- 2.26	0.96			
Content of rectum	0.98	0.31 – 3.10	0.97			
Mesenteric lymph nodes	1.33	0.46 – 3.85	0.60			
Content of duodenum						
Lairage area	4.16	0.05 – 16.41	0.04			
Content of ileum	6.38	2.57 – 15.85	<0.01	6.36	2.48 – 16.29	<0.01
Content of rectum	4.18	1.39 – 12.57	0.01	4.56	1.56 – 13.32	<0.01
Mesenteric lymph nodes	4.66	1.71 – 12.69	<0.01			
Content of ileum						
Lairage area	1.84	0.46 – 7.34	0.38			
Content of duodenum	4.10	1.57 – 10.70	<0.01	3.97	1.46 – 10.80	0.01
Content of rectum	1.34	0.49 – 1.29	0.57			

Mesenteric lymph nodes	6.60	2.83 – 15.36	<0.01	5.88	2.48 – 13.92	<0.01
Content of rectum						
Lairage area	3.35	0.62 – 18.08	0.16			
Content of duodenum	3.65	1.20 – 11.07	0.02	3.65	1.20 – 11.07	0.02
Content of ileum	1.64	0.62 – 4.36	0.31			
Mesenteric lymph nodes	2.92	1.06 – 8.07	0.04			
Mesenteric lymph nodes						
Lairage area	4.60	1.06 – 19.95	0.04	3.99	0.99 – 16.03	0.05
Content of duodenum	2.93	1.48 – 8.37	0.04			
Content of ileum	5.71	2.43 – 13.26	<0.01	3.15	1.00 – 9.92	0.05
Content of rectum	2.29	0.81 – 6.45	0.12			

Nc: model not able to convert

Salmonella *serotyping*

Of the 276 *Salmonella* isolates, 13 different serotypes were identified (Table 4). *Salmonella* Typhimurium (58.7%) and *Salmonella* Rissen (17.4%) were the predominant serotypes, followed by *Salmonella* Derby (8.3%), *Salmonella* Brandenburg (5.8%) and *Salmonella* Infantis (4.7%). Differences in serotypes were noticed between the different slaughterhouses and between the different slaughterhouse visits (Table 4). The largest variation in *Salmonella* serotypes was noticed in the lymph nodes, rectum and ileum content. *Salmonella* Typhimurium and *Salmonella* Rissen were detected in all kinds of samples whereas *Salmonella* Derby was not detected in the lymph nodes and on the carcass (after polishing, after splitting and after first chill). *Salmonella* Infantis was never isolated from the rectum content, the lymph nodes and the carcass after forced chilling. *Salmonella* Brandenburg was only noticed in overshoes of slaughterhouse E and on carcasses after splitting in slaughterhouse C, whereas *Salmonella* Braenderup could only be detected in slaughterhouse B in samples of the rectum content. Furthermore, *Salmonella* Anatum was only found in rectum content (slaughterhouse B) and in the lymph nodes (slaughterhouse A and B). *Salmonella* Schwartzengrund could only be detected in ileum and rectum content of the same animal. In addition, *Salmonella* Agona, *Salmonella* Nagoya and *Salmonella* Livingstone were only found in samples of the lymph nodes. The serotype Typhimurium was predominant in the *Salmonella* positive overshoe samples (57.1%), followed by Derby (17.8%), Rissen (10.7%), Brandenburg (10.7%) and Infantis (3.6%).

Table 4: Distribution of the 13 *Salmonella* serotypes derived from the positive samples taken at 5 slaughterhouses (A-E) and subdivided per slaughterhouse visit (1 and 2).

	A		B		C		D		E		Total
	1	2	1	2	1	2	1	2	1	2	
Typhimurium	4		5	5	25	54	31	15	8	15	162
Rissen				2	46						48
Derby	3	10		3			5	2			23
Brandenburg					13					3	16
Infantis				8	5						13
Braenderup			1	2							3
Anatum		1	2								3
Schwartzengrund									2		2
O6,7:R:-					2						2
Agona			1								1
Nagoya	1										1
O4:I:-					1						1
Livingstone					1						1

Macrorestriction profiling

In total, 276 *Salmonella* isolates were characterized using PFGE with the restriction endonuclease *XbaI*. For three isolates (one *Salmonella* Typhimurium, one *Salmonella* Derby and the only *Salmonella* Livingstone serotype), no PFGE-generated pattern could be obtained. Forty-six different PFGE profiles were obtained, with 32 profiles for *Salmonella* Typhimurium, four for *Salmonella* Derby, two for *Salmonella* Infantis, *Salmonella* Brandenburg, *Salmonella* Anatum and O6,7:R:-. No distinguishable patterns from the *Salmonella* Rissen isolates were obtained when using the restriction endonuclease *XbaI*, therefore, samples were again analyzed using PFGE with the *BlnI* enzyme, resulting in 2 distinct clusters according to slaughterhouse B and C, respectively.

A detailed description of the serotypes and genotypes from the only five animals positive on the carcass after forced chilling can be found in Table 5.

Table 5: Description of the individual animals positive on the carcass after first chill: serotypes and genotypes recovered

SH	SH visit	Farm	Animal	Lairage area	Swabs oral cavity	Carcass after polishing	Carcass after splitting	Carcass after chilling	Content of ileum	Mesenteric lymph nodes
C	1	18	101	Rissen R1 Typh T13		Rissen R1		Typh T9		Livingst
C	1	18	108	Rissen R1 Typh T13		Typh T6		RissenR1		
C	1	19	115	Rissen R1 Typh T13	Rissen R1		Typh T9	Typh T9		
C	1	19	116	Rissen R1 Typh T13	Rissen R1	Infant I2	Brand 1	Rissen R1		
D	2	30	198	?		Typh T22	Typh T22	Typh T22	Typh T22	Typh T22

SH: slaughterhouse /SH Visit: slaughterhouse visit / Typh: Typhimurium / Brand: Brandenbrug / Infant: Infantis / Livingst: Livingstone / ?: herd number not known / Duodenum and rectum content not included (all negative for *Salmonella*)

Table 6 shows the number and percentage of pigs sampled with the same genotype in the different samples taken in the lairage area as well as along the slaughter line. If pigs were positive in the lairage area as well as in the oral cavity, then in 95% of the cases, the same genotype was found. Of the genotypes detected in the content of the ileum, 50% could also be found in the lymph nodes. Of all the samples positive in rectum and in the lairage area, 31% harbored the same genotype. If pigs were positive in the lymph nodes and in the gut content, then in 83% of the cases, the same genotype could be found in both samples. Further, in 32% of the genotypes found in carcass swabs after splitting, the same were found in the gut content.

In slaughterhouse A, genotyping showed that the occurrence of a particular genotype was herd dependent. In slaughterhouse B, the genotypes found in the lairage area could be related to those found on the carcass and in half of the cases to those found in the gut contents of the slaughtered pigs. In slaughterhouse C, the genotypes detected in the lairage area were not recovered from the lymph nodes, but were mainly found in the oral cavity, the rectum and duodenum content and on the carcasses along the slaughter line. On these carcasses also a particular Typhimurium genotype was detected that could not be found in the other isolates. Serotyping and genotyping revealed that after polishing and after splitting, different sero- and genotypes could be isolated. In slaughterhouse D, distinction should be made between the first and the second visit. During the first visit, a high degree of similar genotypes was found in the lairage area and on the carcass and intestines of slaughtered pigs, while during the second visit, the genotypes of the lairage were not found in other samples. In slaughterhouse E, only one of the strains found in the lairage area was also found in the ileum content of one slaughtered pig. The strain detected on the only positive carcass was also found in the lymph nodes of a previous slaughtered animal originating from the same herd.

Table 6: Number of pigs with the same genotype in the different samples taken in the lairage area and along the slaughter line, gut = duodenum + ileum + rectum, carcass swabs = carcass swabs taken after polishing + after splitting + after first chill (with : not performed)

		Lairage area	Swabs oral cavity	Mesenteric lymph nodes	Gut content	Carcass swabs
Lairage area			18/19 (95%)	0/16 (0%)	19/29 (65%)	11/19 (58%)
Swabs oral cavity		18/19 (95%)		2/10 (20%)	11/26 (42%)	10/21 (48%)
Gut content:	Total	19/29 (65%)	11/26 (42%)	24/29 (83%)		10/18 (55%)
	duodenum	10/19 (28%)	3/11 (11%)	4/24 (10%)		2/12 (9%)
	ileum	2/19 (6%)	3/11 (11%)	20/24 (50%)		4/12 (18%)
	rectum	11/19 (31%)	5/11 (19%)	9/24 (23%)		6/12 (28%)
Mesenteric lymph nodes		0/16 (0%)	2/10 (20%)		24/29 (83%)	4/9 (44%)
Carcass swabs:	Total	11/19 (58%)	10/21 (48%)	4/9 (44%)	10/18 (55%)	
	after polishing	6/11 (27%)	6/10 (22%)	1/4 (7%)	4/12 (18%)	
	after splitting	5/11 (22%)	6/10 (22%)	4/4 (30%)	7/12 (32%)	
	after chilling	2/11 (9%)	1/10 (4%)	1/4 (7%)	1/12 (5%)	

Discussion

This study showed a large variation in *Salmonella* contamination level of the lairage area and the carcasses between the different slaughterhouses. This is in accordance to the results of Botteldoorn et al. (2003) indicating that sampling results depend on the slaughterhouse (hygienic parameters and qualification of personnel), the sampling day and the origin and number of infected pigs delivered during the sample period.

In general, overshoes taken at the lairage area were highly contaminated, as well as ileum content and mesenteric lymph nodes. A large variation between the slaughterhouses was noticed in positive samples recovered from the lairage area, with a fully slatted floor having a lower risk. As animal welfare legislation (EC No 1099/2009) states that the floor type in slaughterhouses must be non-slippery, rough or grooved floor types are recommended. However, such animal welfare friendly floor types are more difficult to clean than slatted floors. Roughened slatted floors might be a good solution to keep the animals clean, to minimize contact with feces, and to prevent animals from falling or slipping. However, this kind of floor type is more expensive because a cleanable manure pit must be provided underneath the floor. This study further shows that cleaning and disinfection procedures performed in the slaughterhouses with solid flooring in the lairage area are insufficient to prevent contamination.

In literature, several authors emphasize the role of the lairage area in causing contamination and cross contamination of *Salmonella* in pigs. The inadequate cleaning and disinfection, as well as the time spent in lairage are hereby important factors. In Belgium, slaughter pigs are kept in lairage on average 126 minutes (min 5- max 720 minutes) (De Sadeleer at al., 2008). This time period is long enough for animals to become infected with *Salmonella*. Hurd et al. (2001) demonstrated that exposure times as short as 30 minutes could lead to isolation of *Salmonella* from the intestines. In the present study, a contaminated lairage area increases the risk of having a positive carcass after polishing, but it was not related to the inner contamination of the pig (gut and lymph nodes). This is logical as the pig carcasses just pass through the flaming device and the belly of the pig has not yet been opened at this stage. The contamination after polishing could be due to contamination from the environment as the polishing equipment is difficult to clean (Borch et al., 1996) and the water used is mostly cold (De Sadeleer at al., 2008). A second flaming device after polishing could be helpful to avoid that contaminated carcasses enter the clean part of the slaughterhouse.

Furthermore, the risk of carcass contamination after splitting is influenced by the contamination of the oral cavity of pigs at bleeding and by the carcass contamination after polishing. The latter can be explained by the lack of a contamination decreasing procedure in the clean part of the slaughterhouse. Carcasses after forced chilling have more risk of being *Salmonella* positive if the carcasses were already positive after polishing. This can again be explained by the fact that in the clean part of the slaughterhouse, no step is available for decreasing the carcass contamination. The importance of the carcass contamination level at previous events was also confirmed by the five pigs that were positive after forced chilling. All of these animals were positive after polishing or after splitting or both. These findings correspond to those of Berends et al. (1997) who estimated that 5-15% of all carcass contamination occurred during polishing. The effect of a contaminated lairage area, content of duodenum and content of rectum on the carcass contamination after forced chilling could not be assessed in the statistical model used in this study. This is probably due to a lack of statistical power as only 5 carcasses were positive after forced chilling.

A *Salmonella* contaminated lairage area influences the contamination of the external pig (the carcass) more than the internal pig (gut content and lymph nodes). Rossel et al. (2009) demonstrated that carcass contamination is directly related to skin contamination of live pigs before stunning. This skin contamination was connected with the contamination of the lairage area, highlighting the need of effective cleaning and disinfection.

Looking at the similarity of the genotypes, this study shows that the same genotype was detected in the lairage area as in 94.7% of the positive swabs of the oral cavity, 65.5% of the positive gut contents and 52.6% of the positive carcass swbas. The high level of corresponding genotypes found in the lairage area and in the oral cavity of pigs demonstrates the uptake or the oral shedding of *Salmonella*. Furthermore, genotypes found in the lairage area were often detected in the rectum content of pigs. As only 23% of the genotypes found in the rectum content and in the lymphnodes was similar, part of the rectum contamination could be due to contamination occurring during dehairing and polishing as the anal sphincter is relaxed post mortem and contaminated water can seep through the anus (Boudry et al., 2002). Furthermore, the *Salmonella* strain detected in the lairage area was not always found in other pig samples as for instance the lymph nodes. This indicates that pigs were not shedding that specific genotype and consequently, the lairage area had already been contaminated (as the lairage area was sampled in the morning just before slaughter activities started).

The content of the ileum (23.0%) was highly contaminated. This percentage is slightly higher than percentages found by Botteldoorn et al. (2003) (19%) and by De Busser et al. (2009) (17.4%). Animals harboring *Salmonella* in the gut content can be infected at the herd, during transport from the herd to the slaughterhouse and in the lairage, or can be carriers who start reshedding due to stress. It seems that carriers shed *Salmonella* preferably in the ileum as the same genotype could be found in 50% of the pigs with a positive ileum and lymph nodes. Avoiding stress at all times is crucial, not only regarding animal welfare, but also regarding meat quality and food safety. The results of this study underline this issue, as more than 80% of the pigs revealed the same genotype in their lymph nodes as in the gut content indicating that carriers started re-shedding. The high number of *Salmonella* positive gut contents further emphasize the need to prevent accidental cutting into these organs, as this may result in contamination of the carcass but also in cross-contamination of subsequent carcasses. It is therefore important to implement a good fasting procedure (12-16 h before transport) at the farm and to have well trained personnel at the slaughterhouse.

The contamination level of the mesenteric lymph nodes (17.7%, n= 226) was slightly higher than the prevalence (13.9%, n=601) reported in the EFSA study (EFSA, 2008a), but lower than the results found by Botteldoorn et al. (2003) (21%, n=346) and by De Busser et al. (2009) (20.0%, n=70).

The EFSA study, also, showed a *Salmonella* prevalence of 18.8% (n=381) on the carcasses (EFSA, 2008a). This percentage however should be compared with the number of positive swabs after splitting (13.7%, n=226) in our study, since the carcasses in the EFSA study were sampled after evisceration but before chilling.

Characterization of the sampled carcass swabs (after polishing, splitting and forced chilling) further showed that most genotypes found on the carcass were not present in other pig samples. Similar results were obtained in the study of Wonderling et al. (2003) showing that half of the genotypes found on carcasses were distinct from those detected in the feces. Also Botteldoorn et al. (2004) demonstrated that carcass contamination did not solely result from infection of the corresponding pig, but also from previously slaughtered positive pigs and the slaughterhouse environment. Aerosols generated by frequent washing of the carcasses were considered an important vehicle for transmission of *Salmonella* to the carcasses (Botteldoorn et al., 2004).

The slaughtering stage is considered to have the highest impact on the number of contaminated carcasses as shown by Swanenbrug et al. (2001), van der Gaag et al. (2004) and Alban and Stärk (2005). Special attention should be paid to the dehairing and polishing equipment, evisceration technique and splitting device. Furthermore, this study showed that in samples taken from the same pig, different *Salmonella* serotypes and genotypes could be identified. This finding is in contrast to the study results of Vieira-Pinto et al. (2006) where it was shown that in all samples from the same pig (except for one animal), the same *Salmonella* serotype and genotype was identified.

Performing macrorestriction profiling using *XbaI* was not suitable to distinguish different PFGE profiles for the serotype Rissen. However PFGE has been widely used for *Salmonella* DNA fingerprinting (Lukinmaa, 1999; Lyytikäinen, 2000; Valdezate, 2000; Bender et al., 2001). The serotype of the isolates has a considerable influence on the outcome of the typing method. As shown by Liebana et al. (2001), limited success was obtained when PFGE was applied to serotype Livingstone isolates. This is in accordance with our results as the only *Salmonella* Livingstone isolate was non-typeable. Molecular typing of the serotype Rissen using *XbaI* in a PFGE (Vieira-Pinto et al., 2006) revealed a homogenous genotype (17 out of 19 isolates showed the same MRP). The study of Hendriksen et al. (2008) however demonstrated the molecular diversity of *Salmonella* Rissen. By using the restriction enzyme *XbaI*, 63 unique patterns could be identified among the 112 isolates originating from humans, food and pig or pork products. The predominant pattern in this study was further subtyped using *BlnI* and revealed 6 patterns. As in our study, no differentiation could be obtained by using *XbaI*, isolates were analyzed once more by using *BlnI*, revealing two distinct patterns according to the two different slaughterhouses where these isolates originated from.

In conclusion, this study elucidated major differences in contamination level between slaughterhouses. Although pigs can harbor *Salmonella* before stunning, it seemed that the slaughterhouse itself is crucial in the further contamination level along the slaughter line. This study further documented the importance of the contamination level of the lairage area. Contamination and cross contamination can indeed occur inside the slaughterhouse. Still, avoiding the entrance of *Salmonella* positive pigs in the slaughterhouse remains crucial as this can significantly increase the contamination level in the slaughterhouse.

Acknowledgements

This research was funded by the Belgian Federal Public Service of Health, Food Chain Safety and Environment.

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CHAPTER 6

GENERAL DISCUSSION

GENERAL DISCUSSION

Introduction

Since the implementation of a successful mandatory *Salmonella* control program in poultry, the relative importance of pork as source for human salmonellosis has increased. The European Food Safety Authority (EFSA) carried out surveys to map the *Salmonella* prevalence in pigs throughout the European Union (EU). Results show that the prevalence in breeding and production holdings as well as in slaughtered pigs varies widely between different Member States (MS). The average EU prevalence of *Salmonella* positive breeding and production holdings established by bacteriological analysis of pooled fecal samples was 28.7%, and 33.3%, respectively (EFSA, 2009). The average EU prevalence in slaughtered pigs was 10.3% (based on positive lymph nodes) and 8.3% (based on positive carcasses) (EFSA, 2008). According to these surveys, the *Salmonella* prevalence in Belgian breeding and production holdings was 18.8% and 36.4%, respectively. At the slaughterhouse, 13.6% of the sampled lymph nodes and 18.8% of the sampled carcasses were *Salmonella* positive. Moreover, Belgium was one of the only EU countries where the carcass prevalence was higher than the prevalence in the lymph nodes.

To monitor and control *Salmonella* in the EU and to decrease the proportion of human salmonellosis due to contaminated pork and pork products, the MS are asked to set up monitoring and control programs at the pre-harvest stage before July 2009. These control programs classify pig herds in different categories based on serological (blood or meat juice) and/or bacteriological results. As these control programs largely count on good diagnostic results, it is of great importance that the diagnostic techniques used are of good quality.

Salmonella diagnostics

The bacteriological isolation protocol of *Salmonella* in samples of the primary production is standardized in the ISO 6579, Annex D (ISO, 2007). MSRV is used as enrichment medium as it is developed for the detection of motile *Salmonella*, which all porcine *Salmonella* theoretically should be. The results described in Chapter 3 show, however, that not all *Salmonella* strains were motile. The non-motile ones could easily be missed in the standard isolation protocol. To increase the chance of detecting less motile

Salmonella bacteria, the additional analysis of a sample taken close to the inoculation drop on semi-solid enrichment media after incubation is advised.

Further, different sero- and genotypes originating from the same animal were found between, but also within a given enrichment medium (Chapter 3). This is in agreement with other studies demonstrating the appearance of multiple *Salmonella* sero- and genotypes in one pig or in a single pig sample (Chapter 5, Gomes-Neves et al., 2012; van Hoek et al., 2012). In epidemiological studies, more than one presumptive colony on the selective agar after enrichment should be tested. As several serotypes were only detected after enrichment in broths (Chapter 3), enrichment in different media should be considered when more detailed epidemiologic information is warranted.

Although Harvey and Price's demonstrated already in 1967 that different *Salmonella* serotypes had different growth characteristics in the same enrichment medium, not much research has yet been done to elucidate the responsible factors. In literature, some authors describe the nature of the tested sample (Singer et al., 2009), the presence of bacteriophages (Muniesa et al., 2005) and the amount of sample added to the culture (Funk et al., 2000) as influencing the distribution and diversity of the recovered serotypes. Rostagno et al. (2005) and Gorski (2012) demonstrated that asynchronous growth curves among serotypes were also due to the selective enrichment media used. To improve the accuracy of diagnostic procedures, a better understanding of the factors related to the growth of serotypes in certain selective media is necessary.

As multiple genotypes can be detected within the same serotype, genotyping is necessary in more advanced epidemiological studies where the contamination source or the clonal relationships of the recovered isolates have to be assessed. Pulsed-field gel electrophoresis (PFGE) has remarkable discriminatory power and reproducibility (van Belkum et al., 2007), and is often considered as the "gold standard" for comparative typing of many bacteria (Tenover et al., 1995; Olive and Bean, 1999). However, the detection of different profiles depends on the *Salmonella* serotype as well as on the restriction enzyme used. The results described in Chapter 5 show that the restriction enzyme *XbaI* was not able to detect different PFGE profiles for the serotype Rissen. Van Hoek et al. (2012) and Gomes-Neves et al. (2012) also reported this finding. When plugs were restricted with 10U of *XbaI* at 37°C for 14h, Vieira-Pinto et al. (2006) could distinguish three different profiles. Because of the high similarity (one band difference) found among the three profiles, it was concluded that

Salmonella Rissen was a homogeneous genotype. This is contradictory to the results obtained by Hendriksen et al. (2008) demonstrating 63 unique patterns among 112 *Salmonella* Rissen isolates. These isolates originated from humans, food and pig or pork products. The predominant *XbaI* pattern was further differentiated by PFGE following digestion with *BlnI*, revealing six different patterns. In the study described in Chapter 5, two distinct clusters separating isolates according to their origin (slaughterhouse B and C) were obtained after restriction of the *Salmonella* Rissen isolates with 25U *BlnI*. It is therefore recommended to include secondary restriction with *BlnI* when there is more than one isolate with indistinguishable *XbaI* patterns. To achieve full restriction of DNA, Ribot et al. (2006) recommended the use of 30 units *BlnI* per plug slices, although the 25 units used in our study were sufficient to obtain distinct patterns.

Results described in Chapter 4 strongly emphasize the need for adaptation of the standard *Salmonella* isolation protocol according to the aims and the nature of the performed study. If the *Salmonella* status (positive or negative) has to be defined, the use of MSRV with or without other enrichment media (depending on the wanted sensitivity) is recommended. When more detailed information is necessary, multiple parts of the semi-solid enrichment media, additional liquid enrichment media and multiple colonies per selective plate should be tested and isolates should additionally be sero- and genotyped.

***Salmonella* control at the primary production**

Besides having a good *Salmonella* control program at the primary production, it is at least equally important to be able to advice on how *Salmonella* infections and spread of *Salmonella* can be prevented. Several control measures can be advised to decrease the *Salmonella* infection level in pig herds, as described in the General Introduction of this thesis. In this section of the General Discussion the focus will be on the administration of organic acids.

The antibacterial effect of organic acids is based on their ability to disrupt cellular pH gradients and intracellular pH regulation, leading to disruption of vital metabolic processes of the bacteria (Cherrington et al., 1990; Van Immerseel et al., 2006).

Besides administering organic acids to feed during processing or in finished feed, it might also be added to the drinking water. Benefits of the usage in drinking water are the

treatment of animals during periods of feed withdrawal (particularly pre-slaughter), the strategic use and dosing flexibility as well as the reduction or elimination of vegetative pathogens in the water itself (Wales et al., 2010). The pH of the drinking water can further be lowered to a pH<4, at which *Salmonella* cannot multiply (Ekperigin and Nagaraja, 1998).

A study performed by van der Wolf et al. (2001) showed a marked reduction in *Salmonella* serum titers when the drinking water of finishing pigs was acidified during the entire finishing period to a pH of 3.8-3.9. Although a beneficial effect was seen on *Salmonella* seroprevalence, considerable problems with clogging of the drinking nipples were reported. Additionally, the calculated running costs per pig for the acid mixture were high (2.49€/pig). The study described in Chapter 4 aimed to assess the effect of the acidification of drinking water during the last fourteen days prior to slaughter. This limited treatment period would considerably lower the costs involved. However, no beneficial effect of the treatment (drinking water pH of 3.6-4) was observed on the *Salmonella* prevalence in samples taken at the slaughterhouse. Most probably the administration period was too short, although cross-contamination and/or infection during transport, in the lairage area or along the slaughter line might have occurred as well.

Creus et al. (2007) compared the use of different dosages of lactic and/or formic acid in the feed of fattening pigs during 14, 9 and 8 weeks prior to slaughter. When using a dosage of 0.6% lactic and 0.6% formic acid during 14 weeks, the *Salmonella* prevalence in lymph nodes declined to zero (compared to eight positive lymph nodes in the control group). When the administration lasted for 9 weeks, differences in fecal and lymph node prevalence were observed between control and treatment groups but when the administration period was limited to 8 weeks, no differences between groups were noticed.

The lack of assessing a clear beneficial effect when acidification is shortened to 8 (Creus et al., 2007) or 2 (Chapter 3) weeks prior to slaughter, suggests that once a *Salmonella* infection has been established, the *Salmonella* status of the pig cannot be altered with the described treatment strategy. This might further be explained by the fact that organic acids are metabolized and absorbed by epithelial cells in the upper part of the gastro-intestinal tract (Van Immerseel et al., 2006; Louis et al., 2007) and do not reach the major sites of *Salmonella* colonization, namely the ileum, caecum and colon (Boyen et al., 2008).

No consistent data are available regarding the type of organic acid that is most effective against *Salmonella*. *In vitro* studies showed that formic acid (C_1) seems to be more

potent than acetic (C_2), propionic (C_3), lactic (C_3) or citric acid (C_6) (Martin and Maris, 2005; Diebold and Eidelsburger, 2006), while other studies demonstrated that butyric (C_4) and valeric (C_5) acid (Khan and Katamay, 1969) or caprylic acid (C_8) (Skrivanova et al., 2006) were most effective against *Salmonella*.

Antimicrobial activity depends on the characteristics of the organic acid (chain length, side-chain composition, pK_a value and hydrophobicity) (Van Immerseel et al., 2006), the used concentration but also on the pH of the environment (Baik et al., 1996). Boyen et al. (2008) demonstrated that the minimal inhibitory concentrations (MIC) of formic acid are highly pH-dependent, while MIC values of propionic acid are less influenced by changes in pH. These authors further showed that even non-bacteriostatic concentrations of caproic, caprylic, butyric and propionic acid considerably decreased virulence gene expression and epithelial cell invasion by *Salmonella* Typhimurium *in vitro*. *In vivo*, coated butyric acid in feed was the most promising in decreasing the level of fecal shedding and intestinal colonization, although the colonization of tonsils, spleen and liver was not influenced (Boyen et al., 2008). De Ridder et al. (2012) demonstrated that the administration of feed supplemented with coated calcium-butyrate significantly decreased the number of infected organs (ileum, caecum and ileocaecal lymph nodes), but no difference was seen in the *Salmonella* prevalence of tonsils.

Comparison between coated and uncoated fatty acids in feed showed that coating is necessary to obtain beneficial effects on *Salmonella* shedding and colonization (Boyen et al., 2008). A recent study performed by De Ridder et al. (2012) showed a significant reduction of the *Salmonella* transmission ratio in weaned pigs fed for 11 weeks with coated calcium-butyrate. The use of coated organic acids in drinking water has not yet been described in literature and is limited to in-feed application.

To conclude, it can be stated that administration of organic acids in drinking water can reduce *Salmonella* prevalence when the treatment period involves the whole fattening period (van der Wolf et al., 2001). Further research has to be conducted to evaluate the effect of the strategic use of acidified drinking water during risk periods (sows after weaning, piglets at weaning, fattening pigs after moving) (Nollet et al., 2005) in order to lower the costs involved. Attention has to be paid on possible clogging of drinking nipples (van der Wolf et al., 2001) and decreased water intake when the pH is lowered to 4 (De Busser et al., 2011).

The major benefit of in-feed administration of organic acids is the possible use of coated organic acids, of which coated calcium-butyrate might be most promising. Studies

under natural conditions have to be performed in order to establish the effect of these acids on existing *Salmonella* infection and persistence in pigs.

A final point of attention in the use of organic acids is the so called acid tolerance response (ATR). The ATR is a complex defense system that can minimize the lethal effects of extreme low pH (pH=3) (Foster and Spector, 1995) due to habituation after prior exposure to acids. Some concern has been risen as selection for acid tolerant organisms might occur by using organic acid feed treatments which may lead to the development of *Salmonella* clones which are more likely to survive gastric acidity in humans consuming contaminated food (de Jonge et al., 2003; Fratamico, 2003; Theron and Lues, 2007).

***Salmonella* control at the slaughterhouse**

Although Member States have to consider whether on farm intervention, slaughterhouse intervention or a combination of both offer the optimum *Salmonella* control strategy (EFSA, 2008), quantitative microbiological risk assessment (QMRA) showed that specific slaughterhouse interventions are at present more likely to produce larger reductions of human illness than interventions in the primary production (Bollaerts et al., 2010; EFSA, 2010). In Belgium and in Ireland, the carcass contamination was found to be higher than the contamination of lymph nodes examined from slaughtered pigs (EFSA, 2008), pointing to an important contamination at slaughterhouse level in these countries. Results described in Chapter 5 emphasize the importance of carcass contamination occurring from other sources than the pig itself and pointed out the large variation in *Salmonella* prevalence at the different slaughterhouses.

The contamination of the lairage area varied from 0 to 100% among the different slaughterhouses. The only slaughterhouse where no *Salmonella* could be detected in overshoes taken at the lairage area, was the one with a fully slatted floor in the lairage area, preventing or minimizing the contact between animals and feces. The importance of clean pigs is demonstrated by other studies (Rossel et al., 2009; Letellier et al., 2009) showing that skin contamination of live pigs was associated with the contamination of the lairage area and with an increased carcass contamination. Results of Chapter 5 confirmed this finding as contamination of the lairage area increased the probability of carcass contamination after polishing. The genotypes recovered from the lairage area were further closely related to those

found on the carcasses (Chapter 5). The strong association between isolates obtained from the lairage area and the carcass has been demonstrated by several other authors as well (Swanenburg et al., 2001a; Letellier et al., 2009; Kich et al., 2011).

Due to a continuous incoming flow of pigs to be slaughtered, lairage pens are re-used throughout the day without cleaning and disinfection in between. Pens can thus be contaminated by different slaughter batches originating from different herds or by residual flora. Cleaning and disinfection procedures are mainly carried out at the end of the day, although no standard procedures are applied as shown in Chapter 5. Cleaning can be performed with cold water under low or high pressure and disinfection can be carried out on a daily, weekly or monthly basis. Boughton et al. (2007) showed that daily cleaning with cold water under high pressure was not effective in reducing *Salmonella* prevalence on lairage pen floors. Only when cleaning is followed by disinfection a reduction of the *Salmonella* load could be obtained (Swanenburg et al., 2001b; Schmidt et al., 2004; Boughton et al., 2007). The survival of *Salmonella* in lairage pens can be facilitated by the presence of organic matter on the floor, walls or drinkers (Mannion et al., 2007), by the survival within biofilms (De Beer et al., 1994; Stewart et al., 2001) and within certain protozoa species (Tezcan-Merdol, 2004). Improvements in cleaning procedures, using hot instead of cold water (Boughton et al., 2007) and increasing the frequency of disinfection may help in reducing the *Salmonella* prevalence at the lairage area. To evaluate the effectiveness of the cleaning and disinfection procedure, a routine analysis of samples should be implemented in the auto-control program of the slaughterhouse.

Slaughter personnel working at the lairage area, should be familiar with the natural behavior of pigs and with the current welfare legislation. Lairage pens and stunning corridors should be designed to allow minimal human interference when pigs are moved (Barton Gade et al., 1993) in order to avoid stress as the latter enhances *Salmonella* shedding (Hurd et al., 2001).

After stunning and bleeding, pig carcasses are moved through a scalding tank or steam tunnel to facilitate the removal of the hair. The presence of *Salmonella* in scalding water increases the risk of carcass contamination (Letellier et al., 2009) and a time-temperature combination of 1.4 min at 60°C is required to achieve a 1 log reduction (Bolton et al., 2003). The results described in Chapter 5 showed that no *Salmonella* could be detected in the scalding tank water from all the sampled slaughterhouses before and during slaughter

activities. A previous Belgian study (Botteldoorn et al., 2003) demonstrated that in the scalding tank water (temperature of 60-62°C) during slaughter activity, no *Salmonella* could be detected. De Sadeleer et al. (2008) described however, that the temperature of the scalding tank water varied from 58.5-63.7°C in the ten largest Belgian slaughterhouses, indicating that continuously monitoring of the temperature of the scalding tank water remains necessary.

As dehairing and polishing contribute to contamination and cross-contamination of the carcasses, a good maintenance and cleaning and disinfection of this equipment is necessary. Singeing is performed before polishing, although an additional singeing step might be available after polishing. Bacterial reduction after singeing is to be expected as the surface temperature may increase to 100°C during the singeing process (Borch et al., 1996), although uneven exposure to flames could lead to differences in bacterial reduction among different carcass sites (Spescha et al., 2006). Although hand-held singeing is thought to be more comprehensively and consistently applied to all areas of the carcass (Borch et al., 1996), da Silva et al. (2012) demonstrated the opposite. Eight of the seventeen carcasses that were contaminated with *Salmonella* before singeing remained positive after hand-held singeing, whereas only 1 of 13 contaminated carcasses was positive after singeing with the automated system (da Silva et al., 2012). Yu et al. (1999) further demonstrated that a second singeing step is not as effective as the first one, probably because the lower time-period spent in the second singeing step. In the study described in Chapter 5, one slaughterhouse performed additional singeing and showed a low carcass contamination level. The exact contribution of the additional singeing step could not be established in this study (Chapter 5). Further research should be conducted to evaluate the actual benefit of a second singeing step in the slaughter process.

In the clean part of the slaughterhouse, evisceration and splitting are critical points as accidental cutting into intestines can occur and splitting devices are difficult to clean and disinfect. Cross contamination can further occur by the bung cutter, hands and knives of the employees. Proper training of slaughterhouse personnel regarding hand- and personal hygiene and regarding slaughter procedures is important (Arguello et al., 2012), as well as establishing the correct temperature (82°C) of the water used for disinfecting knives. In a previous Belgian study, the temperature of the water used for cleaning the bung cutter and evisceration knives varied from 47°C to 81°C (De Sadeleer et al., 2008). This implies that significant improvements can be made and that a stricter monitoring is necessary.

A high slaughter speed further tends to increase the risk of carcass contamination (Chapter 5, Letellier et al., 2009; Gomes-Neves et al., 2012). This can partially be explained by the failure of washing the knives and equipment between every carcass as demonstrated by De Sadeleer et al. (2008). Therefore, it is useful to work with a set of 2 knives/bung cutters, one can be used while the other is sanitized in water of 82°C (Bolton et al., 2002).

Also meat inspectors can contribute to a higher contamination level through incorrect hand and knife hygiene. Yet, in the near future, risk based visual meat inspection will replace the traditional meat inspection in the EU (Blaha et al., 2007; EFSA, 2011). In this new way of inspection, palpation and incisions will be kept to a minimum, preventing cross contamination in this step of the slaughter process.

Forced chilling followed by cold room storage reduces the *Salmonella* prevalence on the carcass (Gonzales Barron et al., 2008) as a result of the cold and osmotic shock induced by the low temperature and by the drying of the carcass surface. This was confirmed by the study described in Chapter 5, which demonstrated that only 2% of the carcasses was positive after chilling. This percentage could however be an underestimation of the true prevalence as the detection rate can be influenced by active attachments to the carcass and bacterial stress during chilling (EFSA, 2011).

The slaughtering stage is considered to have the highest impact on carcass contamination (Swanenburg et al., 2001a; van der Gaag et al., 2004; Alban and Stärk, 2005; van Hoek et al., 2012). Characterization of *Salmonella* isolates obtained from the study described in Chapter 5 confirms this as only a minority of the genotypes recovered from carcasses was indistinguishable from those detected in the intestines (gut and lymph nodes) of the same pig. Large differences between slaughterhouses were noticed in the contamination level of the carcasses (Chapter 5), suggesting that carcass contamination is mainly slaughterhouse specific. Other molecular studies confirm this finding and indicate differences in hygienic parameters and control processes as possible explanation (Botteldoorn et al., 2004; Pearce et al., 2004; da Silva et al., 2012). Botteldoorn et al. (2004) suggested that the aerosol generated by washing the carcasses might be an important vehicle for transmission of *Salmonella* strains to carcasses. Factors influencing the contamination level further include slaughterhouse infrastructure, slaughter speed, education of slaughterhouse personnel, cleaning and disinfection procedures and presence of residual flora.

The results of the study described in Chapter 5 demonstrate that the obtained *Salmonella* reduction at primary production can easily be nullified when pigs are slaughtered in a contaminated environment. Slaughterhouses should therefore take part in the national *Salmonella* control program. The monitoring in the auto-control program should be improved at first as discrepancies are found in the carcass prevalence obtained by FASFC inspectors and the slaughterhouse itself (Anonymous, 2012). When carcass contamination is high, immediate action has to be taken to reveal the contamination source.

Although a slaughterhouse specific approach is necessary, general guidelines describing the critical points for *Salmonella* contamination and spread as well as possible control measures should become available to facilitate and refine the monitoring and control of *Salmonella* at slaughterhouse level.

The implementation of slaughterhouses in the national control program, might further lead to public classification of slaughterhouses into high and low risk categories, enabling farmers to select a slaughterhouse of their choice. The risk classification of herds as well as slaughterhouses might also be part of the risk based meat inspection system in the near future (EFSA, 2011). Carcass decontamination might then be considered for carcasses originating from pigs of high risk herds and slaughtered at high risk slaughterhouses, although one should be aware that the decontamination procedure may not lead to lower hygiene and control standards at the slaughterhouse.

Perspectives for future research

This thesis has provided some useful information on the influence of the enrichment medium used during isolation of *Salmonella*, on the effect of acidified drinking water in slaughter pigs and on the prevalence, distribution and characterization of *Salmonella* in different slaughterhouses and along the slaughter process.

Still some questions remained unanswered or new issues were addressed. Future research should focus on following items:

- Further studies are needed to unravel the factors influencing growth on different enrichment media and to determine the different growth characteristics of specific serotypes,
- Studies should be conducted to evaluate the effect of acidification of drinking water during risk periods and to evaluate the effect of coated organic acids on existing *Salmonella* infection in pigs,
- At herd level, official criteria should be established to define the *Salmonella*-free status,
- Additional research has to be carried out to obtain a sampling strategy that is able to assess the actual *Salmonella* status of pigs prior to slaughter,
- At the slaughterhouse, the effect of auto-evaluation of the cleaning and disinfection procedures at the lairage area should be investigated,
- General guidelines involving *Salmonella* risk factors and control measures at slaughter should be created as an easy and practical aid to support the slaughterhouses in the monitoring and control of *Salmonella* at this level,
- The national *Salmonella* control program should be improved and should also include surveillance and control programs at slaughterhouse level.

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SUMMARY

SUMMARY

Salmonellosis is still a major cause of bacterial gastro-intestinal illness in humans, with 99,020 confirmed cases in the EU in 2010. Eggs were estimated to be the most important source of disease in the EU, followed by pork. In Belgium, 3,231 human *Salmonella* cases were reported in 2011 with an estimated proportion of 74% of *Salmonella* cases that was attributed to the consumption of contaminated pork (data from 2006 to 2009). In January 2005, a national *Salmonella* sero-surveillance and control program (SAP) was implemented in Belgian pig herds, categorizing herds as *Salmonella* risk herds when the average S/P ratio of three consecutive sampling rounds is higher than 0.6. In literature, several risk factors and possible control measures are described to prevent the entrance and spread of *Salmonella* at herd level (**Chapter 1**), although no easy, practical and economical measure to eradicate *Salmonella* from all types of pig herds is currently available.

A critical evaluation of the SAP further demonstrated that little progress has been made in the average S/P ratio of Belgian herds (year 2005-2010) and that sampling error influences the correct allocation of herds. In March 2012, the Scientific Committee of the Federal Agency for the Safety of the Food Chain proposed several possible monitoring scenarios based on bacteriological monitoring at herd and/or slaughterhouse level, although the Belgian Food Agency has, at present, not yet decided when and how the current SAP will be changed.

Quantitative microbiological risk assessment showed however that interventions taken at slaughterhouse level produce larger reductions of human illness than interventions in the primary production. An overview of the slaughter process, current carcass monitoring in Belgium and possible control measures at harvest is given in **Chapter 1** of this thesis.

The general aim of the present thesis (**Chapter 2**) was to obtain more knowledge on the epidemiology, diagnostics and control of *Salmonella* in the primary production as well as at the slaughterhouse.

In **Chapter 3**, the use of different enrichment media in the standard protocol for isolation of *Salmonella* from animal feces and samples from the primary production was evaluated. Besides the currently described MSRV medium, two other semi-solid media (DIASALM and SMS) and three different enrichment broths (RV, RVS and MKTTn) were investigated. The effect of the enrichment medium on *Salmonella* recovery and diversity

(sero- and genotype) was assessed by using content of duodenum from naturally infected slaughter pigs. Results demonstrated that a significantly higher relative sensitivity was obtained when using semi-solid media compared to enrichment broths. Within semi-solid media and within enrichment broths no significant differences in relative sensitivity were obtained. To obtain a relative sensitivity higher than 83.1% (MSRV), different enrichment media (semi-solid and broth) should be combined. Differences in sero- and genotypes were predominantly found within the same enrichment medium. Including sampling close to the inoculation drop (semi-solid media), and analyzing multiple colonies derived from the enrichment broths increased the number of detected sero- and genotypes. Although increasing the number of colonies analyzed and using multiple enrichment media inherently involve higher costs and more labor, on occasions when detecting the *Salmonella* source is of primary importance, such as in outbreak investigations, it should be definitely considered.

The effect of acidifying drinking water of finishing pigs two weeks prior to slaughter on *Salmonella* prevalence at slaughter was described in **Chapter 4**. A mixture of organic acids was used to lower the pH of the drinking water to a maximum of 4. The pH of the drinking water was checked daily and adjusted if needed. Approximately 600 pigs originating from four farrow-to-finish herds were included in the study. At slaughter, blood, mesenteric lymph nodes, content of ileum and rectum and carcass swabs after chilling were collected. *Salmonella* was isolated from 11.8% of the slaughterhouse samples. The difference between treatment and control groups concerning the number of animals tested positive in at least one sample was variable and small (37.1% and 32.8% respectively). None of the observed differences in *Salmonella* prevalence between treatment and control group were statistically significant. The results of this study indicate that the application of organic acids in drinking water during the last two weeks prior to slaughter was insufficient to decrease *Salmonella* shedding and carcass contamination in pigs at slaughter.

In **Chapter 5** the prevalence of *Salmonella* contamination along the slaughter line in five different slaughterhouses was investigated and possible contamination sources were identified. Therefore, pigs were individually identified at bleeding and sampled at different stages along the slaughter line. Of each pig, swabs of the oral cavity, mesenteric lymph nodes and content of duodenum, ileum and rectum were collected. Carcass swabs were taken after polishing, splitting and forced chilling. Additionally, lairage pens were sampled using overshoes and scalding tank water was collected before and during slaughter activity and at the in- and outlet of the scalding tank. All samples were submitted to *Salmonella* isolation and

Salmonella isolates were serotyped and genotyped by PFGE to define clonal relationships between *Salmonella* strains and to assess the distribution of the recovered strains. In total, 14.1% of all samples taken was *Salmonella* positive. Of the sampled pigs, 48.2% harbored *Salmonella* in at least one sample. The prevalence of *Salmonella* in the lairage area and the level of carcass contamination varied widely among the sampled slaughterhouses. In the scalding tank water, no *Salmonella* could be detected at any occasion. Statistical analysis showed that the contamination of the lairage area was related to an increased carcass contamination after polishing. The carcass contamination after splitting and forced chilling was related to the carcass contamination level after polishing. Genotyping demonstrated that only a minority of the genotypes found on the carcasses corresponded with those found in the pig (mesenteric lymph nodes and gut content). The results of this study illustrate that the lairage area is a primary source of *Salmonella* in slaughter pigs and that carcass contamination originates from the environment rather than from the pig itself. The major differences in contamination level between slaughterhouses further suggest that *Salmonella* control at this level demands a slaughterhouse specific approach.

Based on the results of the studies carried out during the current thesis, it can be stated that the standard *Salmonella* isolation protocol is inadequate when it comes to more advanced epidemiological studies. Multiple enrichment media and multiple colonies per medium should be analyzed to obtain realistic and valid data in such surveys. As multiple sero- and genotypes occur within pigs, characterization of isolates is important when a *Salmonella* source has to be identified. To control *Salmonella* at herd level, the limited administration of organic acids in the drinking water of finishers is not sufficient to influence the *Salmonella* prevalence at slaughter. The strategic administration of organic acids in drinking water during risk periods earlier in life should further be investigated. More promising seems the administration of coated organic acids in feed, although this should be confirmed by *in vivo* studies performed under natural conditions (**Chapter 6**). Other control measures such as purchase policy, biosecurity and management, *Salmonella*-free feed and vaccination are discussed in **Chapter 6**. As interventions taken at the primary production alone are insufficient to rapidly reduce human salmonellosis to an acceptable level, slaughterhouse interventions should be implemented in national *Salmonella* control programs. The results described in this thesis clearly demonstrate that the slaughterhouse environment is a major source of carcass contamination. An accurate surveillance and control program should be put in place at this

level. Only combining efforts at both primary and slaughterhouse level could eventually result in a decrease of the *Salmonella* prevalence on the carcass.

SAMENVATTING

SAMENVATTING

Met 99020 bevestigde gevallen in de Europese Unie (EU) in 2010, vormt salmonellose nog steeds een belangrijke oorzaak van bacteriële gastro-intestinale infecties bij de mens. In de EU worden eieren beschouwd als de belangrijkste bron van ziekte, gevolgd door varkensvlees. In België werden in 2011, 3231 humane *Salmonella* gevallen gerapporteerd, waarvan geschat werd dat 74% van de gevallen toegeschreven kon worden aan de consumptie van besmet varkensvlees (data over de periode 2006-2009). Vanaf januari 2005 werd het *Salmonella* Actie Plan (het SAP) ingevoerd op Belgische varkensbedrijven. In dit programma worden bedrijven als risico-bedrijf aangeduid wanneer de gemiddelde S/P ratio van drie opeenvolgende staalnames hoger is dan 0.6. In de literatuur werden verschillende risicofactoren en mogelijke bestrijdingsmaatregelen beschreven om de intrede en verspreiding van *Salmonella* op bedrijfsniveau te voorkomen (**Hoofdstuk 1**). Tot op heden bestaat er echter geen gemakkelijke, praktische en economisch verantwoorde maatregel om *Salmonella* uit te roeien in al de verschillende varkenshouderijsystemen.

Een kritische evaluatie van het SAP toonde verder aan dat slechts weinig vooruitgang werd geboekt aangaande de gemiddelde S/P ratio van Belgische bedrijven (jaar 2005-2010) en dat een juiste toewijzing van de bedrijven sterk beïnvloed werd door fouten eigen aan de bemonsteringsmethode. In maart 2012 diende het Wetenschappelijk Comité van het Federaal Agentschap voor de Veiligheid van de Voedselketen een advies in waarbij verschillende bewakingsprogramma's gebaseerd op bacteriologische monstername op bedrijfs- en /of slachthuisniveau werden voorgesteld. Tot op heden heeft het Belgische Voedselagentschap nog niet beslist hoe en wanneer het huidige SAP gewijzigd zal worden.

Kwantitatieve microbiologische risicobeoordeling heeft aangetoond dat maatregelen genomen op slachthuisniveau een grotere reductie van humane salmonellose teweeg brengen dan maatregelen genomen in de primaire productie. Een overzicht van het slachtproces, de huidige karkasbemonstering in België en mogelijke bestrijdingsmaatregelen op slachthuisniveau is terug te vinden in **Hoofdstuk 1** van deze thesis.

De algemene doelstelling van deze thesis (**Hoofdstuk 2**) was om meer kennis te verwerven aangaande de epidemiologie, diagnostiek en bestrijding van *Salmonella* zowel in de primaire productie als op slachthuisniveau.

In **Hoofdstuk 3** werd het effect van verschillende aanrijkingsmedia nagegaan in het standaard isolatie protocol voor *Salmonella* uit dierlijke feces en stalen afkomstig uit de primaire productie. Naast het huidig beschreven MSRV medium, werden twee andere half-vaste media (DIASALM en SMS) en drie verschillende aanrijkingsbouillons (RV, RVS en MKTTn) onderzocht. Door gebruik te maken van de inhoud van het duodenum van natuurlijk geïnfecteerde slachtvarkens, werd het effect van het aanrijkingsmedium op het detecteren en de verscheidenheid (sero- en genotype) van *Salmonella* nagegaan. De resultaten toonden aan dat met half-vaste media een significant hogere relatieve sensitiviteit werd bekomen dan met de aanrijkingsbouillons. Tussen de half-vaste media en de aanrijkingsbouillons onderling werden geen significante verschillen in relatieve sensitiviteit waargenomen. Om een relatieve sensitiviteit te bekomen van meer dan 83.1% (MSRV), dienen meerdere aanrijkingsmedia (half-vast en bouillon) gecombineerd te worden. Verschillende sero- en genotypes werden voornamelijk teruggevonden binnen eenzelfde medium. Het aantal gedetecteerde sero-en genotypes nam toe wanneer er ook stalen werden genomen dichtbij de inoculatie druppel (half-vaste media) en wanneer meerdere kolonies afkomstig van de aanrijkingsbouillons werden onderzocht. Hoewel het analyseren van meerdere kolonies en het gebruik van meerdere aanrijkingsmedia ongetwijfeld meer kosten en arbeid met zich meebrengen, moet het zeker overwogen worden wanneer het vinden van de *Salmonella* bron, zoals in het onderzoek van uitbraken, erg belangrijk is.

In **Hoofdstuk 4** werd het effect beschreven van het gedurende twee weken voor slacht aanzuren van drinkwater bij varkens op de *Salmonella* prevalentie bij het slachten. Een mengsel van organische zuren werd gebruikt om de pH van het drinkwater te doen dalen tot een pH van maximum 4. De pH van het drinkwater werd dagelijks gecontroleerd en indien nodig aangepast. Ongeveer 600 varkens afkomstig van vier gesloten varkensbedrijven namen deel aan de studie. In het slachthuis werden bloed, mesenteriale lymfeknopen, inhoud van ileum en rectum en karkasswabs na koeling verzameld. *Salmonella* werd geïsoleerd uit 11.8% van de slachthuisstalen. Het verschil tussen behandelings- en controlegroep aangaande het aantal dieren dat in minstens één staal positief werd bevonden, was klein en variabel (37.1% en 32.8%, respectievelijk). Geen enkele van de waargenomen verschillen in *Salmonella* prevalentie tussen behandelings- en controlegroep was statistisch significant. De resultaten van deze studie tonen aan dat het gebruik van organische zuren in het drinkwater gedurende de laatste twee weken voor slachten onvoldoende was om de uitscheiding van *Salmonella* en de karkascontaminatie van varkens bij slachten te verminderen.

In **Hoofdstuk 5** werd in vijf slachthuizen de prevalentie van *Salmonella* besmetting langsheen de slachtlijn nagegaan en werden mogelijke contaminatiebronnen geïdentificeerd. Hiervoor werden varkens bij het uitbloeden individueel geïdentificeerd en werden op verschillende plaatsen langsheen de slachtlijn stalen genomen. Van ieder varken werden swabs uit de mondholte, mesenteriale lymfeknopen en inhoud van duodenum, ileum en rectum verzameld. Karkasswabs werden genomen na poetsen, na klieven en na snelkoeling. Bijkomend werden de hokken van de wachtruimte bemonsterd door middel van overschoentjes en werd broeibakwater verzameld aan de in- en uitlaat van de broeibak en dit voor en tijdens slachtactiviteit. Alle stalen werden onderworpen aan het standaard *Salmonella* isolatie protocol. *Salmonella* isolaten werden verder geserotypeerd en genotypering werd uitgevoerd door gebruik te maken van PFGE om clonale verbanden tussen *Salmonella* stammen aan te tonen en om de verdeling van de bekomen stammen te beoordelen. In totaal werden 14.1% van alle stalen *Salmonella* positief bevonden. Bijna de helft (48.2%) van de bemonsterde varkens was *Salmonella* positief in minstens één staal. Het voorkomen van *Salmonella* in de wachtruimte en het niveau van karkascontaminatie varieerde sterk tussen de bemonsterde slachthuizen. In het broeibakwater kon geen enkele keer *Salmonella* teruggevonden worden. Statistische analyse toonde aan dat de besmetting in de wachtruimte gerelateerd was aan een verhoogde karkascontaminatie na poetsen. De karkascontaminatie na klieven en na snelkoeling was gerelateerd aan de karkascontaminatie na poetsen. Genotypering toonde aan dat slechts een minderheid van de genotypes die werden teruggevonden op de karkassen overeenstemden met deze gevonden in het varken (mesenteriale lymfeknopen en darminhoud). De resultaten van deze studie illustreren dat de wachtruimte een primaire bron van *Salmonella* bij slachtvarkens is en dat karkascontaminatie eerder afkomstig is van de omgeving dan van het varken zelf. De grote verschillen in besmettingsniveau tussen de slachthuizen toont verder aan dat bestrijding van *Salmonella* op dit niveau een slachthuis-specifieke aanpak vergt.

Gebaseerd op de resultaten beschreven in deze thesis, kan gesteld worden dat het standaard *Salmonella* isolatie protocol ontoereikend is in het kader van meer geavanceerde epidemiologische studies. Meerdere aanrijkingsmedia en meerdere kolonies per medium zouden onderzocht moeten worden om realistische en waardevolle data te verkrijgen. Aangezien meerdere sero- en genotypes voorkomen binnen varkens, is verdere typering belangrijk wanneer de *Salmonella* bron dient achterhaald te worden. Als bestrijdingsmaatregel op bedrijfsniveau is de beperkte toepassing van organische zuren in het drinkwater van

varkens onvoldoende om de *Salmonella* prevalentie bij slachten te beïnvloeden. De strategische toepassing van organische zuren in het drinkwater gedurende risico-perioden dient verder onderzocht te worden. Het gebruik van gecoate organische zuren in het voeder lijkt veelbelovend, hoewel dit bevestigd moet worden aan de hand van *in vivo* studies onder praktijkomstandigheden (**Hoofdstuk 6**). Andere controle maatregelen zoals aankoopbeleid, bioveiligheid en management, *Salmonella*-vrij voeder en vaccinatie worden bediscussieerd in **Hoofdstuk 6**. Aangezien het nemen van maatregelen op het niveau van de primaire productie alleen onvoldoende is om humane salmonellose snel te reduceren tot een aanvaardbaar niveau, dienen ook slachthuisinterventies opgenomen te worden in nationale *Salmonella* bestrijdingsprogramma's. De resultaten beschreven in deze thesis tonen duidelijk aan dat de slachthuisomgeving een belangrijke bron is van karkascontaminatie. Op dit niveau dient een nauwkeurig surveillance en controle programma opgezet te worden. Enkel wanneer inspanningen gecombineerd worden op zowel het niveau van de primaire productie als op slachthuisniveau kan dit leiden tot de uiteindelijke daling van de *Salmonella* prevalentie op het karkas.

CURRICULUM VITAE - PUBLICATIONS

CURRICULUM VITAE

Emily Valerie De Busser werd geboren op 31 oktober 1981 te Herentals. Na het behalen van het diploma hoger secundair onderwijs aan het Sint-Jozefinstituut van Herentals (Latijn-Wetenschappen, 6 uur Wiskunde), begon zij in 1999 met de studie Diergeneeskunde. De kandidaturen volgde zij aan het RUCA in Antwerpen, de daaropvolgende drie jaar studeerde zij aan de UGent. In 2005 behaalde zij het diploma van Dierenarts (optie Herkauwers) aan de Universiteit Gent met onderscheiding.

In november 2005 trad zij als wetenschappelijk medewerker in dienst bij de Vakgroep Voortplanting, Verloskunde en Bedrijfsdiergeneeskunde. De veiligheid van een varkensvaccin werd hierbij getest onder praktijkomstandigheden. Vanaf 1 februari 2006 werd zij aangesteld als halftijds assistent bij de Vakgroep Veterinaire Volksgezondheid en Voedselveiligheid en als halftijds assistent bij de Vakgroep Voortplanting, Verloskunde en Bedrijfsdiergeneeskunde. Haar onderzoek handelde over het voorkomen en de diagnostiek van *Salmonella* bij het varken en mogelijke bestrijdingsmaatregelen tegen *Salmonella* in zowel de primaire productie als op slachthuisniveau.

Gedurende de 7 jaren aan de Faculteit was zij ook actief in de Bedrijfsbegeleiding Varken en nam ze deel aan de nacht- en weekenddiensten op de Buitenpraktijk. Voor beide vakgroepen stond ze tevens mee in voor de opleiding van de studenten Diergeneeskunde gedurende hun Masterjaren.

Emily V. De Busser is auteur en medeauteur van meerdere wetenschappelijke publicaties in nationale en internationale tijdschriften en nam actief deel aan diverse nationale en internationale congressen. Tevens is zij reviewer voor diverse internationale tijdschriften.

PUBLICATIONS

International journals

De Busser, E.V., Dewulf, J., Nollet, N., Houf, K., Schwarzer, K., De Sadeleer, L., De Zutter, L., Maes D., 2009. Effect of organic acids in drinking water during the last 2 weeks prior to slaughter on *Salmonella* shedding by slaughter pigs and contamination of carcasses. Zoonoses Public Health 56, 129-136

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