Assessing and managing the risk posed by *Salmonella* in pig feed

by

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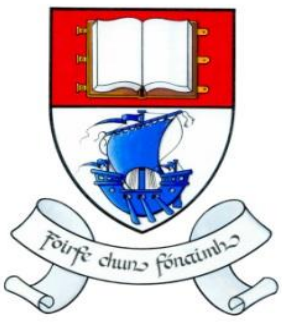

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Abbreviations

^{o}C	Degrees centigrade
a_w	Water activity;
BGA	Brilliant green agar
bp	Base pairs
BPW	Buffered peptone water
BSE	Bovine Spongiform Encephalopathy
CFU	Colony forming unit
CLSI	Clinical and Laboratory Standards Institute
D-Value	Decimal reduction time
DAFM	Department of Agriculture, Food and the Marine
DNA	Deoxyribonucleic acid
EFSA	European Food Safety Authority
FDA	Food and Drug Administration
g	Gram(s)
>	Greater than
h	Hour(s)
HACCP	Hazard Analysis Critical Control Point System
IAC	Internal Amplification Control
ISO	International Organisation for Standardisation
kg	Kilogram(s)
<	Less than
l	Litre(s)
log_{10}	Logarithm to the base to the base 10

min	Minute(s)
ml	Millilitre(s)
Mm	Millimolar
MPN	Most probable number
MLVA	Multi locus variance analysis
MRD	Maximum recovery diluent
n	Total number of samples
NCCLS	National Committee for Clinical Laboratory Standards
NSRL	National *Salmonella* Reference Laboratory
%	Percent
±	Plus or minus
PCR	Polymerase Chain Reaction
PFGE	Pulsed-field gel electrophoresis
R^2	Regression coefficient
RMSE	Root mean squared error
rpm	Revolutions per minute
RT	Reverse Transcription
RT-PCR	Reverse transcription-polymerase chain reaction
RVS	Rappaport-Vassiliadis
s	Seconds(s)
SAS	Statistical Analysis System
SE	Standard error
SED	Standard error of difference
S.I.	Statutory Instruments
TSA	Tryptone soya agar

TSB	Tryptone soya broth
µl	Microlitre
µg	Microgram
UPGMA	Unweighted pair group method with arithmetic averages
VRBGA	Violet red bile glucose agar
VNTR	Variable number tandem repeat.
XLD	Xylose lysine deoxycholate agar
Z-Value	Change in temperature required for one $\log_{10}$ reduction in the D-value

Declaration

I declare that this thesis has not been previously submitted as an exercise for a degree at Waterford Institute of Technology, or any other university. I further declare that the work embodied in this thesis is my own, except where I have received help as stated in the acknowledgement and text. All quotations and summary of the work of others have been acknowledged where appropriate. Permission is granted for this work to be copied, provided that in each case the user acknowledges the source and his or her indebtedness to the author.

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Date_____

Abstract

Salmonella in pigs and derived pork is a significant food safety concern in Ireland. During commercial pig production, it is proposed that a number of risk factors including commercial feed can introduce *Salmonella* to fattening pig herds and may thereby cause human infections via consumption of contaminated pork. The objective of this work was to carry out an in-depth study on ten high *Salmonella* sero-prevalence pig farms to firstly identify which production stages were the principal harbours of *Salmonella* infection and secondly, to assess the occurrence of *Salmonella* in feed throughout the different production stages on these farms and thereby assess potential risks as well as epidemiological relationships. Isolates were characterized by serotyping, antibiotic resistance profiling, pulsed field gel electrophoresis (PFGE) and multiple locus VNTR analysis (MLVA). Eleven serotypes were recovered, with a monophasic variant of Typhimurium (4,[5],12:i:) accounting for 41.1% of all isolates recovered. Five *Salmonella* 4,[5],12:i:- isolates were recovered from the commercial feed mills supplying our pig farms. Typing of all isolates resulted in two common distinct MLVA profiles for both mills and farms. The *Salmonella* 4,[5],12:i:- variant that predominated in the pigs and feed is an emerging strain in Europe and are of increasing food safety concern. The objective of the final study was to determine the survival characteristics of *Salmonella* 4,[5],12:i:- strains. The thermal inactivation of the five feed strains at 55, 60 and 65°C was investigated using an immersed heating coil apparatus. The ability of the five strains to survive during storage on weaner pig feed premixed with Sodium Butyrate was also assessed over 28 day storage. The key findings in this study is the confirmation of monophasic *Salmonella* Typhimurium occurrence in new geographical settings and the indication of its possible role in the transmission of *Salmonella* from contaminated feed ingredients and feed to pigs.

Chapter 1

Literature Review

1. Introduction

1.1 The Bacterium *Salmonella*

Salmonellae belong to the *Enterobacteriaceae* family. The genus *Salmonella* contains two species; *S. bongori* and *S. enterica,* the latter being classified into six subspecies on the basis of biochemical and genomic characteristics (Table 1). Each species/subspecies is then further classified into serovars. *Salmonella* are Gram negative straight rods, with peritrichous flagellae when motile. They are mesophilic, growing at temperatures of between 5.2 and 47°C, with an optimum growth temperature of 35-37°C. In addition, *Salmonella* are facultative anaerobes and are capable of growing at pH 4-9 and at a water activity (a_w) of greater than 0.94 (Mani-López et al. 2012).

Salmonella enterica is adapted to live in the intestine of man and warm-blooded animals, whereas *S. bongori* is found in the intestine of cold-blooded animals. As regards food safety, *S. enterica* subsp. *enterica* is the subspecies of most concern, as serovars from within this group are known to cause 99% of *Salmonella* infections in humans (Brenner et al. 2000). Historically, *Salmonella* serovars were named according to syndrome (e.g. *S.* Typhi), relationship (e.g. *S.* Paratyphi A, B, C), host-specificity (e.g. *S.* Choleraesuis) or geographical origin of their first isolation (e.g. *S.* Dublin). However, nowadays *Salmonella* is classified using the White-Kauffmann-Le Minor scheme (Grimont and Weill, 2007). Serotyping is performed by slide agglutination based on antigenic variability of lipopolysaccharides (O antigens), flagellar proteins (phases 1 and 2 H antigens, encoded by *fliC* and *fljB*) and capsular polysaccharides (Vi antigens) (Switt et al. 2009). In most *S. enterica* subsp. *enterica* serovars, the antigenic formula is composed of two flagellar phases, however there

are monophasic variants that lack this second flagellar phase S**.**1,4,[5],12:i:- (Switt et al. 2009) In addition, strains within a particular serovar i.e. *S.* Typhimurium, may be further differentiated into phage types according to their susceptibility to lysis by a set of bacteriophages with different specificities.

Table 1.1: Details of the two *Salmonella* species and six *S. enterica* subspecies (Grimont and Weill 2007)

***Salmonella* Species**	***S. enterica***						***S. bongori (V)***
Subspecies	*Enterica (I)*	*Salamae(II)*	*Arizonae (IIIa)*	*Diarizonae (IIIb)*	*Houtenae (IV)*	*Indica (VI)*	
Tests							
Dulcitol	+	+	-	-	-	d	+
ONPG (2 h)	-	-	+	+	-	d	+
Malonate	-	+	+	+	-	-	-
Gelatinase	-	+	+	+	+	+	-
Sorbitol	+	+	+	+	+	-	+
Growth with KCN	-	-	-	-	+	-	+
L(+)-tartrate[a]	+	-	-	-	-	-	-
Galacturonate	-	+	-	+	+	+	+
γ-glutamyltransferase	+[b]	+	-	+	+	+	+
ß-glucuronidase	d	d	-	+	-	d	-
Mucate	+	+	+	- (70%)	-	+	+
Salicine	-	-	-	-	+	-	-
Lactose	-	-	- (75%)	+ (75%)	-	d	-
Lysed by phage O1	+	+	-	+	-	+	d
No. of Serovars	1,531	505	99	336	73	13	22

+ = 90% or more positive reactions.
– = 90% or more negative reactions.
d = different reactions given by different serovars.
[a] *d*-tartrate.
[b] Typhimurium d, Dublin –.

1.2 Disease symptoms

1.2.1 *Salmonella* Infection in Humans

The principal clinical syndromes associated with *Salmonella* infection in humans are enteric (typhoid) fever and gastroenteritis. Enteric fever is a protracted systemic illness that results from infection with the exclusively human pathogens, *S.* Typhi and *S.* Paratyphi (Ohl and Miller, 2001). It generally occurs in developing countries, such as those in South America, Africa, and parts of Asia (Crump et al. 2004). Clinical manifestations include fever, abdominal pain, nausea, vomiting, transient diarrhoea or constipation, and occasionally a maculopapular rash. The pathological hallmark of enteric fever is mononuclear cell infiltration and hypertrophy of the reticuloendothelial system, including the intestinal Peyer's patches, mesenteric lymph nodes, spleen, and bone marrow. Without treatment, mortality rates are 10-15%.

On the other hand, non-typhoidal *Salmonella* serovars are important food-borne pathogens that cause gastroenteritis, bacteraemia, and focal infections in humans. The incubation period ranges from 5 h to 7 days, but clinical signs usually begin 6-72 h after consumption of food containing *Salmonella*. The infectious dose of *Salmonella* was thought to be >10,000 cells, but several outbreaks have indicated that as few as 1-10 cells can constitute an infectious dose (EFSA, 2010). However, several factors including strain virulence, age and immune status of the individual, as well as the composition of the food in which the pathogen is found can influence infectivity (Lloyd-Smith et al. 2005). Symptoms of food-borne salmonellosis include diarrhoea, nausea, abdominal pain, mild fever and chills (Nørrung and Buncic, 2008). Vomiting, prostration, anorexia, headache and malaise may also occur.

Severity of the symptoms varies, depending on the age and immune status of the individual, ranging from mild pain and barely detectable diarrhoea to debilitating pain and severe, even bloody diarrhoea. Infection can also give rise to chronic diseases, including localised infections in specific tissues or organs and reactive arthritis, e.g. Reiter's syndrome, as well as neurological and neuromuscular illnesses.

Serovars such as Typhimurium and Enteritidis are the most common cause of food-borne salmonellosis, accounting for 60 to 65% of outbreaks worldwide (EFSA, 2014). Although the occurrence of monophasic variant cases of *Salmonella* Typhimurium (4,[5],12:i:-) that lack the expression of flagellar phase 2 antigens has increased in humans from 360 in 2007 to 5932 in 2012 along with the number of countries reporting this type (EFSA, 2014). In 2012, 92,916 cases of food-borne salmonellosis were reported in the EU alone, with worldwide incidence estimated at 1.3 billion cases causing 3 million deaths per year (EFSA, 2011, 2014). However, these data often underestimate the magnitude of the problem, as many cases are not reported (Majowicz et al. 2010). The majority (90-95%) of sporadic cases and outbreaks of salmonellosis result from the ingestion of contaminated foods, both fresh produce and ready-to-eat products, such as poultry meat, pork, beef, eggs, milk and seafood (EFSA, 2008). However, infection through direct contact with carrier animals and person-to-person transmission can also occur.

1.2.2 Antibiotic Resistance

Multidrug resistance (MDR) is defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories despite earlier sensitivity to it (Magiorakos et al. 2012). Multidrug resistance in bacteria may be attained through

intrinsic or acquired mechanisms. Intrinsic refers to resistance that is a consequence of a large selection of genetically-encoded mechanisms resulting in increased expression of genes that code for multidrug efflux pumps. Acquired resistance refers to resistance that is achieved through the accumulation of genes via the acquisition of additional mechanisms (transduction, conjugation, and transformation (Alekshun and Levy, 2007)) or is a consequence of mutational events under selective pressure.

Since the early 1990s, the frequency of isolation of multidrug-resistant (MDR) *Salmonella* phenotypes has been increasing worldwide. In particular, the DT104 phage type of *S.* Typhimurium has been of concern since it was first recognized in the UK in 1984, as it often displays a typical penta-resistance pattern (ACSSuT). However, in recent years there has been a reduction in the isolation of penta-resistant *S.* Typhimurium DT104 in Europe. This has been counteracted by an increase in prevalence of monophasic *S.* Typhimurium (4,[5],12:i:) isolates of resistance (R) type ASSuT (EFSA, 2013).

1.2.3 *Salmonella* Infection in Pigs

In pigs, salmonellosis is typically caused by *S.* Choleraesuis which causes swine paratyphoid (fever). *S.* Choleraesuis is an uncommon serotype in Europe and North America, possibly as a result of *Salmonella* control programs in these countries; however, the epidemiological pattern differs greatly in Asian countries (Chiu et al. 2004). Clinical manifestations include septicemia, enterocolitis or bacteremic localisation as pneumonia and hepatitis or occasionally as meningitis, encephalitis and abortion (Haesebrouck et al. 2004). Pigs will exhibit clinical signs 36-48 h after infection (Chiu et al. 2004). The disease is usually seen in pigs post-weaning but can

occur at any stage. Clinical signs include lack of appetite, depression, huddling, weakness, temperatures of up to 41.6°C and red to purple discoloration of the skin of the extremities, known as cyanosis. Diarrhoea is not present initially but occurs after a few days of illness. Morbidity is usually low to moderate but mortality is high among pigs that become ill. Duration of illness is unpredictable but is prolonged without successful intervention.

On the other hand, the enterocolitic form of salmonellosis in pigs can be caused by *S.* Typhimurium and/or monophasic variants (Haesebrouck et al. 2004). This tends to occur in weaned pigs, due to stresses caused by changes in feed, commingling and deprivation of the sow's milk (Nollet et al. 2005). Initial signs include moderate anorexia and diarrhoea that may be watery to yellow and intermittent. In chronic cases, following an acute episode, fever is intermittent and watery diarrhoea persists, resulting in progressive dehydration and weight loss. Recovery may be slow and death rates are difficult to predict because animals are often culled because of failure to thrive and poor condition.

However, asymptomatic carriage of *Salmonella* (as described in the next section) is much more common in pigs than clinical disease.

2. *Salmonella* Carriage in Pigs

The biological cycle of *Salmonella* is complex and involves many variables such as animals, environment and food, with animals acting as the most important reservoir for its conservation (Giaccone et al. 2012). As outlined in Section 1.3.2, a limited number of *Salmonella* serotypes can lead to clinical infection in pigs. However, more

commonly, a broad range of serotypes can occur as subclinical infections which constitute a potential source of *Salmonella* infection for humans. This is because *Salmonella* is carried asymptomatically by these animals, mainly in the gastrointestinal tract (principally the caecum) and the associated lymphatic tissues (tonsils and mesenteric lymph nodes), is shed in the faeces and can therefore be transferred onto the carcass at slaughter (Oliveira et al. 2012). The role of "healthy carrier" pigs is also important on-farm, as even if they do not show any disease symptoms (due to the fact that the *Salmonella* infection is latent), they readily become a continual, intermittent source of environmental contamination because of faecal shedding.

2.1 *Salmonella* Monitoring and Detection in Pig Herds

2.1.1 Bacteriology

Despite the availability of DNA-based strategies, serology and bacteriology still form the basis of *Salmonella* detection procedures. Culture-based methods are the most widely used technique and remain the gold standard for the detection of *Salmonella*. *Salmonella* detection in pigs/carcasses can be performed using standard culture-based isolation techniques on samples of faeces, caecal content, mesenteric lymph nodes, etc. *Salmonella* has been shown to persist in the intestinal mucosa, mesenteric lymph nodes or tonsils following infection (Berends et al. 1997; Vieira-Pinto et al. 2006, Methner et al. 2011), whereas pigs exposed to an infected population shedding $\leq 10^3$ colony forming units/gram of faeces can become infected with, and shed *S.* Typhimurium within 48 h post-exposure (Fedorka-Cray et al. 1994). For the monitoring of *Salmonella*, both within pig herds and in feed and feed ingredients, a culture-based ISO method is routinely used (ISO 6579, 2002/Amd

1:2007). This follows a basic strategy of non-selective pre-enrichment that allows the proliferation and regeneration of dehydrated and stressed bacterial cells, followed by selective enrichment, differential plating and biochemical or serological confirmation (Patel and Bhagwat, 2008). This method is labour-intensive (both in terms of operation and data collection), time consuming (require 4-5 days to obtain presumptive results) and expensive (significant usage of media and reagents). In addition, sensitivity can be affected by antibiotic treatment, inadequate sampling, and low numbers of viable microorganisms in samples. Despite these limitations, due to the high selectivity and sensitivity of standard culture methods they provide the greatest chance of isolating *Salmonella* organisms, if present. They express the actual infection status of the animal, including recent transmission or contamination and detect all serovars (Methner et al. 2011). The actual infectious agent is isolated, which makes further characterisation (e.g. typing and AMR profiling; see Section 2.2) possible.

2.1.2 Serology

National programmes to reduce *Salmonella* in pigs are based on serological tests on finisher pigs as a basis for classifying pig herds. The enzyme-linked immunosorbent assay (ELISA) test which was originally developed in Denmark detects anti-*Salmonella* antibodies and can be applied to meat juice (slaughterhouse level) and serum (farm level). The ELISA system offers the most sensitive and economical method of monitoring the incidence of exposure of pigs to *Salmonella*. The rate of exposure can be monitored at the slaughterhouse, and at various stages of the production cycle, in order to establish the point of exposure. This can then be used to facilitate the introduction of control procedures designed to reduce the exposure rate.

However, serology is of limited use in individual animals, as antibodies do not appear until two weeks after infection and as a result it may overlook recently-infected pigs that have been exposed but have not seroconverted (EFSA, 2010). In addition, it cannot differentiate between current and past infections and may identify animals that are already clear of infection that pose no further risk (Ball et al. 2011). Only the most common O-antigens are included in the test and because of this new emerging serovars may not be detected (Farzan et al. 2007; Forshell and Wierup, 2006).

2.1.3 Molecular Methods

Polymerase chain reaction (PCR) is a nucleic acid amplification technology which involves repeated cycles of DNA denaturation, primer annealing and extension (Sue et al. 2014). PCR methods developed for bacterial detection include multiplex PCR and real-time quantitative PCR (qPCR). However, few reports exist on the use of PCR-based methods for the detection of *Salmonella* in pigs, as many of these methods have not been fully validated for faecal and intestinal samples. To date, the technique is principally only reliable for pork meat products and animal feeds where PCR inhibitors are not so problematic. PCR may complement traditional microbiological methods by increasing speed, sensitivity, and specificity for detecting *Salmonella*. DNA is always present whether the cell is dead or alive and as a result PCR methods cannot discriminate between viable and non-viable cells (Malorny and Hoorfar, 2005; Keer and Birch, 2003). Despite this, PCR results have been shown to have a >95% correlation with culture-based results for faecal samples (Maddox, 2008; Pusterla et al. 2010; Wilkins et al. 2010).

2.2 Typing of *Salmonella* isolates

The capability to serotype or fingerprint any *Salmonella* isolates recovered is of importance for surveillance and investigation of outbreaks. As *Salmonella* is a diverse genus a single method may not work for all isolates. However, combinations of typing methods that are capable of differentiating clones of a particular serovar or phage type may overcome this.

2.2.1 Typing of *Salmonella* by phenotypic methods

2.2.1.1 Serotyping by slide agglutination

The White-Kauffmann-Le Minor scheme is the most commonly used and extensive conventional serotyping method for the identification of *Salmonella* isolates beyond the subspecies level (Popoff and Le Minor, 1997). Serotyping is performed by slide agglutination based on antigenic variability of lipopolysaccharides (O antigens), flagellar proteins (H antigens) and capsular polysaccharides (Vi antigens) (Switt et al., 2009). The combination of the antigens, referred to as the antigenic formula, is unique to each *Salmonella* serotype. Groups were originally designated by letters and the numbers 51-67 but they are now classified using the characteristic O factor. Although serotyping requires the use of over 150 specific antisera and carefully trained personnel, it is still the reference method by which *Salmonella* are identified following isolation. However, it is not sufficient for tracking isolates or to define phylogenetic relationships and is therefore usually followed by molecular subtyping.

2.2.1.2 Phage typing

Bacteriophages (phages) are viruses that can only grow or replicate within a bacterial cell. Phage typing may be used to differentiate *Salmonella* strains according to their

susceptibility to lysis by a set of bacteriophages with different specificities. This relies on the presence or absence of particular receptors on the bacterial surface which are used by the virus to bind to the bacterial wall. Phage typing schemes for *S.* Typhi, *S.* Paratyphi A and B, *S.* Typhimurium and *S.* Enteritidis and additional serovars of local importance are used in most reference laboratories. Phage typing in conjunction with antimicrobial susceptibility testing has been shown to be successful in the identification of the multidrug-resistant *S.* Typhimurium DT104, referred to in Section 1.2.2.

2.2.2 Typing of *Salmonella* by molecular methods

2.2.2.1 Pulsed-field gel electrophoresis (PFGE)

PFGE is often considered the ‘‘gold standard’’ of molecular typing methods for *Salmonella* and is characterized by a high degree of reproducibility both within and between laboratories (Swaminathan et al., 2001). The procedure involves cutting the intact bacterial chromosome with site-specific restriction endonucleases and separation of the resultant DNA fragments by changing the polarity of the current running through the gel at regular intervals. This generates a banding pattern that forms the basis for assessing similarity of isolates. However, serovars such as Typhimurium can be so genetically similar that even PFGE fails to discriminate strains because the low rate of genetic variation does not significantly impact the electrophoretic mobility of the restriction fragments (Leekitcharoenphon et al. 2014; Foley et al. 2006). These serovars can, however, be further classified by sequence-based molecular typing methods, such as multilocus variable number tandem repeat analysis (MLVA) or multilocus sequence typing (MLST).

2.2.2.2 Multilocus variable number tandem repeat analysis (MLVA)

MLVA utilises the naturally occurring variation in the number of tandem repeated DNA sequences found in the microbial genome. This is achieved by first amplifying the target loci by PCR followed by the subsequent sizing of the amplified DNA segments (amplicons) by either gel or capillary electrophoresis or on an automated DNA sequencer. The calculated number of repeats of the variable number tandem repeat (VNTR) loci (alleles) are represented as a strain-specific profile which consists of a series of numbers, each of which represents the number of repeats at one of the loci in a standard order, which can then be used for comparison. MLVA, in comparison to PFGE, is reproducible, faster and easier to perform and can be completely automated. Additionally, the data generated is easily analysed and shared via data-bases (Hopkins et al. 2011). In addition, MLVA has been shown to have a high discriminatory power for several *Salmonellae* serovars in comparison to other molecular methods such as PFGE and may provide an alternative to phenotypic assays and current molecular methods (Barco et al. 2013).

2.2.2.3 Multi Locus Sequence Typing (MLST)

Multi Locus Sequence Typing (MLST) is used to determine the nucleotide sequence (alleles) from a portion of housekeeping genes, which is performed by amplification by PCR followed by sequencing. The generated allelic profile or sequence types are then compared against publicly available databases to determine sequence types (Achtman et al. 2012). The great advantage of MLST is that all data produced are presented in an internationally standardised nomenclature which is available worldwide through the publicly accessible databases, making it highly reproducible and allowing for the comparison of data collected in different laboratories (EFSA,

2013). MLST is, however, unsuitable for routine laboratory testing or in outbreak investigations due to its relatively low discriminative power, although it is useful for the examination of the evolution of bacterial populations. It is also expensive and labour intensive (Feil and Enright, 2004).

2.3. On-Farm *Salmonella* Risk Factors

Producing pigs for meat production includes several production stages, as outlined in Figure 1; gestation (pregnancy of the sow), farrowing (giving birth to the piglets and suckling them up to weaning), weaner (growth of weaned pigs to 25-30kg normally broken into 2 stages, weaner 1 and weaner 2) and grow-finish (growth of pigs from 25-30kg to slaughter weight).

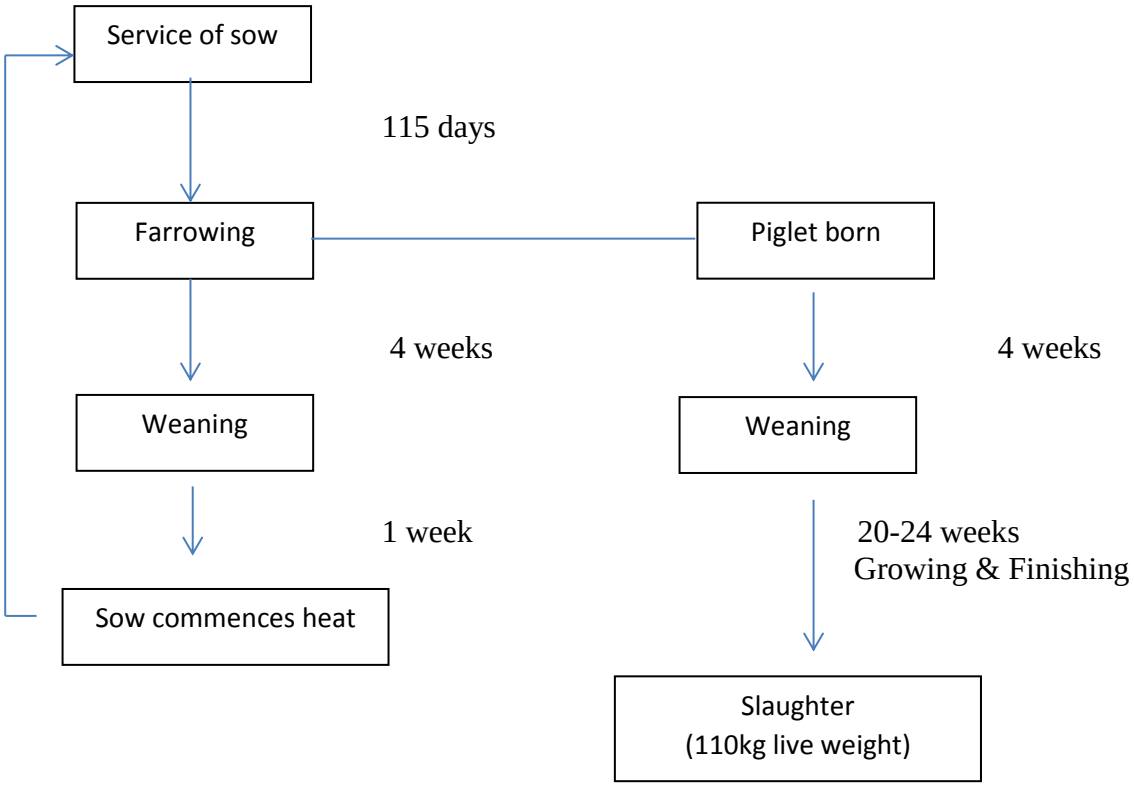


Figure 1: Schematic diagram of pig production cycle

Existing research on the epidemiology of *Salmonella* in pigs has primarily focused on finishing pigs due to their proximity to the consumer; however, other production stages can play an important role in the maintenance and dissemination of *Salmonella* on-farm (Wilkins et al. 2010). In particular, vertical transmission from breeding stock to progeny has been shown to be a potential route of infection (Davies and Hinton, 2000; Letellier et al. 2009). Therefore, pig flow management practices i.e. "all in-all out" should be practised to reduce stress within the herd and control cross-contamination of *Salmonella* between batches (Lo Fo Wong et al. 2004). Pigs should move in a one-way flow from relatively uncontaminated areas (dry sow/farrowing) to areas of higher *Salmonella* contamination (i.e. grower/finisher areas) to prevent the transmission of *Salmonella* to younger more susceptible pigs. However, a study by Dahl et al. (1997) questioned the importance of vertical transmission via the strategic removal of weaners off-site to clean premises, the removal of weaners was also proven to be successful in reducing *Salmonella* infection. The low susceptibility of the piglets to *Salmonella* infection from the sows may have been due to the absence of infection in the sows or may be attributed to the protective effect of maternal antibodies in the colostrum. However, when maternal antibodies decrease, the piglets are no longer protected and they then become susceptible to contamination with *Salmonella* from the environment.

Effective cleaning and disinfection are vital steps in *Salmonella* control. *Salmonella* may enter a herd via the introduction of a carrier animal; therefore, insufficient cleaning of excreted *Salmonella* within the pen environment may be sufficient to serve as a source of infection for subsequent pigs (Fedorka-Cray et al. 1994; Hurd et al. 2001). Poor biosecurity-related practices regarding farm personnel and visitors

have also been associated with increased risk of infection of pigs with *Salmonella*. Humans are able to spread far greater numbers of *Salmonella* than any other vector via boots, overalls and implements contaminated with faeces (Berends et al. 1996). Other external sources of the pathogen include transient vectors such as rodents, wild birds, pets, animal vehicles and (re)contaminated feed or water (Griffith et al. 2006). The most important risk factors regarding the prevalence of *Salmonella* in the pre-harvest stages of pork production according to a study by Berends et al. (1996) included the misuse of broad spectrum antibiotics (i.e. growth promotion), a positive *Salmonella*-status of animals prior to transport, lack of transport hygiene and transport stress.

2.4 *Salmonella* Prevalence in Pig Herds

A survey by the EFSA established the prevalence of *Salmonella* in slaughter pigs across the 24 EU member states and Norway for the years 2006-2007 (EFSA, 2008). In each participating country, a representative sample of carcasses was randomly selected in slaughterhouses, representing at least 80% of domestic production. In order to assess the infection status of the pigs, a 25g sample from an aggregate of ileo-caecal lymph nodes was collected from each carcass. Thirteen member states also performed carcass swabs in addition to sampling lymph nodes and nine member states also collected a muscle or blood sample for serological analysis. In total, eight member states collected all three sample types.

Salmonella-positive lymph node samples were found in 24 of the 25 member states. Finland was the only country to have no lymph node test positive, whereas only one pig tested was positive in Norway. This was not unexpected considering that the

Nordic countries (Finland, Sweden and Denmark) have virtually *Salmonella*-free pig production as a result of intervention strategies implemented some time ago, which proposed zero tolerance for *Salmonella*. Overall, the lymph node results showed that one in every 10 slaughter pigs in the EU (10.3%) was *Salmonella*-positive. EU prevalence ranged from 0-29%, with Ireland having a prevalence of 16.1%. In total, 87 different *Salmonella* serovars were isolated from the lymph nodes with the most prevalent being *S.* Typhimurium (40.0%), *S.* Derby (14.6%), *S.* Rissen (5.8%), S. 4,[5],12:i:- (4.9%) and *S.* Enteritidis (4.9%). All with the exception of *S.* Rissen have been shown to be frequent causes of *Salmonella* infections in humans. Ireland had the highest *Salmonella* prevalence (20%) on pig carcasses of all 13 member states that participated in the carcass survey, which was well above the EU average of 8.3%.

In Ireland, a number of studies have been conducted to determine the occurrence of *Salmonella* in pigs and to assess the risk factors contributing to its transmission. Duggan et al. (2010) tracked the *Salmonella* status of pigs from selected herds of different serological categories from the farm through transport and lairage to intestinal, carcass and pork primal cut samples taken at slaughter. Herds were graded as category 1 when ≤10% of the herd was serologically positive (ELISA cut-off 40% OD) for *Salmonella*, category 2 when >10% but ≤50% are sero-positive, and category 3 when > 50% are sero-positive. The study highlighted the lack of correlation between historical serological categorisation of the *Salmonella* status of a herd and actual bacteriological status of an individual pig at the time of slaughter, with category 2 pigs having the highest overall bacterial prevalence (72%). Genetic fingerprinting of all isolates recovered by PFGE showed that cross contamination

within the slaughter plant environment accounted for up to 69% of contamination on carcasses and pork cuts. In both the Republic of Ireland and Northern Ireland, *S.* Typhimurium and *S.* Derby were the predominant isolates recovered, accounting for ~50 and ~20% isolates, respectively, which is much higher than the EU prevalence for each of these serovars (EFSA, 2008).

2.5 Pig *Salmonella* Control Programmes

There are several points in the food chain, from farm to fork, at which control measures can be implemented to prevent the spread of *Salmonella* from slaughter pigs via meat products to man. Control of *Salmonella* in pork can be implemented on the farm, at slaughter and during processing. Pre-harvest control consists of monitoring *Salmonella* at the herd level, and implementing *Salmonella* reduction measures in infected herds through biosecurity, management and feeding strategies. The European regulation for the control of *Salmonella* and other specified food-borne zoonotic agents (EC No 2160/2003) states that 'the protection of human health against diseases and infections transmissible directly or indirectly between animals and humans (zoonoses) is of paramount importance' (Hotes et al. 2011). This regulation obliges Member States to set up and implement national control programmes for poultry and pigs for *Salmonella* serovars deemed to be of particular importance for public health. EFSA and the European Centre for Disease Prevention and Control (ECDC) annually collect data from the member states to assess the prevalence of zoonoses and zoonotic agents. Despite control programmes in each member state having the same objective, the variance in the levels of *Salmonella* found in pigs between member states (see Section 2.3) may be due to inter-laboratory variability, bioclimatic characteristics, differences in rearing and

production systems, different feeding practices, etc. (Vico et al. 2011). Pig *Salmonella* control programmes tend to be based on bacteriological or serological testing or a combination of both. Both approaches are outlined in Section 2.1.

2.5.1 *Salmonella* Control Programmes in Europe

Not all European countries have pig *Salmonella* control programmes. Some of those that do will be discussed here. A revised National Pig *Salmonella* Control Programme was implemented in Ireland in January 2010 (SI 521 2009; SI 522 2009). Monitoring is by determination of the *Salmonella* status of pig herds via serological testing of meat juice at slaughter. This new program consists of two categories based on a serological prevalence (≤50% or >50%). The original programme was introduced in 2002 (Mannion et al. 2007a, b) and was based on that developed some years earlier in Denmark (Alban et al. 2002; Mannion et al. 2007a), in which herds were assigned a category of 1, 2 or 3 based on a serological prevalence of ≤10%, 10-50% or >50%, respectively. In the new program, the first consignment of pigs every month from each herd to enter a slaughter plant must be sampled. This involves taking 6 samples per herd each month, up to a maximum of 72 samples per year. The *Salmonella* status is calculated on a weighted mean of the previous three results. If sero-prevalence is >50% for the previous 3 results (3x3 months), then the farm will lose its Bord Bia quality assurance status. Pigs that have never been tested and therefore have no valid certificate are also excluded from the Bord Bia quality assurance scheme. However, those herds that can demonstrate by on-farm bacteriological sampling that the *Salmonella* serovars present are unlikely to be of public health significance will not be excluded from the scheme.

In April 2008 the UK *Salmonella* Control programme referred to as the Zoonoses Action Plan (ZAP) was replaced by the Zoonoses National Control Programme (ZNCP) for *Salmonella* in pigs, to focus on a whole chain risk-based approach to tackling *Salmonella* and other zoonoses. This programme is implemented by an industry steering group, unlike other countries where programmes are government-implemented. Serological testing of meat juice for *Salmonella* antibodies was suspended in July 2012 and *Salmonella* is now monitored bacteriologically by measuring the prevalence of *Salmonella* organisms on carcases in abattoirs. A *Salmonella* risk assessment tool is used to allow producers to identify the most effective control methods for their herds. Unlike the Irish scheme, no penalties are implemented against units with high ZNCP status.

The Nordic countries (Sweden, Norway and Finland) have very low prevalence's (~<1%) of *Salmonella* in pigs. These countries implement pre-harvest surveillance programs along with an eradication strategy program. Unlike the Irish control programme, the Danish, Finnish and Swedish programmes operate at all stages of production involving mandatory testing of feeds and feedstuffs, monitoring serological and/or bacteriological prevalence across breeding/multiplier and finishing herds and control measures within the abattoirs (Mousing et al. 1997). According to Swedish legislation (Zoonosis Act, SFS 1999:658), action must be taken to eliminate the infection or contamination with *Salmonella* spp. whenever the bacterium is detected in the food chain (Österberg et al. 2010). Restrictions are placed on herds when *Salmonella* spp. is detected, prohibiting any animals to leave the farm before all infected animals have either been culled or tested negative in two faecal samples taken one month apart. In addition, all contaminated surfaces on the

premises must be thoroughly cleaned and disinfected before the restrictions can be lifted.The total number of samples tested in each country is calculated to be sufficient to detect *Salmonella* at a prevalence level of 0.1 % in the whole pig population with a confidence interval of 95% (Rautiainen et al. 2001). In Denmark, herds sending <200 pigs to slaughter each year are not tested, leaving 1.6% of the slaughter pigs outside the monitoring scheme (Wegener et al. 2003). Financial penalties (2 and 4% reduction in final payment, respectively) are applied to Level 2 and 3 herds along with follow-up bacteriological testing to establish the serovar present on-farm. In Sweden and Finland the targeted annual sample size is 6000, and in Norway it is 3000 (Maijala et al. 2005; Rautiainen et al. 2001). If *Salmonella* is detected, legislative measures are taken including epidemiological identification of serovar, restrictions on sales or purchases of pigs and products, disinfection procedures and special arrangements for slaughter. In Sweden and Finland, the costs of sampling and analyses are paid by the industry and the pig producers. Although the schemes are expensive to implement, using cost-benefit ratios for meat production with and without market adjustments it was estimated that for every Euro invested in Finland there is a return of between 5.4-258.1 Euros (Maijala et al. 2005).

3. Pig feed as a source of *Salmonella*

When discussing on-farm control of *Salmonella* in pigs, it is important to consider the full production chain, beginning with the feed. Prior to reviewing pig feed as a risk factor for transmission of *Salmonella*, feed ingredients, feed production processes and on-farm delivery practices will first be discussed. Annually the total feed requirement of the Irish pig industry is around 925,000 tonnes of which around 30% is home compounded (Carroll, 2006). Currently there are over 20 feed

manufacturers producing pig feed in Ireland and almost 40 suppliers of raw materials to the Irish pig feed sector (Teagasc Pig Newsletter, 2006)

3.1 Feed Production

3.1.1 Feed ingredients

Commercial pig feeds can be produced as complete feed to cover the total nutrient requirements of pigs, or as complementary feed to provide part of the ration such as protein or energy, or as pre-mixtures to provide minerals, vitamins, amino acids and trace elements. Commercially produced pig feed can be produced in either meal (mash) or pelleted form. Complete pig diets/feeds are formulated from a variety of basic ingredients to provide a nutritionally balanced ration/diet. This is normally done on a least-cost basis, with feed companies tending to purchase ingredients from domestic sources and overseas so that specific ingredients can be chosen or substituted according to availability and price (Wierup, 2013). Approximately 1.5 million tonnes of home-grown cereals are used in the production of feed in Ireland (2025 Agri-Food Strategy). However, the risk of *Salmonella* contamination is greater with imported ingredients as they may originate in countries with different regulations and there is an opportunity for contamination during transit. In 2014, ~3 million tonnes of cereals were imported to meet 65% of Ireland annual requirements, ~55% of which came from countries outside the EU (2025 Agri-Food Strategy). Currently, the main protein sources for animal feed are fish meal, processed animal proteins, and soybean meal, of which the use of processed animal proteins in pig and poultry diets is now prohibited in the EU due to Bovine Spongiform Encephalopathy (BSE) (Commission Regulation (EC) No 163/2009). Rapeseed, palm kernel meal and sunflower meals are also used, but to a lesser extent. The main energy

ingredients used are cereals and by-products; however, fats and oils of both animal and vegetable origin are also very important energy sources in pig diets, although their inclusion level is normally low.

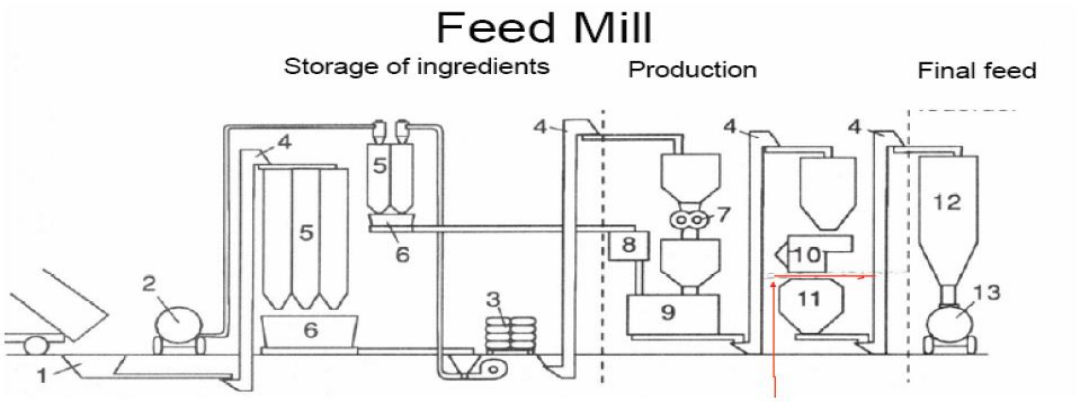
Both commercial pig feed and feed produced on-farm utilize products, by-products/co-products from primary production (agriculture), food processing industries, breweries and industrial processors, such as biofuel manufacturers as ingredients (Animal Feed Controls Programme, 2012–2014). Such by-products/co-products, when sold as feed ingredients, create an additional income stream for the processor. Many of these by-products are utilized regularly in manufactured feeds due to their ability to provide nutrients at a reduced cost and abundant supply from nearby sources. Commonly used by-products/co-products from brewing and distilling industries include dried distiller's grain with solubles (DDGS) (Wong et al. 2004) which is the dried residue left after fermentation of the starch fraction of maize to produce ethanol (Jacob et al. 2009). Oil extraction produces by-products such as rapeseed, palm kernel meal/expeller, whereas sugar beet and corn (maize) starch production produces beet pulp and maize gluten feed. Wheat feed/pollard (flour manufacture); citrus pulp (extraction of orange from citrus fruits) and whey (a by-product of cheese manufacture) are also used as feed ingredients.

Minerals (macro and micro) and vitamins are included in pig diets to avoid deficiencies, thereby maximising growth and feed efficiency. They are usually incorporated into feed, because common feed ingredients are limiting. Incorporation rates usually represent less than 3% of the feed but this depends on the diet formulation (Gaudré and Quiniou, 2009). Other components that are included in pig

diets include fats, such as tallow and lard. The number of ingredients used for on-farm mixed diets is usually less than that used for compound feed manufacture due to limitations in on-farm storage. In addition, the cereal ingredients used in pig diets are usually Irish or European in origin, while maize can be either European or American in origin.

3.1.2 Feed Production Processes

Feed ingredients are delivered to the feed mill and deposited in the intake pit (Figure 2). From there, they are conveyed, before or after grinding (normally before), to storage bins (Figure 2). There may be limited carry-over of material in auger systems, particularly in the chutes. Cross-contamination of ingredient storage bins is more likely. Although storage bins in modern mills are closed/sealed, problems with *Salmonella* contamination can arise when low-risk ingredients, such as cereals, are stored after a high-risk ingredient, or, more seriously, when finished products are stored in ingredient bins if there is a shortage of storage space. Ingredients may be mixed either before or after grinding. If a meal ration is produced, then the feed is transferred to bulk finished product bins or is bagged at this stage. Pelleting is rarely carried out during on-farm feed preparation, although some farms do pellet rations, with or without heat treatment.



1. Intake pit for trucks, **2.** Pneumatic intake, **3.**Intake pit for bags, **4.** Elevators, **5.** Storage bins, **6.** Scales, **7.** Mill, **8.** Pre-bin for premixes, vitamins etc., **9.** Mixer, **10.** Conditioner and pellet press, **11.** Pellet cooler, **12.** Storage bin for compound feedingstuffs, **13.** Bulk truck

Figure 2: Schematic diagram of a feed mill (EFSA, 2008)

The creation of dust is a feature of manufacturing. Therefore, adequate dust collection systems are important to control dust and to keep the feed mill clean.

3.2 Transport and on-Farm Storage of Feed

Feed is usually delivered from the feed mill to the farm by delivery trucks. Once delivered, there are many options for on-farm storage. These include; storage bins/silos for bulk materials which may be sealed or unsealed; hoppered bins which can be fitted with aeration and auger systems; liquid feed tanks; farm sheds/stores for bagged ingredients (Kavanagh et al. 2009). There is a wide range of storage systems available for pig farms, due to the variety of pig diets used across production stages, some with the capability of being automated. In the case of feeds that require mixing on-farm i.e. liquid feed, storage is needed both before and after mixing. In addition to the physical nature of the feed (i.e. dry versus liquid), the feeding system must also be considered when determining the type of storage facilities required.

3.3 On Farm Feed Delivery Systems

3.3.1 Feed Mixing Systems for Pigs

To ensure that pigs consuming the feed receive a nutrient-balanced diet, the feed ingredients must be thoroughly mixed, particularly those added at low levels (e.g. minerals, vitamins and medicinal or zootechnical feed additives).

The main feed mixing systems used are dry mixing and liquid mixing. Dry mixing typically takes place on small farms which home-produce and mix feed entirely for their own use. Home millers tend to produce feeds in meal form, as very few have the facilities to manufacture pelleted feeds. Mixing may involve only a few feed materials to form a complete diet e.g. ground home grown grains combined with a bought-in complementary protein source and a vitamin/trace element premix. This process is performed in a mixer, with feeds being mixed in batches suited to the size of the mixer (e.g. tumble, vertical and horizontal mixers) and stored in storage bins prior to feeding This diet is then delivered to the pigs through a mechanical auger delivery system and self-feeders/ad-libitum hoppers for nursery, growing and finishing pigs, or hand-fed to sows at the barns. There are limited data available on the risks of home milling as regards *Salmonella* contamination; therefore, there may be a significant under-recognised risk associated with home-mixing of feed. Feed manufacture is a capital-intensive venture; therefore, home millers often use less sophisticated facilities in comparison to their commercial counterparts. *Salmonella* prevalence may therefore be expected to be high in home mills due to the lack of heat treatment steps, inferior quality control measures and cross-contamination of equipment (EFSA, 2008)

Liquid feeding involves the use of a diet prepared either from a mixture of liquid food industry by-products and conventional dry food, or from dry raw materials mixed with water (Missotten et al. 2010). Liquid feeding requires specialized equipment, such as a mixing tank and delivery pipelines, which can be expensive to install. As a result, it is normally only found on larger pig units and historically, those sited close to suitable food plants with an abundant source of suitable liquid by-product, such as whey (Brooks et al. 2003). Liquid feeding systems are now virtually all automated, allowing the quantity of feed delivered to each trough, its dry matter content and the number of meals fed each day to be easily adjusted for each pen of pigs. Most farms with liquid feeding systems rarely empty and clean their feed mixing tanks. Instead fresh feed is added to and mixed, either by stirring or recirculation, with feed residues remaining in the tank. Although a microbial inoculum is not deliberately added, fermentation will occur, with the feed residue serving as the inoculum for the next batch of fresh feed (Beal et al. 2002). Fermentation is highly variable due to diurnal and seasonal temperature fluctuations, as the temperature at which liquid feed is held is not regulated. Liquid feeding systems may be responsible for carry-over of *Salmonella* between batches of pigs, as most are composed of feed delivery pipes and feeding valves which are difficult to clean and disinfect because of inaccessible surfaces and pooling of wash water. In addition, bacterial biofilms may remain in mixing tanks despite a cleaning/disinfection cycle being used. A study by Royer et al. (2004) indicated that it takes only 2-3 days for bacteria to colonize equipment surfaces.

The choice of feed type and feeding system is dictated, in part, by the scale and type of housing on individual pig farms.

3.4 *Salmonella* in Pig Feed and Feed Ingredients

There are several routes by which *Salmonella* may contaminate animal feed. These include contamination of feed ingredients, contamination in the feed mill and contamination during transport and on the farm. The physiology of *Salmonella* lends itself well to survival on a wide range of feeds and feed ingredients as well as in the feed mill environment (Maciorowski et al. 2007). For example, it has developed diverse mechanisms to survive at adverse water activity levels and at low concentrations of available carbon, nitrogen, and phosphorus, the latter by means of a starvation stress response (Spector, 1998). Transient low pH is avoided by means of a stationary phase acid tolerance response (Bang et al. 2000). In addition, certain serotypes are isolated more often from feed and feed mills, as a result of their physiology and in particular their ability to survive in dry environments. The following sections will focus on pig feed, although other animal feed and feed ingredients will also be discussed.

3.4.1 *Salmonella* in Pig Feed Ingredients

The main source of *Salmonella* contamination in feed mills is the feed ingredients that enter the mill. *Salmonella* contamination of feed ingredients is of concern, as it is possible to trace specific serovars present in feed ingredients through the production process to the finished feed (Häggblom et al. 2002). *Salmonella* is regularly isolated from animal proteins such as bone meal and vegetable proteins, such as soya, rape seed meal, palm kernel, rice bran and cottonseed, all of which are used as feed ingredients, as outlined in Section 3.1.1. Historically, ingredients of animal origin from the rendering industry were used in animal feed. In general, these had a high prevalence of *Salmonella*, i.e. ranging from 3-81% (Loken et al. 1968; Isa

et al. 1963; Hacking et al. 1978). However, in 2001, to prevent the spread of BSE, a total ban was introduced on the use of animal protein in feeds for any animals intended for human consumption (EFSA, 2008). Exceptions to this ban include fish meal, certain blood products and by-products from the production of gelatine. As a result of the ban, the frequency of *Salmonella* isolation has increased from consignments of vegetable proteins such as soya bean, rapeseed, palm kernel and cottonseed due to their increased use (Wierup and Häggblom, 2010; Salomonsson et al. 2005; Jones and Richardson, 2004; Hald et al. 2006). These by-products of oil extraction are particularly prone to contamination by salmonellae that are endemic in the processing plant (Morita et al. 2006; EFSA, 2006). Cereal and vegetable ingredients may become contaminated with *Salmonella* via contact with wildlife excreta and agricultural effluents during growth and harvesting. Importation of feed ingredients is a particular concern, as new *Salmonella* serovars are often introduced into countries in this way. For example, imported Peruvian fish meal used in animal feed in the United States was contaminated with *S.* Agona as quoted by Clark et al. 1973.

3.4.2 *Salmonella* in the Feed Mill

A study by Davies and Wray (1997) found widespread *Salmonella* contamination in feed mill environments, based on analysis of dust and aggregated fatty material. Mill locations most commonly contaminated were intake pits and augers for raw ingredient receipt, cooling systems for pellets or mash/meal, grinders and finished product bins. The mills with the highest overall prevalence of contamination were those where the inside of the cooling system had become colonised by salmonellae. Interestingly, *Salmonella* serotypes that are frequently isolated from feed processing

environments, such as *S.* agona and *S.* Montevideo, have the ability to form biofilms under laboratory conditions (Vestby et al. 2009). Feed ingredients delivered to the mill are deposited in the intake pit and contamination may be associated with faecal material carried by lorries or contamination by wild animals or birds. Interestingly, rodent and wild bird faeces collected in and around feed mills have been shown to contain *Salmonella* (Davies et al. 1997; Whyte et al. 2003). Enclosed pits with automatic doors offer some protection from the latter. In addition, multiplication of *Salmonella* may occur in bulk storage bins (those storing either feed ingredients or finished feed) in warm, moist conditions. This is most likely to occur in the autumn, when warm days followed by cold nights result in condensation on the sides of bins. *Salmonella* growth can also occur during on-farm storage of feed or feed ingredients (see Section 3.4.3). Contamination in bulk bins can be reduced by implementation of a regular emptying and cleaning programme and appropriate insulation of buildings/bins. For home-mixed pig feed, there is a limited range of ingredients with a relatively slow turnover in comparison to the commercial feed producers and ingredients are purchased in bulk. As a result, the level and infectivity of any *Salmonella* present may diminish during ingredient storage. However, there is a risk of contamination of feed storage and production facilities when there are nearby herds that are infected with *Salmonella.* In addition, studies in Denmark have indicated that the use of feed from commercial compound feed mills tripled the risk for the acquisition of *Salmonella* by pig herds, and have advised farmers to consider home-mixing as part of the national control strategy (Thamsborg, 2002). This is despite the fact that most home-produced pig feed is not heat-treated, has a coarser structure after grinding and is not pelleted.

3.4.3 *Salmonella* in Finished Pig Feed

Contamination of finished feed with *Salmonella* occurs at low prevalence (<10%) and typically exhibits non-homogeneous distribution within the infected batch (Davies et al. 2004). 'Exotic' strains of *Salmonella* spp. are often associated with purchased feed (Jones et al. 1982). An EFSA report from 2008 showed European Union (EU) national prevalence's for *Salmonella* in compound pig feed samples of up to 1.7% (EFSA, 2008). However, this is likely to be an underestimate of the scale of contamination, owing to the difficulty of representative sampling of large consignments of feed (Jones and Richardson, 2004). The report also highlighted that data from different member states were not comparable as sampling schemes, sample sizes and sample origin were not harmonized and the reported prevalence's could therefore not be interpreted as true national prevalence's. Similarly, a recent EU project involving five European countries also reported incomparable data due to differing sampling protocols (Lo Fo Wong, 2001). In this study, the overall prevalence of contamination of feed samples collected at the point of delivery to pigs (6.9%; Germany 0%; Sweden 0.8%; Netherlands 3.5%; Greece 4.8%; Denmark 9%) greatly exceeded the prevalence reported in samples taken from commercial feed mills (usually <1%) (Davies et al. 2004); suggesting possible occurrence of contamination during transport and on-farm.

Feed trucks have been implicated as a source of contamination of feed and feed ingredients (Fedorka-Cray et al. 1997). For this reason, all vehicles used for the transport of ingredients and compound feeding stuffs should be subject to regular cleaning and sanitisation to ensure that they are in a clean state with no accumulation of residual waste material (EFSA, 2008). Ideally, trucks delivering feed to a pig

production unit should be able to unload feedstuffs without entering the unit. This requires that all bulk bins and facilities storing bagged feed are accessible from outside the perimeter of the pig site. However, in reality most farms allow feed trucks to breach biosecurity measures, due to numerous onsite locations of the bulk feed bins.

Once on the farm, there is also potential for contamination of feed and feed ingredients with *Salmonella*. For example, *Salmonella* has been isolated from a range of animals including mice, rats, wild birds, insects and larger mammals (e.g. cats and dogs) that may potentially come into contact with feed material on the farm (Wang et al. 2011). In addition, farm grain stores that are in close proximity to the livestock-housing or manure-storage areas may result in cross-contamination, mediated by animals, birds and human traffic, unless a high level of bird-proofing, rodent control and general biosecurity is practised. Other causes of contamination include the use of empty livestock buildings for short-term storage of grain before drying and trailers and bucket loaders that have not been adequately cleaned and disinfected. Grain in trailers may also become contaminated by *Salmonella* from material thrown up from the wheels passing through effluent on driveways and roads. On-farm, feeding systems may be responsible for carry-over of *Salmonella* between batches of pigs, as mentioned in section 3.3.1. In general, it should be borne in mind that although *Salmonella* is often present only at low numbers in feed and feed ingredients, it can multiply in warm, moist conditions on-farm in feed bins (as outlined for feed mills in section 3.4.2), feed hoppers and feeding equipment (Hilbert et al. 2012; Davies and Hinton, 2000).

3.4.4 Transmission of *Salmonella* to Pigs via Feed

Animal feed is considered a transmission vehicle for *Salmonella* in various food animals. For example, palm kernel has been implicated in one documented outbreak of *Salmonella* in cattle in England (Jones et al. 1982). While *Salmonella* counts in finished feeds are usually very low, there is still a substantial risk for transmission to animals, as the infective dose is much lower in stressed animals (Wales et al. 2010). However, exotic' strains of *Salmonella* spp. often associated with purchased feed are usually transient, whilst ''local'', well-established strains of *Salmonella* spp. are usually the most persistent on-farm (Nørrung et al. 2008). Despite this, in the Nordic countries (Sweden, Finland and Norway), where there is a low prevalence of *Salmonella*, feed is the principal means of introduction of *Salmonella* into pig farms (Wales et al. 2011; Davies et al. 2010). Few studies have tracked Salmonella from pig feed sampled on-farm to pigs. One study by Österberg et al. (2006) did show strong links between *Salmonella* contamination of feedstuffs or feed mills and infection of groups of pigs. Furthermore, Molla et al. (2010) found genotypically related and in some cases clonal *Salmonella* strains in commercially processed pig feed and pig faecal samples. Therefore, it is evident that animal feed should be an important component in pre-harvest pig *Salmonella* control programs.

3.5 Microbiological Testing of Feed

In the EU, microbiological safety of commercial animal feed production is regulated and comprehensive guidelines for the production of microbiologically safe feed have been published in the Codex Alimentarius "Code of Practice on Good Animal Feeding" (Doyle et al. 2012). According to these regulations, it is the feed manufacturer's responsibility to ensure the hygiene of production processes on a

daily basis. In addition, EC Feed Hygiene Regulation 183/2005 requires feed companies to comply with detailed standards concerning facilities and equipment, personnel, storage, transport and record-keeping i.e. good manufacturing practises (GMP). Feed businesses are also required to have a hazard analysis and critical control point (HACCP) plan in place (Nesbakken and Skjerve, 1996; Mead, 1994). Under HACCP, contamination of feed with *Salmonella* is considered a hazard, as its presence could potentially cause harm to human health. In line with EU legislation on control of *Salmonella* and other zoonotic agents (EC No 2160/2003), the following five critical control points along the processing line have been identified in feed mills manufacturing compound animal feed:

1. Intake pit/bottom part of elevator for raw feed materials.
2. Dust from the aspiration system (filter).
3. Top of pellet cooler.
4. Area/room for pellet coolers.
5. Top of bin for final feed (compound feed) (Wierup and Häggblom, 2010)

In accordance with the legislation, a number of dust samples or sweepings must be collected at each of these critical control points, as well as samples of the feed ingredients and finished feed, and these must be tested for the presence of *Salmonella* (Table 1.2). If a sample tests positive a number of corrective actions are taken, such as cleaning and disinfection, increased monitoring, cessation of production and cessation of delivery of compound feed. Most feed mills produce feed on order and finished feed is only transiently stored prior to on-farm delivery, as storage represents a difficulty both in terms of logistics and expense. This means that

feed is commonly delivered and even fed before the results of the lengthy conventional culture-based approaches used for *Salmonella* isolation are available. Therefore, the development of rapid testing methods for *Salmonella* would greatly benefit a mill-based control programme. There is a recognised association between the presence of *Salmonella* and the degree of *Enterobacteriaceae* contamination. This has led to the consideration of the use of *Enterobacteriaceae* counts in feed stuffs as an indicator of hygiene as shown in Table 1.3 (EFSA, 2008).

Table 1.2: Microbiological criteria for control of *Salmonella* in feedstuffs and feed mills (EC Regulation (EC) No 2160/2003)

Feed or sample category	Sampling Plan		Limits	Analytical Reference method	Stage where the criterion apply	Action in case of unsatisfactory results
	n[1]	c[2]				
Raw materials for production of animal feeds	10	0	Absence in 25g	EN/ISO 6579	At any stage until the processing of the feed	• Processing of the feed material using techniques effective to destroy *Salmonella*
Storage and Processing Environment and Transport Equipment in contact with the feed materials and processed feed	10	0	Absence in 25g	EN/ISO 6579	Along the compound feed production chain at a processing plant	• Cleaning and disinfection of the contaminated environment and equipment. • Investigation of the contamination source and improvement of the cleaning and disinfection practices. • Increased monitoring of raw materials and finished feed
Finished compound feed	10[2]	0	Absence in 25g	EN/ISO 6579	At any stage before leaving the manufacturing establishment until to the intended use	• Investigation of the contamination source. • Increased monitoring of raw materials and processing environment and transport equipment. • Review of all Critical Control Points. • Reprocessing of the feed if still at the control of the manufacturer. • Inform the holdings if delivered and if intended for certain species (e.g. Species for which a target for reduction of *Salmonella* has been set in accordance with R 2160/2003) • Inform the competent authority.

[1]n = number of units comprising the sample; [2]c = number of sample units giving values over minimum limit

Table 1.3: Hygiene standard limits used in EU GMP codes for the animal feed sector (EU Commission Regulation No 2073/2005)

Hygiene Standards	Quality Interpretation	Feed levels
Enterobacteriaceae **TEC**[1] **(cfu/g)**	**Good**	**<100**
	Acceptable	**100-1000**
	Questionable	**1000-10000**
	Poor	**>10000**
Salmonella **in 25g**	**Maximum**	**0**
Clostridium **(cfu/g)**	**Maximum**	**10**

Target
Action Limit

[1]**Total *Enterobacteriaceae* counts**

4. Control Strategies for *Salmonella* in Pig Feed

The principal approaches for the reduction and elimination of *Salmonella* in animal feeds and feed ingredients are through process control and monitoring which are major components of the HACCP program and chemical and thermal treatments of feed applied at various stages of manufacture or storage (Koyuncu et al. 2013; Jones, 2011). These approaches may be to varying extents, but all have their associated costs and technical weaknesses; hence, a range of methods may be needed to suppress, eliminate, or prevent *Salmonella* contamination (Wales et al. 2010). Some of these approaches will be discussed in the following sections.

4.1 Heat Treatment

During their manufacture, pig feeds undergo an array of processes such as grinding, mixing, conditioning, thermal treatments (extrusion/expansion) and pelleting as mentioned in section 3.1.1. Each one of these processes can have either a negative or positive effect on feed quality and subsequent pig performance. Heat treatments are used to improve the nutritional, hygienic, physical and chemical properties of feed. The efficacy of the heat treatment in terms of reducing *Salmonella* contamination is influenced by factors such as temperature, duration of heat treatment, the *Salmonella* serotype/strain and its physiological state and the matrix in which the bacterium is found (Finn et al. 2013).

The production process begins with the ingredients being weighed out, followed by grinding in a hammer/roller mill fitted with a sieve as mentioned in section 3.1.1. The feed then enters a conditioner where the raw materials are prepared for pelleting or extruding/expanding. Basic methods of conditioning are: water, steam (short and extended) and mechanical conditioning (Lević, 2010). For some feeds, an expansion process is included after the conditioner and prior to the pelleting process. Extruders can be broadly classified as wet or

dry and single or twin screw (Chae and Han, 1998). The temperature within the extruder increases to above 100°C due to friction and pressure caused by a screw that presses the feed through a die, with feed humidity reaching about 30% in dry extrusion and up to 80% in wet extrusion (Lević, 2010). Dry extrusion, however, does not employ pre-conditioners and therefore is limited in its ability to process a wide range of raw materials. Extruders and expanders are based on the same principles, except for different operating conditions, i.e. temperature range of an expander is between 100 and 130°C (Fancher et al. 1996), whereas the temperature in extruders varies from 80-200°C (Lević, 2010). After the extruding/expansion process, the feed is pressed through a die of a particular length and diameter and thus pellets are formed. Retention time in the pellet press is 30 seconds maximum. The friction involved in the pelleting process will also raise the temperature of the feed by an additional 3-6ºC (EFSA, 2008). The pellets are thereafter cooled in a cooler by air at an ambient temperature.

Salmonella may be completely eliminated by the pelleting process at temperatures exceeding 83°C with a 99% (2 $\log_{10}$) reduction (Maciorowski et al. 2004) or even 4 $\log_{10}$ reductions having been achieved at 85°C for 90s with 15% moisture content (Himathongkham et al. 1996). The conditions required to eliminate *Salmonella* are, however, rarely obtained in practice due to the high energy cost involved, the likelihood of heat damage to vitamins, amino acids and other nutrients and the adverse effect on the integrity of the pellets (De Busser et al. 2013; Jones et al. 1995). *Salmonella* in naturally contaminated feed may be more resistant to heat inactivation than those in artificially contaminated feed as a result of tolerance to a range of biotic stresses such as heat, cold, starvation, and desiccation. Although heat-treated feed may help to prevent the introduction of *Salmonella* into a *Salmonella*-

negative herd, it does not have a controlling effect in herds where *Salmonella* is already present, and can be readily re-contaminated after treatment.

4.2 Chemical Treatment

4.2.1 Organic acids

Acidification of feed and water as an intervention treatment for pigs has been studied extensively as an alternative to subtherapeutic antibiotics (Doyle and Erickson, 2012). The bacteriostatic activities of acids are thought to be achieved via the disruption of pH gradients and intracellular pH regulation (Van Immerseel et al. 2006). Inorganic acids such as phosphoric acid and hydrochloric acid are both efficient and cost effective in reducing water pH (Friendship et al. 2009). On the other hand, the principal agents used for treatment of feed are organic acids although blended products may additionally use surfactants, bacterial membrane-disrupting compounds and other elements (Wales et al. 2010). The magnitude of antimicrobial effects varies from one acid to another and is dependent on concentration and pH. The concept of incorporating chemical agents into feed is based on the notion that the acids will decontaminate the feed itself and prevent *Salmonella* uptake in food-producing animals (Van Immerseel et al. 2006). Organic acids are generally metabolized by recipient animals, or if stabilized they pass through the GIT unabsorbed; therefore, leaving no residues in foods of animal origin (Wales et al. 2010). The anti-*Salmonella* efficacy of formic acid and different blends of formic acid, propionic acid and sodium formate was investigated by Koyuncu et al. (2013) in different feed materials. Pelleted and compound meal feed showed the largest reduction in *Salmonella* counts, with a 2.5 log10 reduction obtained after 5 days exposure in comparison to rapeseed meal (1 $\log_{10}$ reduction). Effects in soybean meal, which represents two-thirds of the total world output of protein feedstuffs, were limited (< 0.5 $\log_{10}$ reduction) even after several weeks' exposure. The use of high concentrations of organic

acids in feed and feed ingredients may result in improved *Salmonella* control but there are also negative implications. Problems associated with acidification of feed or water include; cost, clogging of drinkers, corrosion of metal pipework and concrete, safety of workers and diet palatability (EFSA, 2008). Organic acids may provide a level of residual protection against post-production contamination/recontamination of the feed (Papadopoulou et al. 2009). However, they can mask the presence of *Salmonella* in feed when assessed by standard culture techniques (Carrique-Mas et al. 2007). In addition, the reduction of salmonellae in feed by organic acids may take several days and it is possible that feed may have been consumed and colonisation established before sufficient inactivation has had time to occur (Hinton and Linton, 1988). There are also concerns that the use of organic acids in feed may lead to selection for acid tolerant *Salmonella* strains as a result of insufficient or sub-lethal treatments, which may enable subsequent survival through the gastric acidity of humans (Mani-López et al. 2012; Álvarez-Ordóñez et al. 2012 and Kwon et al. 2000).

4.2.2. Formaldehyde

The greatest efficacy and lowest masking has come from chemical treatment of feed with formaldehyde which resulted in no apparent loss of palatability or growth reduction in pigs (Carrique-Mas et al. 2007). Formaldehyde is a highly effective in inactivating *Salmonella* as it causes irreversible cross linking of proteins in bacteria (Trampell et al. 2014). Although formaldehyde is a volatile substance that may evaporate in open systems (Khan et al. 2003) unless feed is held in a closed bin. To minimize the effects of evaporation on antimicrobial activity some commercial formaldehyde-based products may also contain organic acids (e.g., propionic acid) or other antimicrobial compounds (e.g., terpenes) (Carrique-Mas et al. 2007). This produces a synergistic combination allowing lower levels of formaldehyde and acids to be used which minimizes fuming, operator hazard, and corrosiveness. There is a concern that

formaldehyde might not be safe in humans (U.S. EPA, 1999) and therefore people working with this substance should avoid overexposure.

4.3 Alternative Anti-*Salmonella* Control Strategies

4.3.1 Essential oils

Essential oils (EO) are volatile components of plants that possess antibacterial, antifungal, antiviral, insecticidal, and antioxidant properties. EO containing phenolic compounds such as carvacrol and thymol, have strong antimicrobial activity which is attributed to their hydrophobic nature (Nazzaro et al. 2013). The oils initiate damage to the cell membrane, which compromises pH homeostasis and equilibrium of inorganic ions across the bacterial cell membrane (Bajpai et al. 2012). EO may increase the palatability of the diet due to their characteristic odours/flavours as well as controlling enteric pathogens (De Lange et al. 2010). To date, only a limited number of studies have investigated the anti-*Salmonella* activity of EO in pigs and these have failed to show efficacy (Peňalver et al., 2005; Turner et al., 2002). Studies investigating the anti-*Salmonella* effects of EO in feed have shown that they have strong anti-microbial activity but appear not to be effective in controlling bacterial pathogens when fed to pigs i.e. they are effective *in vitro* but not *in vivo* (Bento et al., 2013; De Lange et al. 2010 and Janczyk et al. 2009).

4.3.2 Bacteriophages

Bacteriophages (phages) are naturally occurring viruses that infect bacteria and reproduce within them, with the result that the bacterium is killed. The genome of a temperate phage integrates into the host bacterial genome where it replicates along with the host cell DNA without lysing the bacterial host. Lytic phages, on the other hand, destroy the host cell DNA, replicate within the cell, and then lyse the host cell. Studies by Callaway et al. (2011) have

shown that anti-*Salmonella* phage isolated from porcine faeces have the ability to reduce caecal *Salmonella* populations and faecal shedding in weaned pigs. However, the effectiveness of phages has been shown to be compromised by several factors. For example, the generation time for most bacteria are typically short enough that mutants with bacteriophage resistance can emerge within hours (Lowbury and Hood, 1953). In addition phage lack stability in acidic environments and hence they may not survive gastric transit (Colom et al. 2015). However, no studies to date have investigated the use of bacteriophage for *Salmonella* reduction in feed but have been used in food packaging systems to reduce *Salmonella* in raw meats intended for human consumption.

4.3.3 Probiotics and Prebiotics

Probiotics are defined as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (FAO/WHO, 2001). The addition of probiotics to pig diets is based on feeding viable beneficial bacteria (e.g., *Lactobacillus, Bifidobacterium, Propionibacterium, Bacillus*, *Strepococcus*), antagonistic toward pathogens in the gut (Nørrung et al., 2008). Gebru et al. (2010) and Casey et al. (2007) reported that *Salmonella* shedding was reduced in pigs fed *Lactobacillus* and a *Lactobacillus/Pediococcus* mixture, respectively. The possibility of anti-*Salmonella* effects of probiotics in feed during storage has not been investigated to date; however, they are unlikely to have any effects as they will most likely be in a dormant state and therefore not metabolically active.

A prebiotic is 'a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/ or activity of one or a limited number of bacteria in the colon that have the potential to improve host health' (Holzapfel et al, 1998, Rolfe, 2000, Marinho et al. 2007, O' Sullivan et al. 2010, Gibson and Roberfroid, 1995). Combinations of probiotics and

prebiotics can result in synergistic effects on gastrointestinal function (Naughton et al, 2001; Burns and Rowland, 2000). These combinations, known as synbiotics, are defined as ‘a mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract, by selectively stimulating the growth and/or activating the metabolism of one or a limited number of health-promoting bacteria, and thus improving host welfare’ (Kailasapathy and Chin, 2000). Modulation of the gut microbiota in pigs through the use of prebiotics may prove useful as a strategy for eliminating intestinal pathogens, both those that cause illness in the pig, and those that are carried asymptomatically but have human health implications (i.e. *Salmonella*) (O’ Sullivan et al. 2010; Ojha et al. 2007). However, these effects will only be seen in the live animal and prebiotics are unlikely to be of any benefit in terms of feed decontamination/protection.

5. Feed Intervention Strategies to Control *Salmonella* in Pigs

5.1 Feed form and particle size

Several researchers have shown that the composition and structure of feed are associated with *Salmonella* prevalence and that offering pigs meal as opposed to pellets significantly reduces *Salmonella* prevalence. It has been suggested that increased particle size of the meal and hence, increased overall surface area per unit volume (Lawrence et al. 2003), lower viscosity of the liquid phase of digesta (Yasar, 2003), longer retention time and more acidic conditions due to increased gastric acid production within the intestinal tract, all make the intestinal environment more inhospitable to *Salmonella*. The lower pH also promotes the growth of beneficial bacteria, such as *Lactobacillus*, which can kill *Salmonella* (Hotes et al. 2010). Jones et al. (1995) found that pelleting of broiler feed reduced *Salmonella* isolation rates by 82-81.1%. The importance of particle size of the feed is further reinforced by Jorgensen et al. (1999) who reported that pigs fed finely ground diets (1/16 inch screen) were 3.3 times more likely to test positive for *Salmonella* compared to pigs fed a coarsely ground meal diet (5/32 inch screen). While similar studies have shown that coarsely ground feed can be an effective *Salmonella* control measure (Hedemann et al. 2005; Rajtak et al. 2012), the results are not always consistent. Laitat et al. (2004) showed that pigs had a preference for meal feed, as the time spent at the feeder by each pig was 1.6 times greater when they were fed meal rather than pellets, and this parameter was not influenced by group size. Mikkelsen et al. (2004) showed that feed conversion ratio in pigs was improved (2.53 versus 2.69) due to pelleting the feed; however, pigs fed pelleted feed had a higher diarrhoea score.

Although the use of meal is recommended for the reduction of *Salmonella* prevalence over pellets, its use can increase feed wastage, thereby reducing feed efficiency and hence increasing the cost of production (Hedemann et al. 2005). Finely ground feed, in addition, has

been linked to the development of ulcers in the oesophageal region of pigs (Behnke, 1996). Pelleting on the other hand agglomerates smaller feed particles with the help of mechanical pressure, moisture and heat to larger particles. Pelleting is associated with improved feed conversion ratio and improvements in animal performance due to less feed wastage, improved palatability and digestibility of nutrients along with starch gelatinization (Mikkelsen et al. 2004; Rajtak et al. 2012).

Several studies have focused on the pelleting conditions that result in the elimination of *Salmonella* from feed. *Salmonella* reductions are, however, dependent on the duration of heat exposure, temperature obtained, and the moisture content of the feed at pelleting. There is also the possibility of re-contamination with *Salmonella* after the pelleting process (Jones and Richardson, 2004). The effect of feed form on *Salmonella* infection tends to be more pronounced in the later rearing and finishing stages, when less digestible, least-cost rations are fed (Wales et al., 2011).

5.2 Feed type

There is some evidence to suggest that different feed ingredients can alter the *Salmonella* status of pigs. Jorgensen et al. (2001) found that substitution of around 25% wheat by barley in the diet lowered *Salmonella* prevalence. The inclusion of barley showed no clear effect on the intestinal microbial ecosystem but had a marked effect on the physico-chemical properties of the digesta, increasing retention times in the stomach with growth and feed efficiency being only moderately affected. Pieper et al. (2009) showed that the intestinal microbial community composition could be modified using the variability in the β-glucan content of barley, thereby altering the total amounts of short-chain fatty acids (SCFA) within the gut. To date, there is limited research on the effect that different feed ingredients may play in

reducing *Salmonella* prevalence in pigs; therefore, it is difficult to assess the effectiveness of this as a control measure.

5.3 Feed Delivery

As outlined in Section 3.3.1, non-fermented liquid feed (NFLF) is defined by Canibe and Jensen (2003) as feed mixed with water immediately before feeding or in the trough at feeding, whereas fermented liquid feed (FLF) is feed mixed with water that is stored in a tank at a certain temperature ~30°C and for a certain period of time before it is fed (Beal et al. 2002). The fermentation in FLF can occur spontaneously or be induced by including material from a previous successful fermentation as inoculum, a process known as backslopping (Canibe and Jensen, 2012). The benefits of liquid feed include an increase in daily feed intake, live weight gain and a reduction in the number of days required to reach market weight compared with pigs receiving pelleted feed/dry feeding (Ojha et al. 2007; Mikkelsen et al. 2004; Wong et al. 2004 and Van der Wolf et al. 2001). Current fluctuations in feed prices and the availability of cheap by-products from the food/pharmaceutical/biofuel industries that can be incorporated into liquid feed have made it an attractive alternative (Canibe and Jensen, 2012). However, liquid feeding systems are only economical for large herds due to the high capital investment needed for storage capacity, mixers, pumps, pipelines and computers (Van der Wolf et al. 2001). Studies performed by Van der Wolf et al. (1999) and Farzan et al. (2006) have shown significantly lower *Salmonella* prevalence in pigs fed fermented by-products in feed in comparison to pigs fed soaked compound feed (liquid feeding without fermentation) which was shown to increase *Salmonella* prevalence. Some researchers (Canibe et al. 2007b; Canibe and Jensen, 2003; Demeckova et al. 2002; Van Winsen et al. 2001), however, found variable growth performance results for animals fed FLF compared to those fed with non-FLF or dry feed. Some possible reasons for the lack of

beneficial effects of FLF is that successful application of FLF in pig feeding systems requires a correct balance of lactic acid bacteria, feed substrates and fermentation conditions (Niba et al. 2009), as uncontrolled fermentation can result in the growth of undesirable bacteria and yeasts and moulds, the latter potentially causing problems such as the production of mycotoxins (Lawlor et al. 2002).

6. Conclusions

Arising from the literature review, it is apparent that there is a substantial problem regarding *Salmonella* carriage in pigs in Ireland. While there is an Irish Department of Agriculture, Food & the Marine (DAFM) *Salmonella* control programme at the pig herd level, research is needed to support the implementation and validation of this programme and to address knowledge gaps. This issue should be addressed at the primary production part of the chain in order to lower the burden of *Salmonella* contamination entering the slaughterhouse and the overall risk. Following a "farm-to-fork" model, animal feed is at the beginning of the food safety chain and the EFSA has highlighted that feed is a risk factor for *Salmonella* prevalence in pigs (EFSA, 2008).; therefore in order to estimate the risk posed by the introduction of *Salmonella*-infected pigs into the slaughterhouse and consequently into the food chain, an understanding of the dynamics of on-farm *Salmonella* infection is of vital importance. This information will allow for the identification of risk factors for herds infected or contaminated with *Salmonella* and will allow for the evaluation of control measures for risk mitigation. Therefore, the overall objective of this thesis was to assess the role of feed in transmission of *Salmonella* to pigs and to investigate the survival of *Salmonella* during production and storage of feed. The specific objectives are outlined below.

7. Overall objectives of the research

The specific objectives of this thesis are:

1. To investigate *Salmonella* carriage at each stage of pig production (farrow to finish) on commercial pig farms with a historically high *Salmonella* sero-prevalence to identify which production stages are the principal harbours of infection.

2. To determine the epidemiological relationship between *Salmonella* isolates recovered from feed and pig faeces on these commercial pig farms, in order to assess the importance of feed in the transmission of *Salmonella* to pigs.

3. To assess the occurrence and characteristics of *Salmonella* and to determine *Enterobacteriaceae* counts in a range of feed ingredients and compound feeds sampled from feed mills supplying the high *Salmonella* sero-prevalent pig farms, where on-farm bacteriology had confirmed *Salmonella* presence in both pigs and feed.

4. To examine the survival of monophasic variants of *S.* Typhimurium isolated from feed ingredients and finished feed sampled at Irish feed mills in terms of their thermal tolerance and ability to persist on stored feed treated with a sodium butyrate feed additive.

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Chapter 2

Assessing the role of feed as a risk factor for the transmission of *Salmonella* in Irish pig production

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2.1 Abstract

The objective of this study was to determine the relatedness of *Salmonella* isolates recovered from feed, pig faeces and the environment on 10 Irish commercial pig farms identified as having a high *Salmonella* sero-prevalence by the Irish National Pig *Salmonella* Control Programme, in order to assess the role, if any, of feed in the transmission of *Salmonella* to pigs. Each farm was sampled on two occasions between March 2012 and June 2013, with pooled faecal samples, feed, water and environmental samples collected from all stages of production. *Salmonella* isolates recovered were characterized by serotyping, antimicrobial susceptibility testing, pulse field gel electrophoresis (PFGE) and multilocus variable number tandem repeat analysis (MLVA). The highest proportion of *Salmonella*-positive faecal samples (21.5%) was found in 2nd stage weaners, closely followed by finishers and gilts (19.5 and 19.1% faecal samples *Salmonella*-positive, respectively. Eleven serovars were identified across all sample types, with monophasic variants of Typhimurium (4,[5],12:i) predominating, accounting for ~43% of faecal, environmental and feed isolates and 32.6% of water isolates. From a total of 585 feed samples, *Salmonella* was detected in 14 feed samples that originated from 6 herds, with a prevalence of 2.4%. These *Salmonella* isolates recovered from these-feed samples were identified as 4,[5],12:I, Typhimurium, Typhimurium Copenhagen, Derby and Tennessee. PFGE genotyping grouped the non-Typhimurium/Typhimurium Copenhagen/monophasic variant isolates into five genotypic clusters, of which four consisted of genotypically related isolates recovered from feed and pig faecal samples. MLVA classified the remaining isolates into twelve main clusters, with the monophasic *Salmonella* isolates being classified into four clusters. In conclusion, the occurrence of genotypically related, multidrug-resistant isolates in commercially produced

feed and pig faecal samples, suggests the significance of commercial feed as a potential vehicle of *Salmonella* transmission.

2.2 Introduction

Pigs are susceptible to subclinical infection with *Salmonella,* which constitutes a potential source of human exposure and illness, as pork can become contaminated with faeces and/or intestinal content containing *Salmonella* during slaughter and evisceration and subsequent processing. A baseline study on slaughter pigs showed that the prevalence of *Salmonella* on intestinal lymph node samples within the EU was 10.3%, whereas the observed prevalence in Ireland was much higher at 16.1% (EFSA, 2008). Furthermore, Ireland had the highest *Salmonella* contamination rate (20%) on pre-chill carcasses. Despite the efforts of the National Pig *Salmonella* Control Programme, *Salmonella* carriage rates in pigs in Ireland remain high (Duggan et al. 2009).

The European Food Safety Authority noted in a scientific opinion that feed is a risk factor for *Salmonella* carriage in pigs (EFSA, 2008). Furthermore, in countries with low on-farm *Salmonella* prevalence, human infection linked to pork consumption has been traced back to *Salmonella*-contaminated feedstuffs (Hald et al. 2006, Wierup et al. 2010; Crump et al. 2002). Several studies have proved that animal feed and feed ingredients can be contaminated with *Salmonella* (Burns et al. 2015; Jones, 2011). Others have shown that pigs can become infected as a result of consuming *Salmonella*-contaminated feed (Smith, 1960; Österberg et al. 2006) The presence of *Salmonella* in feed can lead to the introduction of *Salmonella* into pathogen-free herds, an increase in the prevalence of *Salmonella* shedding and the spread of *Salmonella* in pigs (EFSA, 2007). Even minor *Salmonella* contamination of feed has the potential to affect many herds. Moreover, even if feed is *Salmonella*-free on being introduced to the farm, it can act as a vehicle for on-farm *Salmonella* spread, as on-farm contamination is possible.

However, in order to make a definite link between *Salmonella*-contaminated feed and *Salmonella* carriage in pigs, molecular tracking of *Salmonella* isolates from feed to pigs is necessary. Using pulsed field gel electrophoresis (PFGE) fingerprinting Molla et al. (2010) demonstrated the presence of genotypically related and in some cases clonal *Salmonella* strains in commercially processed pig feed and pig faecal samples taken from the same farm, suggesting the dissemination of *Salmonella* via feed. These type of data are, however, lacking for Ireland, as, although studies have tracked *Salmonella* across Irish farms and at the slaughterhouse and meat-producing plants (McCarthy et al. 2013; Duggan et al. 2010; Prendergast et al. 2011), feed samples were not included in the analysis.

Therefore, the objective of this study was to determine the relatedness of *Salmonella* isolates recovered from feed, pig faeces and the environment on 10 Irish commercial pig farms, each with a history of high *Salmonella* sero-prevalence, in order to assess the role, if any, of feed in the transmission of *Salmonella* to pigs.

2.3 Materials and Methods

2.3.1 *Herd Selection*

Pig herds were selected based on their *Salmonella* sero-prevalence as recorded by the Irish Department of Agriculture, Food and the Marine's (DAFM) National Pig *Salmonella* Control Programme (SI 521 2009; SI 522 2009). Farrow-to-finish farms (n=10) with a history of high (>50%) *Salmonella* sero-prevalence were identified for sampling and each farm was coded A to J. The herds ranged in size from ~120 to 1400 sows. Most of the farms were specialised pig farms; however, some had mixed enterprises (e.g. dairy, beef, etc.). Depending on the age of the farm and the production stage, pigs were housed on solid, partially slatted or fully slated floors. Some farms had dry feed delivery and feeding systems, while others had computerised liquid feeding systems. A questionnaire was completed by each farmer/farm manager in order to obtain information on on-farm management practices. Each farm was sampled on two occasions between March 2012 and June 2013; once during Winter/Spring (November to April) and once during Summer/Autumn (May to October).

2.3.2 *Sampling Procedure*

The number of farms sampled and the number of samples taken per farm and production stage were in accordance with statistical advice. The sampling plan was developed to cover each pig production stage across each of the 10 selected farms. The sampling plan for each farm was determined based on its *Salmonella* sero-prevalence and the overall sample size was based on the number of sampling/observational units with standard assumption of a 95% confidence interval and an accuracy of +/- 5% for Bernoulli trials. The sampling procedure was a stratified scheme based on numbers per pig farm and numbers per farm per production stage. As a secondary focus of the study was to determine, insofar as possible, estimates of

Salmonella prevalence per farm and per stage of production, a simulation was conducted to examine the achievable accuracies, given the minimum sample size already established. Based on this, the minimum sample size for the overall prevalence was augmented to provide a balance between resources and effort required and the accuracy of prevalence estimates in the cells of the stratified scheme. No information was available about heterogeneous variance across the sampling grid so the distribution of samples was un-weighted and proportional to stocking rates. A final constraint was introduced to ensure that no sampling cell had less than three samples.

From each production stage on each farm, freshly voided faecal samples were collected at random from at least 3 pigs per pen and pooled. Sterile pre-moistened gauze socks (Sodibox, Névez, France) were also used to swab the pen floor and this sample was analysed separately. Freshly voided faecal samples were also collected from one boar per farm during the second sampling visit (if a boar was present) and analysed separately. Environmental samples (i.e. swabs of feed troughs, feed bins and nipple drinkers/water troughs) were also taken using sterile pre-moistened swabs. Water samples (500ml) were collected into sterile bottles from nipple drinkers, water troughs and associated header tanks from each pen identified for sampling. Feed samples (50-100g), both liquid and dry feed (meal and pellets), depending on the farm and production stage, were taken from troughs, hoppers and storage areas (feed bins, feed tanks) on each farm. All samples were immediately placed on ice and transported to the laboratory, where they were stored at 4˚C until analysis (within 24 h of collection).

2.3.3 *Microbiological analysis of samples*

The presence/absence of *Salmonella* in 10g faecal and feed samples taken from composite samples was determined according to standard microbiological procedures (EN ISO

6579:2002/Cor 1:2004) with modified brilliant green agar (Oxoid, Basingstoke, Hampshire, UK) used for additional selective plating. Swabs and gauze socks were transferred into buffered peptone water (BPW) so that a 1:10 dilution was achieved and homogenized in a stomacher for 60 seconds prior to testing for *Salmonella* using the procedure outlined above. Water samples were filtered through 0.45µm cellulose acetate filters (Sartorius, Dublin, Ireland) using 'sterifil' filtering funnels (Millipore, Merck, Darmstadt, Germany) fitted onto a filtering manifold (Millipore) and a vacuum applied using a vacuum pump (Millipore). The filters were added to 90ml of BPW and tested for *Salmonella* using the procedure outlined above. Presumptive *Salmonella* isolates (identified based on the results of biochemical tests) recovered from any sample were tested using a *Salmonella* latex agglutination kit (Oxoid).

2.3.4 Confirmation of Salmonella isolates by Real-Time Polymerase Chain Reaction (PCR)

DNA was extracted from isolates identified as *Salmonella* by the latex agglutination kit using a DNAeasy Tissue Kit for Gram-negative bacteria (Qiagen, Crawley, UK) according to manufacturer's instructions. The primer and *Taq*Man probe sequences used for the confirmation of *Salmonella* spp. were based on those of McCabe et al. (2011). pUC18 plasmid DNA was used as the internal amplification control (IAC) instead of pUC19, as described by Fricker et al. (2007), as it is identical except that the multiple cloning site (nt 397-454) is reversed. pUC18 DNA was isolated from *E. coli* using a QIAprep Spin Miniprep kit (Qiagen) according to manufacturer's instructions. Labels for the *hilA* and pUC18 were as described by Fricker et al. (2007). All primers and probes were manufactured by Sigma-Aldrich (St. Louis, USA). Internal Amplification Control (IAC) template DNA was isolated from *E. coli* using a QIAprep Spin Miniprep kit (Qiagen) according to manufacturer's instructions. PCR amplification and detection were performed using a Roche LightCycler 480 (Roche Diagnostics Limited, Burgess Hill, West Sussex, United Kingdom) using LC480

probe master mix kit (Roche Diagnostics Limited). Isolates confirmed as *Salmonella* were grown on standard plate count agar (Oxoid) overnight at 37°C and a loopful of colonies was suspended in 0.5 ml 80 % glycerol, added to cryoprotectant beads and stored at -80°C .

2.3.5 Serotyping and antimicrobial susceptibility testing of Salmonella isolates

All confirmed *Salmonella* isolates were serotyped according to the White-Kauffmann-Le Minor classification scheme (Grimont et al. 2007). *Salmonella enterica* subsp. *enterica* serovar (4,[5],12:i:-) that lack expression of the second-phase flagellar antigens were designated as monophasic variants of *S.* Typhimurium. Antimicrobial susceptibility testing was performed according to the broth dilution method of the Clinical and Laboratory Standards Institute (formerly NCCLS) (CLSI, 2008) using a broth microdilution assay (Sensititre, TREK Diagnostic Systems Inc., Sussex, England). The panel of antimicrobials included Azithromycin (Azt), Ampicillin (A), Amoxicillin/Clavulanic acid (Am), Ceftriaxone (Ax), Chloramphenicol (C), Ciprofloxacin (Cp), Trimethoprim/Sulfamethoxazole (Tm), Cefoxitin (F), Gentamicin (Gm), Kanamycin (Km), Nalidixic acid (Na), Sulfisoxazole (Su), Streptomycin (S), Tetracycline (T) and Ceftiofur (Ce). The cut-off values (mg/l) were as specified in EU Commission Decision 2007/407. *Escherichia coli* ATCC 25922 was used as a control. Isolates were either designated as "fully susceptible", "intermediate" or if resistance to any antimicrobial was found, this was indicated by using the abbreviation for the antimicrobial to which the strain was resistant.

2.3.6 Confirmation of monophasic Salmonella Typhimurium by multiplex Real-Time PCR

All isolates identified phenotypically as monophasic *S. enterica* subsp. *enterica* serovar Typhimurium 4,[5],12:i:− were tested by multiplex PCR with oligonucleotides specific to the antigenic genes *fliC* (H:i antigen), *fljB* (H:1,2 antigen) and *fljB*/IS200 as recommended by

EFSA (2010). The primers used were those outlined by Prendergast et al. (2013) and were obtained from Eurofins MWG Operon, Dublin, Ireland. The probes were *Taq*man hydrolysis probes (TIB MolBiol Berlin, Germany), also as outlined by Prendergast et al. (2013) except that different fluorescent dyes were used. The primer sequences, probes and target genes are listed in Table 1. PCR amplification was performed in a final volume of 10 µl containing 9 µl master mix and 1 µl template DNA. The master mix consisted of 2× Lightcycler 480 probe master, PCR-grade water and optimized concentrations of the primers and probes. PCR amplification was performed in a Roche Lightcycler 480 96-well instrument with an initial denaturation step of 95 °C for 10 min followed by 30 cycles of 95 °C for 10 s, 60 °C for 1 min, 72 °C for 1 sec, followed by an extension step of 72 °C for 10 min. Fluorescence signals were detected in 6FAM (fliB/IS200), LC670 (fliC) and YAK (fljB.1, 2) channels. The reaction was considered positive when Ct values were ≤30.

2.3.7 Pulsed-Field Gel Electrophoresis (PFGE)

All *Salmonella* isolates, apart from those identified as *S.* Typhimurium, *Salmonella* Typhimurium Copenhagen and monophasic variants of *S.* Typhimurium (1,4,[5],12:i, were grown overnight on plate count agar. Pulsed-field gel electrophoresis (PFGE) was performed using the restriction endonuclease *Xba*I (New England Biolabs, MA, USA) according to the standardised CDC PulseNet protocol developed for *Salmonella* (CDC, 2002). Gels were stained in a 1µg/mL solution of ethidium bromide and visualized with UV transillumination using a Gel Doc imaging system (BIO-RAD, Munich, Germany). Macrorestriction patterns were compared using the BioNumerics fingerprinting software (Version 5.10, Applied Maths, Austin, TX). The similarity index of the isolates was calculated using the Dice correlation coefficient with a band position tolerance of 1%, an optimization of 1% and the unweighted

pair group method with arithmetic averages (UPGMA). Isolates were grouped based on similarity, with a correlation coefficient of >80% demonstrating a clonal relationship.

2.3.8 Multilocus Variable Number Tandem Repeat Analysis (MLVA)

All *Salmonella* isolates confirmed by serotyping and multiplex PCR as *S.* Typhimurium and/ or monophasic variants of *S.* Typhimurium (1,4[5], 12:i) were subjected to multilocus variable number tandem repeat analysis (MLVA) using capillary electrophoresis methods as described previously by Prendergast et al. (2011). The variable number tandem repeat (VNTR) loci selected, along with the primers and dyes used, were as outlined by Lindstedt et al. (2004) and are listed in Table 2. Essentially, the loci were amplified in separate PCRs by using fluorescent primers. Raw fragment lengths for each locus were manually discarded using a minimum threshold of ± 3 bp to distinguish alleles. *S.* Typhimurium LT2 ATCC 29946 was used as a positive control during the analysis of each batch of samples on the ABI 3500 genetic analyser (Applied Biosystems, Foster City, California, US). Each peak was identified according to colour and size using GeneMapper (version 4.1) software (Applied Biosystems) and a set of 5 alleles for each isolate was defined as the MLVA profile. MLVA profiles were assigned based on the fragment size amplified from each locus, with 'NA' used to denote a locus not present.

2.3.9 MLVA data analysis

Diversity of variable number tandem repeats (VNTR) was calculated using Simpson's Diversity index formula

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} x_j(x_j - 1)$$

The diversity index for VNTR data is a measure of the variation of the number of repeats at each locus. Simpson's diversity ranges from 0.0 (no diversity) to 1.0 (complete diversity).

Diversity of MLVA was assessed using the Shannon-Weiner (Shannon) diversity (H') index formula

$$H' = -\sum_{i=1}^{S} p_i \ln p_i$$

where P_i is the proportion of individuals in the population belonging to the i^{th} species.

The equitability (E_H) indices were calculated using the formula

$$E_H = H / \ln S$$

where H is the H′ index calculated as previously mentioned and S is the total number of species in the community. Shannon's index calculates separate indices for the diversity index (H'), which is an indicator of the number of subtypes (i.e. value increases as diversity increases) and an equitability index (E), which is a measure of the evenness of subtype distribution and has a maximum value of 1 (Boxrud et al. 2007).

The MLVA profile using the five loci ST9-ST5-ST6-STTR10-ST3 (Lindestadt et al. 2004) was used for cluster analysis using BioNumerics v5.10 software. When deviations from the MLVA profile were minimal, a loss or gain in a repeat at the contingency locus was observed; losses and gains in one repeat at loci are more likely to occur in related isolates. Using this rule of thumb the isolates were assigned to groups (a cluster is when five or more

isolates had the same MLVA profile). Feed isolates were included in the analysis even if not belonging to a group big enough to form a cluster.

2.3.10 Statistical Analysis

For the purposes of determining *Salmonella* prevalence, faecal sample data included combined data from composite faecal samples and pen swabs for all production stages and individual boar faecal samples across the 10 farms, where taken. Environmental sample data included data from swabs taken from water drinkers, feed troughs and feed bins for each production stage across the 10 farms. Feed sample data included data from both liquid and dry feed (meal and pellets) samples taken from troughs, hoppers and storage areas (feed bins, feed tanks) for each production stage across the 10 farms. In all cases, data from the two sampling trips were combined. For the analysis of *Salmonella* prevalence in these pig faecal, environmental, feed and water samples, logistic regression models were fitted to the binomial data of positives and total test numbers to statistically test source, stage, herd and season together, with conditional effects of each and testing for interactions between factors. Marginal models were then fitted to assess the prevalences of each factor ignoring all others. The Glimmix procedure in SAS 9.3 (2011) was used to fit all the logistic regression models.

2.4 Results

2.4.1 Salmonella prevalence in pig faecal, environmental, feed and water samples taken on-farm

In total, 2,975 samples were taken over the course of the 15-month study. *Salmonella* was detected in 138/926 (14.9%) pig faecal samples across all production stages on 9 farms. It was also detected in 9.0% of environmental (water drinker, feed trough and feed bin) swabs and 9.5% of water samples (Table 1). One farm (H) had no *Salmonella*-positive pig faecal samples, another (F) had no positive environmental samples and two farms (C and F) had no *Salmonella*-positive water samples (Table 1). One farm (E) had notably higher faecal prevalence than the other farms (37.2%), which correlates with the fact that it had the second highest sero-prevalence (46.9%; Table 1). This farm also had the highest number of *Salmonella*-positive environmental and water samples (20 and 28.3% samples *Salmonella*-positive, respectively) but no *Salmonella*-positive feed samples; Table 1). In general, the highest proportion of *Salmonella*-positive faecal samples (21.5%) was found in 2nd stage weaners, closely followed by finishers and gilts (19.5 and 19.1% faecal samples *Salmonella*-positive, respectively; Table 2). This was reflected in the environment, with 2nd stage weaner samples also having the highest *Salmonella* prevalence. The lowest faecal and environmental prevalence was in farrowing sows (1.5 and 2%, respectively; Table 2).

Only 14/585 (2.4%) feed samples taken on-farm across all production stages were *Salmonella*-positive (Tables 1 and 2). Six farms (A, B, D, G, I and J) had at least one *Salmonella*-positive feed sample. However, the farm with highest *Salmonella* prevalence in pig faecal samples (E; 37.2%) had no *Salmonella*-positive feed samples and the farm with the highest prevalence in feed samples (D; 6.3%) had very low *Salmonella* prevalence in

pig faecal and environmental samples (2.6 and 2.4%, respectively; Table 1). Six of the *Salmonella*-positive feed samples originated on farms using liquid feed (farms A, B and J). Positive feed samples were generally recovered at one stage of production only, although on farms B and D they were found at more than one stage (1st stage weaner and finishers on farm B and dry sows, farrowing and gilts on farm D; data not shown). In general, feed sampled from dry sows had the highest *Salmonella* prevalence (6%; Table 2) and from finishers the lowest (0.8%; Table 2)

2.4.2 Salmonella serotypes recovered from samples

Eleven different serotypes were identified among the 287 *Salmonella* isolates recovered from the pig faecal, feed, environmental and water samples taken across the 10 pig farms (Table 3). Serotype 4,[5],12:i: (monophasic variant of *Salmonella* Typhimurium) predominated, with 118 strains isolated, accounting for 41.1% of all isolates recovered. It also predominated in all sample types, accounting for ~42% of all pig faecal, environmental and feed isolates and 32.6% of water isolates (Table 1). The other serotypes recovered in order of decreasing prevalence were Derby [77 strains (26.8% of all isolates recovered)], Typhimurium [38 strains (13.2%)]), London [18 strains (6.3%)], Infantis [15 strains (5.2%)], Typhimurium Copenhagen [8 strains (2.8%)], Dublin [5 strains (1.7%)], Tennessee [4 strains (1.4%)], Anatum [2 strains (0.7%)], Stanley [1 strain (0.4%)], and Orion [1 strain (0.4%)]. This order of recovery applied to all sample types except water samples, where London was the 6th instead of the 4th most frequently recovered serotype (Table 1). Although some serotypes were detected in all sample types (faecal, water, feed and environment), Orion and Stanley were exclusively found in the environment and Dublin was only found in water samples. In addition to these serotypes, *S.* London, Infantis and Anatum were also absent from feed.

2.4.3 Antimicrobial resistance of *Salmonella* isolates

Table 4 summarizes the frequency of susceptibility/resistance of the *Salmonella* isolates recovered from the pig faecal, feed, environmental and water samples taken across the 10 pig farms to a particular antibiotic. The antibiotics to which the isolates showed most frequent resistance were tetracycline, sulfisoxazole, streptomycin and ampicillin, with resistance to tetracycline most common and involving all serotypes except Orion and Stanley (data not shown). On the other hand, all of the isolates were susceptible to ceftriaxone. Seventy different antimicrobial resistance (AMR) profiles were observed among the *Salmonella* isolates and those of all serotypes except Typhimurium, Typhimurium Copenhagen and the monophasic variants of *S*. Typhimurium (1,4,[5],12:i) are shown in Figs. 1 and 2. Resistance ranged from one to 12 antibiotics, with 61% of the isolates recovered (n=175) resistant to four or more antibiotics (data not shown). The classic penta-resistant pattern ACSSuT (resistance to ampicillin, chloramphenicol, streptomycin, trimethoprim/sulfamethoxazole and tetracycline) which is common among *Salmonella* Typhimurium DT104 strains, was detected in 24 isolates belonging to the monophasic (4,[5],12:i: [n=12]), Typhimurium (n=6), Derby (n=4), Infantis (n=1) and Tennessee (n=1) serotypes (data not shown). The 118 monophasic *Salmonella* 4,[5],12:i: isolates displayed 26 AMR profiles, with 106 of the isolates showing resistance to four or more antibiotics. Two patterns (ASSuT and ASSuTTm) predominated and were observed in 37 (31%) and 40 (33%) of the monophasic *Salmonella* 4,[5],12:i: isolates, respectively.

2.4.4 Molecular subtyping of non-Typhimurium *Salmonella* isolates by PFGE

A total of 123 non-Typhimurium/Typhimurium Copenhagen/monophasic variant isolates (5 of 14 feed isolates; 57 of 138 faecal isolates, 22 of 43 water isolates and 40 of 92 environmental isolates) which showed similar phenotypic characteristics were subjected to

molecular subtyping by PFGE. A total of 54 different PFGE patterns were seen among the 123 isolates, which belonged to eight different serovars. To analyse clonal relationships and track isolates across samples and farms, two dendrograms were generated; one for the 77 *S.* Derby isolates (Figure 1) and the other for the 46 remaining non-Typhimurium/Typhimurium Copenhagen/monophasic variant isolates (Figure 2). Using an 80% genetic relatedness threshold, the PFGE macrorestriction profiles indicated that the *S.* Derby isolates belonged to four distinct clusters (denoted A to D), while the remaining isolates grouped into two clusters. The PFGE data were not always consistent with phenotypic findings; for example, isolates with indistinguishable PFGE profiles sometimes differed in their AMR profile e.g. the majority of the *S.* Derby isolates within cluster D have a susceptible AMR profile with intermediate resistance to cefoxitin; however, there are isolates within this cluster that have additional resistance (Figure 1).

PFGE results were, however, consistent with serotyping data and, for the most part, farm of origin and sampling time point. For example, *S.* Infantis cultured from farm E on the first visit had indistinguishable PFGE profiles (some clustered together in cluster E) while *S.* Derby was the main serotype recovered from the second sampling trip and the majority of these isolates were also indistinguishable and were assigned to clusters A-C (Figures 1 & 2). Similarly, *S.* Derby isolates recovered from farm I during the second sampling trip had indistinguishable PFGE profiles (most clustered in cluster D; Figure 1) while *S.* London isolates from the same farm but from the first sampling trip were also indistinguishable (most were assigned to cluster F; Figure 2). In some cases, the same strain (as determined by PFGE profiling) was recovered from both sampling trips to farms E and I (Figures 1 & 2). In addition, sometimes the same strain was found on different farms e.g. a *S.* London strain was

common to farms I and J and the same Infantis strain was recovered from farms A and E (Figure 2).

On some farms, the same strain was found across multiple production stages. This was most evident for *S.* Infantis isolates recovered from Farm E, which were found in breeding stock (dry and farrowing sows and gilts) as well as 1st stage weaners and finishers (isolates 712-832; Figure 2). These isolates also illustrate the fact that the same strain was often recovered from multiple sample types i.e. pig faecal, environmental and water samples in this case. Looking specifically at feed isolates, the PFGE profiles of two *S.* Derby isolates recovered from dry sow feed on Farm I (within cluster D were indistinguishable from isolates recovered from pig faecal, environmental and water samples on the same farm (Figure 1). The other *S.* Derby feed isolate for which a PFGE profile was obtained (isolate number 580 found in dry sow feed on farm E) was also found in a feed trough and a water sample from the dry sow stage on the same farm, as well as in faecal samples and a feed trough from the gilt stage on another farm (farm C; Figure 1). In addition, a S. Tennessee isolate recovered from dry sow feed was also recovered from a dry sow faecal sample taken on the same farm, as well as a water sample taken from the finisher stage, also on the same farm.

2.4.5 MLVA profiling of *S.* Typhimurium, *S.* Typhimurium Copenhagen and monophasic variants of *S.* Typhimurium

All *S.* Typhimurium, Typhimurium Copenhagen and monophasic *Salmonella* isolates (n=166; 9 of 14 feed isolates; 81 of 138 faecal isolates, 21 of 43 water isolates and 52 of 92 environmental isolates) were subtyped using MLVA profiling, as it is more discriminatory than PFGE for these serotypes. The discriminatory ability of the technique was determined by calculating Simpson's index of diversity (D) for the 166 typed isolates. MLVA profiling

differentiated 43 strains and showed a D value of 0.9203. Shannon-Wiener's index calculated values of 3.04 and 0.81 for the diversity index (H') and the equitability index (E), respectively.

The discriminatory power of each VNTR was estimated by the number of alleles detected and the allele diversity. VNTR6 was the most diverse loci with 12 different alleles, while VNTR9 and VNTR10 showed the least amount of diversity, with 6 alleles. VNTR5 and 3 generated 9 and 7 alleles, respectively. Absence of a PCR amplicon occurred most often at the Typhimurium-specific virulence plasmid pSLT-bound loci VNTR10 (71.1% of the sample population) correlating with the high numbers of monophasic Typhimurium isolates, followed by VNTR3 (5.4%), VNTR6 (1.8%), VNTR9 (1.8%) and VNTR5 (1.2%).

Based on the five VNTR loci, the 166 isolates were further analysed and grouped into clusters (Table 5) to deduce the relatedness of the isolates. If neighbours differed in no more than one of the five VNTR loci, they were assigned to the same cluster. The MLVA profiles were also used for categorical clustering in BioNumerics, and a minimum spanning tree was constructed (Figure 1, supplementary material). Overall, 23 different MLVA profiles were found and, based on these, the non-*S.* Typhimurium, Typhimurium Copenhagen and monophasic *Salmonella* isolates were classified into 12 main clusters; the monophasic isolates grouped into seven main clusters (A-G), whereas *S.* Typhimurium was grouped into 4 clusters (H-K) and *S.* Typhimurium Copenhagen into only one (L) (Table 5).

Within these clusters the number of isolates with the same profile ranged from 2 to 32. Isolates with identical MLVA profiles originating from the same source at different time points were observed on several occasions. For example, in the largest cluster (cluster E;

n=42), isolates belonging to profile 3-11-9-NA-0211 (n=32) were mainly recovered from farm A on two different sampling visits, but three isolates from farm G (second visit) also had the same profile (Table 5). These isolates were recovered from all samples types (faecal, environmental, feed and water samples) taken from the 1st and 2nd stage weaner and finisher production stages on Farm A and from finishers and gilts on farm G. Two feed isolates from a previous study (Burns et al. 2015) also had the same MLVA profile, one recovered from a dry sow compound meal feed sampled from a commercial feed mill suppling farm H and the other from a finisher whey based meal sampled from a commercial feed mill supplying farms A,C and F. Among the isolates with this 3-11-19-NA-0211 profile, ten different AMR profiles were observed: ASSuT (n=26), AT (1), ASSuTTm (5), ASSuTF (1), SuTTmGmCe (1), ASSuTTmNa (1), ASSuTTm(F) (1), ASuT (1), TGm (1) and T (3). Isolates from different sources with identical MLVA profiles were also frequently observed in some of the other clusters.

Looking at the isolates of feed origin alone, the five monophasic variant isolates recovered from feed had four different MLVA profiles and originated on three different farms (A, B and D). Isolates with profiles 3-14-10-NA-0211 (Cluster B), 3-11-9-NA-0211 and 3-11-10-NA-0211 (both cluster E) were also recovered from pig faecal samples found on the same farms, often during the same sampling visit, and often across different production stages (Table 5). The most notable of these is 3-11-9-NA-0211, which was also recovered from feed and feed ingredient sampled from commercial mills, as outlined above. Of note, is also the fact that, although no feed isolates with the profile 3-13-16-NA-0211 were found on any of farms, three monophasic variant isolates with this profile were previously found in a whey based finisher meal along with a meal and pelleted diet for dry sows sampled from mills C and D

which supplied farms A, C, F and H, respectively (Table 5). One isolate with the same profile was, however, recovered from a finisher faecal sample taken on farm J.

2.5 Discussion

Salmonella was recovered from faecal samples on 9 of the 10 commercial farms sampled. This was expected, as all farms in the study were selected because of their history of high *Salmonella* sero-prevalence. Failure to recover *Salmonella* from any production stage on farm H may be because this farm had a low sero-prevalence during the study period, highlighting the cyclical nature of *Salmonella* contamination on farms (White et al. 2006). *Salmonella* prevalence varied from herd to herd. The mean faecal bacterial prevalence for the 10 study farms taking all stages of production into account was 14.9%; however, when calculated using only samples from the finisher stage, as in the National Control Programme, it was 19.5% which was closer to the 25.8% sero-prevalence determined by the National Control Programme. In general, at an individual farm level, there was good correlation between the faecal bacterial prevalence (for all production stages) for six of the 10 study farms. On some however, there was a lack of correlation between bacteriological and serological data e.g. farm D. In addition, only four farms (A, E, G and I) had bacteriological prevalence's >20%, with prevalence's for the remaining farms ranging from 0 to 17.5%. This further illustrates the lack of correlation, considering that all of the study farms were identified from the >50% sero-prevalence category using data from the National *Salmonella* Control Programme

The role of feed as a risk factor for *Salmonella* carriage may be called into question and is substantiated by the fact that the farm with the highest faecal *Salmonella* prevalence had no *Salmonella*-positive feed samples and the farm with the highest prevalence in feed samples had very low faecal and environmental prevalence. High carriage rates are commonly seen in weaners and finishers (Rowe et al. 2003; Davies et al. 1999); however, few studies have investigated *Salmonella* carriage from farrow to finish. The high prevalence within the replacement breeding stock (gilts) in the present study indicates that these animals may be an

important source of on-farm *Salmonella* infection and this could be of particular importance on farms where replacement breeding stock are purchased onto the unit. This is backed up by the PFGE data which showed that *Salmonella* strains recovered from breeding stock were often found in weaners and finishers on the same farm, indicating that certain strains may be endemic on particular farms. The lowest faecal *Salmonella* prevalence was found in farrowing sows, in agreement with data from a previous Irish study (Rowe et al. 2003).

Faecal samples had the highest *Salmonella* prevalence (14.9%), followed by water (9.5), environmental (9.0%) and feed (2.4%) samples, indicating the widespread occurrence of *Salmonella* in pig production environments. The ability of *Salmonella* spp. to survive outside the host and to multiply over a wide temperature range means that even a very low level of these bacteria has the potential to be infectious. High prevalences of *Salmonella* have previously been seen in water but studies have focused on water used for irrigation purposes (Jones et al. 2014). To our knowledge no other studies have looked at water within pig farms as a risk factor. In the present study, it should be borne in mind that some of the *Salmonella*-positive water samples were taken from nipple drinkers and troughs within pens, to which the pigs had access. In fact, in a number of cases, isolates with indistinguishable PFGE profiles were recovered from pig faecal and environmental samples from the same stage, indicating that the pigs are most likely a source of contamination. However, *Salmonella* may be found in private wells that have been contaminated with faecal matter from sewage overflows, polluted storm water runoff or agricultural runoff, particularly after flooding (Uyttendaele et al. 2015)

Salmonella prevalence in feed samples taken on-farm was relatively low. However, the PFGE patterns of *S.* Derby feed isolates were indistinguishable from those of faecal isolates

recovered from pigs from the same production stage (dry sow) on the same farm on the same day. The fact that the same *S.* Derby strain was also recovered from troughs, water samples and water drinkers from the dry sow stage suggests widespread environmental contamination with this strain. The recovery of genotypically related isolates from feed and pig faeces highlight the importance of feed as a possible source of *Salmonella* infection on pig farms. Likewise, Davis et al. (2003) used PFGE to provide evidence for a role of cattle feed as a vehicle in the transmission of *S.* Typhimurium DT104 and *Escherichia coli* O157:H7 to cattle. In addition, a study by Molla et al. (2010) showed genotypically related and in some cases clonal *Salmonella* strains in commercially processed pig feed sampled from feed bins and pig faecal samples on the same farm. However, as all except one of the *Salmonella*-positive feed samples in the present study originated in troughs within the animal pens, the possibility of on-farm contamination by the pigs is highly likely. On the other hand, the fact that a *S.* Derby strain recovered from dry sow feed was also recovered from faecal samples taken from a different production stage (gilts) on the same farm on the same day, and a *S.* Tennessee isolate recovered from dry sow feed was also recovered from a water sample taken from the finisher stage on the same farm indicates that they share a common vector for *Salmonella* transmission on-farm.

More definitive evidence for the role of feed as a source of *Salmonella* for pigs comes from the fact that two monophasic *Salmonella* isolates found in feed ingredients and compound feed sampled from commercial feed mills in a previous study by our group (Burns et al. 2015) were found to have identical MLVA profiles to monophasic isolates recovered from farms A and G in the present study. Of these, the MLVA profile associated with the *Salmonella* isolate recovered from a finisher meal manufactured at mill C was the same as that of an isolate recovered from on farm A found in weaners (1^{st} and 2^{nd}) along with

finishers, which was supplied with feed for the duration of the study by Mill C. As the feed mill isolate was recovered from feed aseptically sampled in the mill prior to contact with the pig herd, there was no possibility of cross-contamination from the farm or via transport. Although the same MLVA profile was found for monophasic isolates recovered from soyabean meal sampled from mill E and farm G, the same link does not exist as this mill was not the feed supplier for that farm. Despite this, as soyabean is the most frequently used protein-rich feed ingredient in all pig feed diets (Wierup and Widell, 2014), it is plausible to suggest that the same batch of contaminated ingredient may have affected more than just one feed mill and subsequently the farms supplied by these mills.

The isolates recovered from the *Salmonella*-positive feed sampled on-farm in the present study were identified as 4,12:i:-, Derby, Typhimurium, Typhimurium Copenhagen and Tennessee. According to Li et al. (2012) the most common serotypes associated with animal feed are *S.* Seftenburg, Montevideo, Mbandaka, Tennessee and Typhimurium. However, serotypes differ in their ability to invade pigs, and some are recovered more frequently than others. The same serotypes recovered from feed in the present study were also recovered from pigs but some of the main pig-derived serotypes were not found in feed i.e. London and Infantis. A previous Irish study identified *S.* Typhimurium, *S.* Derby, *S.* Infantis, *S.* Livingstone and *S.* London as the five serotypes most commonly isolated from Irish pigs (Rowe et al. 2003). This is in agreement with the present study, except that Livingstone was not recovered Furthermore, the *Salmonella* 4,[5],12:i:- variant that predominated in the pigs and feed, in the present study was not found in the Rowe et al. (2003) study. *Salmonella* 4,[5],12:i:- is one of a number of monophasic variants of the serovar Typhimurium, that have been emerging in Europe and are of increasing food safety concern (EFSA, 2010). It is now reported as the fourth most common serovar in slaughtered pigs (EFSA, 2008) and the third

most common in humans in the EU (EFSA, 2014). Human illness linked to this monophasic variant looks set to keep rising, with human cases of associated illness increasing from 360 in 2007 to 5932 in 2012 (EFSA, 2014). In the present study the main multidrug resistance types exhibited among these monophasic *Salmonella* isolates was a tetra-resistant pattern ASSuT, with or without additional resistances, but lacking resistance to chloramphenicol, as seen previously in *S.* Typhimurium DT104. The detection of serotypes *S.* Dublin, Orion and Stanley in the environment only, indicates the ability of these serovars to survive extra-intestinally. The ubiquitous nature of these serovars may suggest an adaption to ensure passage through a host into the environment and back into a new host (Winfield and Groisman, 2003), although without a host, *Salmonella* proliferation is unlikely.

In 1968, *Salmonella* Agona was introduced to the United States in animal feed and has subsequently become a common serotype in human illness, illustrating the potential effects of feed contamination with *Salmonella* (Crump et al. 2002). Previous investigations have reported pig feed and feed ingredients as sources of *Salmonella* transmission (Boyer et al. 1962; Hacking et al. 1978; Jones et al. 1982; Veldman et al. 1995; Davies and Wray, 1997; Jones and Richardson, 2004). Of the *Salmonella* serovars found in pig feed in the present study, *S.* Tennessee (farm J) was the only one that is not commonly associated with pigs. However, this serovar has previously been found in feed where contamination in a feed mill was responsible for a large feed borne outbreak in layer hen and pig farms in Finland in 2009 (Häggblom, 2009). In the six farms (A, B, D, G, I and J*)* that had at least one *Salmonella*-positive feed sample, the contamination of food or feedstuffs may therefore have a large impact on the spread of *Salmonella* spp., provided that the bacterium is given the right conditions to increase in numbers. Likewise, farms with *Salmonella*-negative feed samples but with *Salmonella*-positive faecal samples demonstrate that there were multiple sources of

Salmonella infection on pig farms. The importance of these sources may vary by production stage, farm and over time. The potential for cross-contamination between livestock and feedstuffs in mixed enterprises was also documented on three of the farms visited, where dairy and beef enterprises were also present. *Salmonella* Dublin, for example, a serovar host-adapted to cattle but also infectious in pigs and humans, was found on farm H in water samples (Andino and Hanning, 2015; Hoelzer et al. 2011; Crilly, 2004; Duijkeren et al. 2002)

2.6 Conclusions

Genetic subtyping by PFGE and MLVA identified genetically indistinguishable isolates from both feedstuffs and pig faeces sampled on the same farm and feed/feed ingredients sampled at feed mills. In one cases, the feed mill supplied the farm, from which the related isolates were recovered. These data support the hypothesis that pig feed can be a vector for the introduction of *Salmonella* into pig herds. In addition, our results confirm the presence of monophasic variants of *S*. Typhimurium (1,4,[5],12:i:-) in pig herds in Ireland, demonstrating the serovar switch in *Salmonella* that has occurred in pig feed and faeces in Ireland. Overall, the occurrence of genotypically related strains of *Salmonella* in commercial pig feed and pig faeces dictates the need for sustainable intervention strategies to reduce and eliminate *Salmonella* in animal feeds and ingredients in order to safeguard human and animal health. However, the solution to the problem of *Salmonella* on pig farms is not simple; hence, a range of methods may be needed to suppress, eliminate, or prevent *Salmonella* contamination.

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Tables and Figures

Table 2.1: Number and percentage (%) of *Salmonella*-positive samples detected by sample type on 10 Irish commercial pig farms with a history of high (>50%) *Salmonella* sero-prevalence

Farm	Mean Un-weighted serology (%) from National Pig *Salmonella* Control Programme [a]	Pig faecal samples [b] [No. Positive/No. Tested (% positive)]	Environmental samples [c] [No. Positive/No. Tested (% positive)]	Feed samples [d] [No. Positive/No. Tested (% positive)]	Water samples [e] [No. Positive/No. Tested (% positive)]
A	24.0	28/99 (28.3)	15/103 (14.6)	3/58 (5.2)	3/47 (6.4)
B	15.6	17/97 (17.5)	16/109 (14.7)	2/65 (3.1)	6/48 (12.5)
C	19.8	5/77 (6.5)	1/84 (1.2)	0/49	0/38
D	34.4	2/78 (2.6)	2/85 (2.4)	3/48 (6.3)	2/38 (5.3)
E	46.9	29/78 (37.2)	17/85 (20.0)	0/47	10/38 (28.3)
F	4.2	1/98 (1.0)	0/103	0/62	0/48
G	43.8	17/78 (21.8)	7/86 (8.1)	2/50 (4.0)	4/38 (10.5)
H	5.2	0/73	1/82 (1.2)	0/49	7/36 (19.4)
I	52.1	23/98 (23.5)	20/107 (18.7)	3/63 (4.8)	6/48 (12.5)
J	12.5	16/150 (10.7)	13/177 (7.4)	1/94 (1.1)	5/74 (6.8)
Total		138/926 (14.9)	92/1011 (9.0)	14/585 (2.4)	43/453 (9.5)
Mean	25.8				
Mean faecal bacterial prevalence (%) for finishers [f]	19.5				
Mean prevalence (confidence intervals; %)[g]		14.9 (12.8-17.4)	9.0 (7.4-10.9)	2.4 (1.4-4.0)	9.5 (7.1-12.6)

[a] Mean un-weighted serology (%) (from testing of meat juice from finisher pigs at slaughter) for the duration of the study period.

[b] Faecal samples include composite faecal samples and pen swabs for all production stages and individual boar faecal samples across the 10 farms.

[c] Environmental samples include swabs taken from water drinkers, feed troughs and feed bins for each production stage across the 10 farms.

[d] Feed samples include feed taken from feed bins, hoppers and troughs within pens for all production stages across the 10 farms.

[e] Water samples include water taken from header tanks, nipple drinkers and water troughs within pens for all production stages across the 10 farms.

[f] Bacterial prevalence (%) calculated using only data from faecal samples and pen swabs taken from the finisher stage of production across the 10 pig farms.

[g] Confidence intervals calculated using lower and upper mean values.

Table 2.2: Total number and percentage (%) of *Salmonella*-positive samples from different production stages on 10 Irish commercial pig farms

Stage	Pig faecal [a] [No. Positive/ No. Tested (% positive)]	Environmental [b] [No. Positive/No. Tested (% positive)]	Feed [c] [No. Positive/No. Tested (% positive)]	Water [d] [No. Positive/No. Tested (% positive)]	Overall prevalence (%)[e]	Confidence Interval [f]	Faecal Prevalence (%)[g]	Confidence Interval [f]
Gilts	26/136 (19.1)	13/146 (8.9)	3/76 (4.0)	9/68 (13.2)	12.0	9.2-15.4	19.1	13.3-26.7
Dry Sow	15/144 (10.4)	10/151 (6.6)	5/83 (6.0)	4/70 (5.7)	7.6	5.5-10.4	10.4	6.3-16.7
Farrowing Sow	2/132 (1.5)	3/151 (2.0)	1/87 (1.2)	2/66 (3.0)	1.8	0.9-3.6	1.5	0.37-5.9
1st Stage Weaner	23/144 (16.0)	15/162 (9.3)	1/117 (0.9)	10/72 (13.9)	9.9	7.6-12.9	16.0	10.8-23.0
2nd Stage Weaner	31/144 (21.5)	25/164 (15.2)	3/95 (3.2)	7/72 (9.7)	13.9	11.1-17.3	21.5	15.5-29.1
Finisher	41/210 (19.5)	26/237 (11.0)	1/127 (0.8)	11/105 (10.5)	11.6	9.4-14.3	19.5	14.7-25.5
Boars	0/16	-[h]	-	-	0	-	0	-
Total	138/926 (14.9)	92/1011 (9.1)	14/585 (2.4)	43/453 (9.5)	9.5			

[a] Faecal samples include composite faecal samples and pen swabs for all production stages and individual boar faecal samples across the 10 farms.

[b] Environmental samples include swabs taken from water drinkers, feed troughs and feed bins for each production stage across the 10 farms.

[c] Feed samples include feed taken from feed bins, hoppers and troughs within pens for each production stage across the 10 farms.

[d] Water samples include water taken from header tanks, nipple drinkers and water troughs within pens for each production stage across the 10 farms.

[e] Calculated using bacteriological data from all samples taken from individual stages of production across the 10 farms.

[f] Calculated using only bacteriological data from faecal samples and pen swabs for each individual production stage across the 10 farms.

[g] Confidence intervals calculated using lower and upper mean values.

[h] - = No samples taken

Table 2.3: Distribution of *Salmonella* serovars recovered from 10 Irish commercial pig farms

Serovar	Farm	Production stage [a]	All isolates (%[b])	Pig faecal isolates [c] (%[b])	Environmental isolates [d] (%[b])	Feed isolates [e] (%[b])	Water isolates [f] (%[b])
4,[5],12:i:-	A,B,C,D,G,H,I,J	FW, G, W1, W2, F	118/287 (41.1)	59/138 (42.8)	39/92 (42.4)	6/14 (42.9)	14/43 (32.6)
Derby	B,C,D,E,I	FW,D, G, W1, W2, F	77/287 (26.8)	38/138(27.5)	25/92 (27.2)	4/14 (28.6)	10/43 (23.3)
Typhimurium	D,F,G,H,I,J	FW,D, G, W1, W2, F	38/287 (13.2)	18/138 (13.0)	11/92 (12.0)	2/14 (14.3)	7/43 (16.3)
London	I, J	D; G, W2; F	18/287 (6.3)	9/138 (6.5)	7/92 (7.6)	-[g]	2/43 (4.7)
Infantis	A, E	FW,D, G, W1, F	15/287 (5.2)	7/138 (5.1)	5/92 (5.4)	-	3/43 (7.0)
Typhimurium Copenhagen	G,I	G, W2;F	8/287 (2.8)	4/138 (2.9)	3/92 (3.3)	1/14 (7.1)	-
Dublin	H	G,W1,W2	5/287 (1.7)	-	-	-	5/43 (11.6)
Tennessee	J	D, F	4/287 (1.4)	2/138 (1.5)	-	1/14 (7.1)	1/43 (2.3)
Anatum	A	D, W1	2/287 (0.7)	1/138 (0.7)	-	-	1/43 (2.3)
Orion	A	W2	1/287 (0.4)	-	1/92 (1.1)	-	-
Stanley	B	W2	1/287 (0.4)	-	1/92 (1.1)	-	-

[a] W1;1st stage weaner, W2; 2nd stage weaner, F; finisher, G; gilts, FW; farrowing, D; Dry sow. All boar samples were *Salmonella*-negative.

[b] Percentage of isolates identified as a particular serotype

[c] Faecal samples include composite faecal samples and pen swabs for all production stages and individual boar faecal samples across the 10 farms.

[d] Environmental isolates were recovered from swabs taken from water drinkers, feed troughs and feed bins for each production stage across the 10 farms.

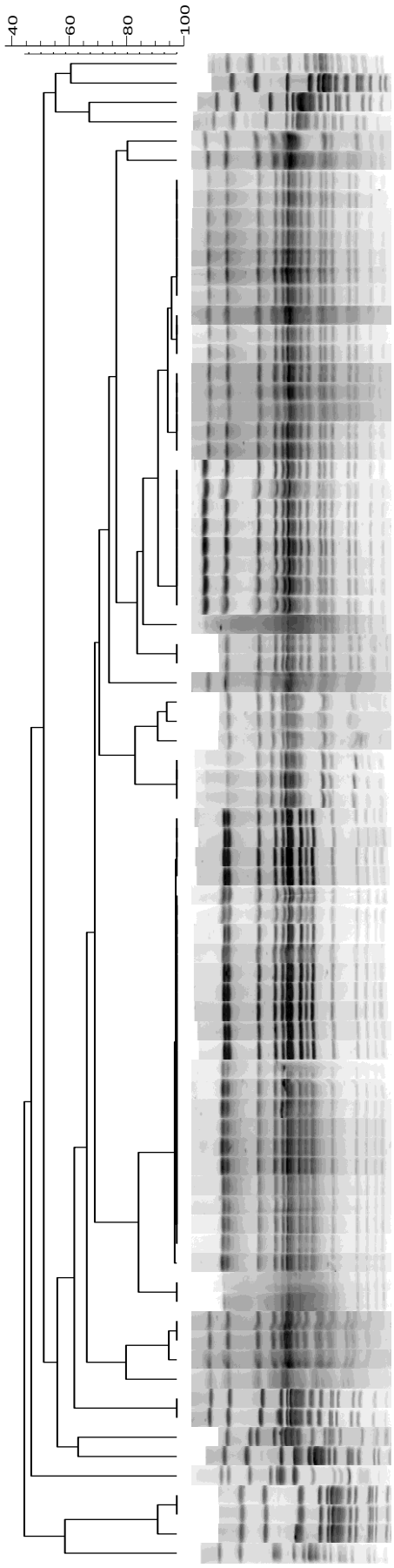
[e] Feed isolates were recovered from feed taken from troughs within pens, hoppers and feed bins for each production stage across the 10 farms.

[f] Water isolates were recovered from water taken from header tanks, nipple drinkers and water troughs within pens for each production stage across the10 farms

[g] - = serotype not recovered

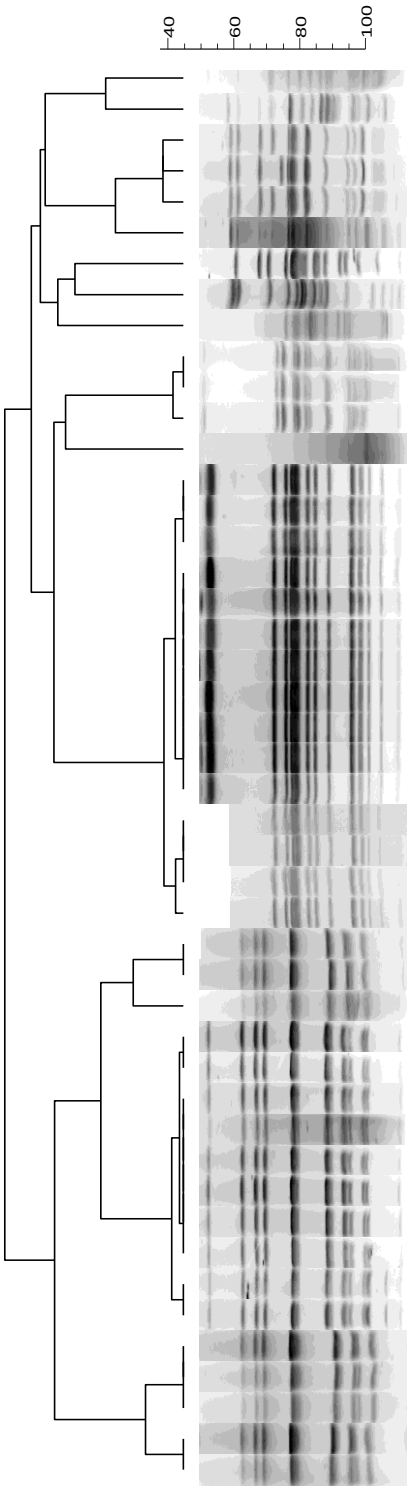
Table 2.4: Antimicrobial susceptibility/resistance of *Salmonella* isolates (n=287) recovered from pig faecal, environmental, feed and water samples taken from 10 Irish commercial pig farms in terms of number of isolates resistant/susceptible to a particular antimicrobial

			Number of Isolates (%)		
Antimicrobial Agents	**Abbreviations**	**Range (µg/ml)**	**Susceptible**	**Intermediate**	**Resistant**
Azithromycin	Azt	0.5–64	271 (94.4)	0	16 (5.5)
Ampicillin	A	1-32	110 (38.3)	1 (0.4)	176 (61.3)
Amoxicillin/Clavulanic Acid	Am	1/0.-32/16	272 (94.8)	8 (2.8)	7 (2.4)
Ceftriaxone	Ax	0.25-64	286 (99.6)	1 (0.4)	0
Chloramphenicol	C	2–32	253 (88.2)	7 (2.4)	27 (9.4)
Ciprofloxacin	Cp	0.0015-4	283 (98.6)	0	4 (1.4)
Trimethoprim/sulfamethoxazole	Tm	0.12/2.38-4/76	210 (73.2)	0	77 (26.8)
Cefoxitin	F	0.5-32	247 (86.1)	32 (11.2)	8 (2.8)
Gentamicin	Gm	0.25-16	268 (93.4)	0	19 (6.6)
Kanamycin	Km	8-64	281 (98.0)	1 (0.4)	5 (1.7)
Nalidixic Acid	Na	0.5-32	279 (97.2)	0	8 (2.8)
Sulfisoxazole	Su	16-256	93 (32.4)	0	194 (67.6)
Streptomycin	S	32-64	100 (34.8)	0	187 (65.2)
Tetracycline	T	4-32	73 (25.4)	2 (0.7)	212 (73.9)
Ceftiofur	Ce	0.12-8	275 (95.8)	4 (1.4)	8 (2.8)



Strain ID	Farm ID		Stage	Sample Type	Resistant to[a]	Visit No.	
1918	Derby	B	Dry Sow	Pen	ASSu(Am)	2	
784	Derby	E	Finisher	Pen	ASSuT	1	
790	Derby	E	Finisher	Pen	ASSuT	1	
810	Derby	E	Gilts	Pen	ASSuT	1	
2158	Derby	C	Dry Sow	Pen	SSuT	2	
2531	Derby	E	Dry Sow	Water	ASSuT	2	
2586	Derby	E	Finisher	Water	ASSuT	2	
2587	Derby	E	Finisher	Water Drinker	ASSuT	2	
2589	Derby	E	Finisher	Trough	ASSuT	2	
2590	Derby	E	Finisher	Pen	ASSuT	2	
2591	Derby	E	Finisher	Faecal	ASSuT	2	
2599	Derby	E	Finisher	Water Drinker	ASSuTTmAzt	2	A
2601	Derby	E	Finisher	Trough	ASSuT	2	
2578	Derby	E	2nd Stage Weaners	Water	ASSuT	2	
2603	Derby	E	Finisher	Faecal	ASSuT	2	
2602	Derby	E	Finisher	Pen	ASSuT	2	
2575	Derby	E	2nd Stage Weaners	Trough	ACSSuT	2	
2576	Derby	E	2nd Stage Weaners	Pen	ACSSuTAzT	2	
2577	Derby	E	2nd Stage Weaners	Faecal	ASSuT	2	
2597	Derby	E	Finisher	Faecal	A(C)SSuT	2	
2598	Derby	E	Finisher	Water	ASSuT	2	B
2607	Derby	E	Finisher	Trough	ASSuT	2	
2623	Derby	E	Gilts	Faecal	ASSuT	2	
2608	Derby	E	Finisher	Pen	ASTTm	2	
2616	Derby	E	Gilts	Pen	ASSuT(Ax)	2	
2617	Derby	E	Gilts	Faecal	ASSuTNa	2	
2618	Derby	E	Gilts	Water	ASSuT	2	
2621	Derby	E	Gilts	Trough	ASSuT	2	C
2622	Derby	E	Gilts	Pen	ASSuT	2	
778	Derby	E	Finisher	Pen	ASSuT	1	
755	Derby	E	1st Stage Weaners	Water Drinker	ASSuT	1	
774	Derby	E	Finisher	Water	ASSuT	1	
2535	Derby	E	Dry Sow	Pen	ASSuTAzT	2	
580	Derby	D	Dry Sow	Feed	ASSuT	1	
693	Derby	D	Farrowing	Trough	SSuT	1	
696	Derby	D	Farrowing	Water	SSuT	1	
540	Derby	C	Gilts	Pen	SSuT	1	
547	Derby	C	Gilts	Faecal	SSuT	1	
551	Derby	C	Gilts	Trough	SSuT	1	
3262	Derby	I	Dry Sow	Trough	Susceptible(F)	2	
3269	Derby	I	Dry Sow	Pen	SuTTmAmAzt(F)	2	
3266	Derby	I	Dry Sow	Water Drinker	Susceptible(F)	2	
3267	Derby	I	Dry Sow	Feed	Susceptible(F)	2	
3255	Derby	I	Dry Sow	Feed	Susceptible(F)	2	
3256	Derby	I	Dry Sow	Trough	Susceptible(F)	2	
3257	Derby	I	Dry Sow	Pen	T(CF)	2	
3259	Derby	I	Dry Sow	Water	Susceptible(CeF)	2	
3260	Derby	I	Dry Sow	Water Drinker	Ce(F)	2	
3261	Derby	I	Dry Sow	Water Drinker	Susceptible(F)	2	
3264	Derby	I	Dry Sow	Faecal	S(F)	2	
3268	Derby	I	Dry Sow	Trough	Susceptible(F)	2	
3274	Derby	I	Dry Sow	Trough	Susceptible(F)	2	
3275	Derby	I	Dry Sow	Pen	Susceptible	2	D
3276	Derby	I	Dry Sow	Faecal	Susceptible(F)	2	
3280	Derby	I	Dry Sow	Trough	Susceptible(F)	2	
3375	Derby	I	Gilts	Faecal	Susceptible(F)	2	
3381	Derby	I	Gilts	Faecal	(F)	2	
3386	Derby	I	Gilts	Pen	Susceptible(F)	2	
3387	Derby	I	Gilts	Faecal	Susceptible(F)	2	
3388	Derby	I	Gilts	Water	Susceptible(F)	2	
3389	Derby	I	Gilts	Water Drinker	Susceptible(F)	2	
3392	Derby	I	Gilts	Pen	Ce(F)	2	
3281	Derby	I	Dry Sow	Pen	Susceptible(F)	2	
3637	Derby	J	Finisher	Trough	Su(F)	2	
3638	Derby	J	Finisher	Pen	ACSSuTTmFCpGmKmNa.	2	
2581	Derby	E	2nd Stage Weaners	Trough	ASSuT	2	
2582	Derby	E	2nd Stage Weaners	Pen	ASSuT	2	
2579	Derby	E	2nd Stage Weaners	Water Drinker	ASSuT	2	
2585	Derby	E	Finisher	Faecal	ASSuT	2	
793	Derby	E	Gilts	Faecal	ASSuT	1	
798	Derby	E	Gilts	Faecal	ASSuT	1	
741	Derby	E	1st Stage Weaners	Water Drinker	SSuTTmNaCeAzt	1	
785	Derby	E	Finisher	Faecal	ASSuTTm	1	
3102	Derby	H	2nd Stage Weaners	Water	Susceptible	2	
780	Derby	E	Finisher	Water	ASSuT(AmCe)	1	
781	Derby	E	Finisher	Water Drinker	ASSuT(Am)	1	
783	Derby	E	Finisher	Trough	ASSuT(AmCe)	1	
1097	Derby	G	Gilts	Water Drinker	ASSuT	1	

Figure 2.1: PFGE dendrogram of *Salmonella* Derby isolates (restricted with *Xba*I) recovered from 10 Irish commercial pig farms with a historically high *Salmonella* sero-prevalence. Percent similarity was calculated by the Dice coefficient using 1% band tolerance and 1% optimization settings and cluster analysis was performed by the unweighted pair group method with arithmetic mean (UPGMA) using Bionumerics version 5.10 software. Four clusters (labelled A, B, C and D) were identified with >80% similarity. [a] See Table 2 for abbreviations for antimicrobials. Isolates were either designated as "fully susceptible", "intermediate" (antimicrobial in parentheses) or if resistance to any antimicrobial was found, this was indicated by using the abbreviation for the antimicrobial to which the strain was resistant.



Strain ID	Farm ID	Stage	Sample Type	Resistant to	Visit No.	
166	Anatum	A	1st Stage Weaners	Water	SSuTmKmNaCe	1	
1714	Anatum	A	Dry Sow	Faecal	STNa	2	
1159	Dublin	H	1st Stage Weaners	Water	Susceptible	1	
3102	Dublin	H	2nd Stage Weaners	Water	Susceptible	2	
3108	Dublin	H	2nd Stage Weaners	Water	T	2	
3143	Dublin	H	Gilts	Water	Susceptible	2	
1758	Orion	A	2nd Stage Weaners	Trough	Susceptible	2	
3254	Dublin	I	Dry Sow	Water	Susceptible(F)	2	
1985	Stanley	B	2nd Stage Weaners	Trough	Susceptible	2	
3447	Tennessee	J	Dry Sow	Feed	SSu	2	
3598	Tennessee	J	Finisher	Water	Susceptible(C)	2	
3426	Tennessee	J	Dry Sow	Faecal	SSu	2	
3149	Dublin	H	Gilts	Water	Susceptible(T)	2	
712	Infantis	E	Dry Sow	Pen	Susceptible	1	
718	Infantis	E	Dry Sow	Pen	SSuF	1	
724	Infantis	E	Dry Sow	Pen	Susceptible	1	
1750	Infantis	A	1st Stage Weaners	Trough	ASSuT	2	
772	Infantis	E	Finisher	Pen	SuCe	1	E
786	Infantis	E	Finisher	Water	SSu	1	E
789	Infantis	E	Finisher	Trough	Susceptible	1	E
799	Infantis	E	Gilts	Faecal	Susceptible	1	E
801	Infantis	E	Gilts	Water Drinker	Susceptible	1	E
804	Infantis	E	Gilts	Pen	SuT	1	E
832	Infantis	E	Farrowing	Water	Susceptible	1	E
2592	Infantis	E	Finisher	Water	ASSuT	2	E
2593	Infantis	E	Finisher	Water Drinker	A(C)SSuTTmCp(KmAzt)	2	
2596	Infantis	E	Finisher	Pen	T	2	
2595	Infantis	E	Finisher	Trough	Susceptible	2	
3373	London	I	Gilts	Trough	Susceptible	2	
3374	London	I	Gilts	Pen	Susceptible	2	
3661	London	J	Finisher	Trough	(A)TAm	2	
3342	London	I	Finisher	Pen	Susceptible	2	
3369	London	I	Gilts	Faecal	Su	2	
3263	London	I	Dry Sow	Trough	Susceptible	2	
3321	London	I	2nd Stage Weaners	Trough	TAmAzT	2	F
3337	London	I	Finisher	Faecal	Susceptible	2	F
3354	London	I	Finisher	Pen	Susceptible	2	F
3360	London	I	Finisher	Pen	Susceptible	2	F
3365	London	I	Finisher	Trough	Susceptible	2	F
1262	London	I	Dry Sow	Water	SSuAmF	1	
1271	London	I	Dry Sow	Trough	T	1	
3380	London	I	Gilts	Pen	Susceptible	2	
3558	London	J	Gilts	Feed Bin	Susceptible	2	
3647	London	J	Finisher	Water Drinker	Susceptible	2	
3376	London	I	Gilts	Water	Susceptible	2	
3657	London	J	Finisher	Faecal	Susceptible(T)	2	

Figure 2.2: PFGE dendrogram of non-*S.* Typhimurium and non-monophasic *Salmonella* isolates (excluding *S.* Derby) (restricted with *Xba*I) recovered from 10 Irish commercial pig farms with a historically high *Salmonella* sero-prevalence. Percent similarity was calculated by the Dice coefficient using 1% band tolerance and 1% optimization settings and cluster analysis was performed by the unweighted pair group method with arithmetic mean (UPGMA) using Bionumerics version 5.10 software. Two clusters (labelled E and F) were identified with >80% similarity. [a] See Table 2 for abbreviations for antimicrobials. Isolates were either designated as "fully susceptible", "intermediate" (antimicrobial in parentheses) or if resistance to any antimicrobial was found, this was indicated by using the abbreviation for the antimicrobial to which the strain was resistant.

Table 2.5: Characterisation of *S.* Typhimurium, *S.* Typhimurium Copenhagen and monophasic variants of *S.* Typhimurium (1,4,[5],12:i) recovered from 10 Irish commercial pig farms by MLVA profiling and the occurrence of these isolates according to sample categories and on-farm production stages. Two isolates recovered in a previous study from pig feed/feed ingredients sampled from three commercial feed mills (B, C and D) and one home compounder (E) supplying four of these farms (farms A, B, C and F, respectively) are also included.

	[a]	No. of isolates (Farm ID [A-J] and visit [1 or 2])[d]										
		Sample category [c]				Production Stage [e]						Feed mill (Mill ID)[f]
Cluster [b] (No. of isolates)	MLVA profile	Faeces	Environment	Feed	Water	Farrowing	1st Stage Weaner	2nd Stage Weaner	Gilts	Dry Sow	Finisher	
Monophasic Isolates												
A (2)	NA-NA-NA-NA-NA		1(J2)	1(D1)			1(J2)		1(D1)			
B (32)	3-14-10-NA-NA		1(B1)					1(B1)				
	3-14-9-NA-0211	1(B2)	1(H2)				2(B2,H2)					
	3-14-10-NA-0211	13(B1,B2)	10(B1,B2)	2(B1,B2)	4(B1,B2)		18(B1,B2)	11(B1,B2)			1(B1)	
C (4)	3-13-16-NA-0211	1(J2)									1(J2)	3(B,D)
D (5)	3-13-9-NA-0211	1(B2)									1(B2)	
	3-12-10-NA-0211	3(A1)			1(B1)	1(A1)	2(A1,B1)	1(A1)				
E (42)	3-11-9-NA-0211	16(A1,A2)	12(A2,G2)	1(A2)	3(A1,G2)		7(A1,A2)	14(A1,A2)	2(G2)		9(A1,A2,G2)	2(C,E)
	3-11-9-NA-0212	1(A2)									1(A2)	
	3-11-10-NA-0211	4(A1,A2)	1(A1)	1(A1)	1(A1)		2(A1,A2)	4(A1,A2)			1(A1)	
F (6)	3-13-10-NA-0211	4(J1,J2)	1(J1)		1(J1)		3(J1)	3(J2)				
G (6)	3-15-10-NA-0211	2(B1)	3(B1,B2)		1(B2)			2(B1)			4(B1,B2)	
Typhimurium												
H (6)	4-11-17-8-0111	4(G2)	1(G2)		1(G2)				4(G2)		2(G2)	
I (11)	4-11-17-9-0111	1(G2)					1(G2)					
	4-11-16-8-0111	6(G1, G2)	4(G1, G2)					5(G1, G2)	1(G1)		4(G1,G2)	
J (2)	3-15-7-12-0311	1(D1)				1(D1)						
	3-15-6-12-0311			1(D2)		1(D2)						
K (6)	4-12-17-8-0111	1(G1)		1(G1)					2(G1)			
	4-13-17-8-0111		2(I2)		2(I2)				1(I2)		3(I2)	
Typhimurium Copenhagen												
L (2)	4-11-14-8-0111	1G2)		1G2)					1(G2)		1(G2)	

[a] Using the five loci ST9-ST5-ST6-STTR10-ST3 (Lindestadt et al., 2004)
[b]The MLVA profile was used for cluster analysis using BioNumerics v5.10 software. When deviations from the MLVA profile were minimal, a loss or gain in a repeat at the contingency locus was observed; losses and gains in one repeat at loci are more likely to occur in related isolates. Using this rule of thumb the isolates were assigned to 12 clusters [A-L; a cluster is when five or more isolates had the same MLVA profile. However, some clusters (i.e. those containing feed isolates) had less than 5 isolates].
[c] Faecal samples include composite faecal samples and pen swabs for all production stages across the 10 farms. Environmental samples include swabs taken from water drinkers, feed troughs and feed bins for all production stages across the 10 farms. Feed samples include feed taken from feed bins, hoppers and troughs within pens for all production stages across the10 farms. Water samples include water taken from header tanks, nipple drinkers and troughs within pens for all production stages across the 10 farms.
[d] Isolates were recovered from farms A-J during two visits over a 15-month period
[e] Stage of production from which the isolates were recovered.
[f] Two isolates recovered in a previous study (Burns et al., 2015) from pig feed/feed ingredients sampled from feed mills B, C, D and one home compounder E supplying farms A, C, F and B, respectively.

Chapter 3

Salmonella occurrence and *Enterobacteriaceae* counts in pig feed ingredients and compound feed from feed mills in Ireland

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3.1 Abstract

The purpose of this study was to assess the occurrence of non-typhoidal *Salmonellae* and Enterobacteriaceae counts in raw ingredients and compound feeds sampled from feed mills manufacturing pig diets. Between November 2012 and September 2013, feed ingredients (n=340) and compound pig feed (n=313) samples were collected from five commercial feed mills and one home compounder at various locations throughout Ireland. Feed ingredients included cereals, vegetable protein sources and by-products of oil extraction and ethanol production. The compound feeds included meal and pelleted feed for all stages of pig production. Samples were analysed for *Salmonella* using standard enrichment procedures. Recovered isolates were serotyped, characterised for antibiotic resistance and subtyped by multi locus variance analysis (MLVA). Total *Enterobacteriaceae* counts were also performed. *Salmonella* was recovered from 2/338 (0.6%) ingredients (wheat and soybean meal), at two of the six mills. *Salmonella* was also detected in 3/317 (0.95%) compound feeds including pelleted feed which undergoes heat treatment. All isolates recovered from feed ingredient and compound feed samples were verified as *Salmonella enterica* subsp. *enterica* serotype (4,[5],12:i:-) that lack the expression of flagellar Phase 2 antigens representing monophasic variants of *Salmonella* Typhimurium (4,[5],12:i:-). Isolates exhibited resistance to between two and seven antimicrobials. Two distinct MLVA profiles were observed, with the same profile recovered from both feed and ingredients, although these did not originate at the same mill. There was no relationship between the occurrence of *Salmonella* and a high *Enterobacteriaceae* counts but it was shown that *Enterobacteriaceae* counts were significantly lower in pelleted feed (heat treated) than in meal (no heat treatment)and that *Enterobacteriaceae* counts would be very useful indicator in HACCP

programme. Overall, although the prevalence of *Salmonella* in pig feed and feed ingredients in the present study was low, even minor *Salmonella* contamination in feed has the potential to affect many herds and may subsequently cause human infection. Furthermore, the recovery of a recently emerged serovar with multi-antibiotic resistance is a potential cause for concern.

3.2 Introduction

Non-typhoidal *Salmonellae* can colonise a wide range of hosts including all the major livestock species (poultry, cattle, and pigs), often asymptomatically, potentially leading to contamination of meat and other food products (Stevens et al. 2009). Following a "farm-to-fork" model, animal feed is at the beginning of the food safety chain. Therefore, the presence of *Salmonella* in animal feed or feed ingredients at the feed mill or on-farm is a cause for public health concern. This is evidenced by a number of incidences where animal infection has been traced back to contaminated animal feed. For example, Österberg et al. (2006) established that contaminated feed was the cause of an outbreak of *Salmonella* Cubana on a number of Swedish pig farms. Furthermore, Molla et al. (2010) found genotypically related and in some cases clonal *Salmonella* strains in commercially processed pig feed and pig faecal samples.

A number of different feed ingredients may potentially harbour pathogenic micro-organisms including non-typhoidal *Salmonellae*. Historically, a number of studies have shown the presence of *Salmonella* in feed ingredients of animal origin (e.g. rendered animal by-products) (Clise and Swecker, 1965; Franco, 2005); however, such ingredients are no longer an issue following their ban in animal feed in the European Union (EU) in 2001 in the aftermath of the Bovine Spongiform Encephalopathy (BSE) crisis (Commission Regulation (EC) No 163/2009). Exceptions have been made for the use of certain animal protein sources including fish meal, milk powders, certain blood products and dicalcium phosphate by-products (e.g. from the production of gelatin) as feed for monogastric animals (Commission Regulation (EC) No 1292/2005). However, these ingredients are not

without risk, as evidenced by the reported introduction of *S.* Agona to the United States (US) food chain via imported Peruvian fish meal, as quoted by Clark et al. 1973.

However, the risk of *Salmonella* contamination of pig feed from ingredients of animal origin may not be an issue, as the protein-rich ingredients currently used to formulate pig diets are principally of vegetable origin. Any ingredient of vegetable origin may become contaminated with *Salmonella* from contact with infected or carrier wildlife or production animals during storage or transit and/or from the use of manure or sludge as fertilizers on the growing crop. However, the risk is greater with imported ingredients as they may originate in countries with different regulations and there is an opportunity for contamination during transit. The Republic of Ireland relies on importing a much higher proportion of its animal feed requirement compared to other EU countries. In 2014, Ireland was importing 65% of its requirements, with ~3 million tonnes of cereals being imported annually, ~55% of which comes from countries outside the EU (DAFM, 2015). The EU in 2014 was 35% deficient in its requirement for protein for animal feed, so third-country imports are unavoidable (Popp et al. 2013, DAFM, 2015). In the EU, these are largely imported in the form of soybean from North and South America (de Visser et al. 2014). The contamination of cereals with *Salmonella* was estimated to range between 0.2 and 0.6% in 2012 in a study by the European Food Safety Authority (EFSA, 2014). This is much lower than for feed ingredients such as soybean meal (3.2-6.7%) and rapeseed (6.8%) which are by-products from other processing operations (EFSA, 2008). In one surveillance study, *Salmonella* was isolated from

14.6% of soybean meal consignments and 10% of rapeseed meal samples (Wierup and Haggblom, 2010).

The reported incidence of *Salmonella* in compound animal feed is generally low and when present, prevalence ranges on average from 0.6 to 1.7% (EFSA, 2008). It is also considered that the reported incidence in both feed ingredients and compound feed is probably lower than the true incidence due to under-reporting, sub optimal sampling procedures and for other reasons such as *Salmonella* detection methods may not offer all *Salmonella* serotypes an equal chance of isolation (Jones, 2011), especially in samples where multiple serotypes are present (De Busser et al. 2013a). A comprehensive sampling plan is therefore required for the monitoring of *Salmonella* in animal feed, as *Salmonella,* when present, is usually in low numbers and unevenly distributed. However, even low numbers of *Salmonella* may be sufficient to cause infection (Finn et al. 2013). This is particularly true for feeds of high fat content in which *Salmonella* can be protected from host gastric defence mechanisms (Jones et al. 1982). *Salmonella,* if present in the feed, also has the potential to multiply in warm, moist conditions, either at the feed mill or on the farm (Davies and Hinton, 2000; Hilbert et al. 2012).

As food-producing animals are the primary source of *Salmonella* infections in humans (Forshell and Wierup, 2006), it follows that contamination of animal feed with this pathogen should not be overlooked as an important origin of foodborne illness and outbreaks. The same *Salmonella* serotypes have been recovered from commercial pig feed and pigs sampled on the same farm (Burns et al. 2013).

However, it remains unclear whether the feed contamination arose on-farm or whether the commercial feed introduced onto the farm was already contaminated.

The total number of *Enterobacteriaceae* can serve as a hygiene indicator in food and feed. *Enterobacteriaceae* have the advantage of being enumerated inexpensively and easily and are useful for quantifying the hygienic performance of a production process, when particular pathogens or spoilage organisms might be difficult to detect (Jordan et al. 2007). In the EU there is legislation (EU 2073/2005) setting microbial process hygiene criteria for *Enterobacteriaceae* counts on foods including carcasses, milk and dairy products, and eggs. Equally, the determination of *Enterobacteriaceae* counts could be used to assess and subsequently improve mill hygiene and the quality of animal feeds (Jones and Richardson, 2004, Veldman et al. 1995). However the relevance of *Enterobacteriaceae* in feed should, however, be assessed and interpreted carefully and recognition given that there is conflicting studies on the correlation between *Enterobacteriaceae* count and the presence of *Salmonella* in feed. Jones and Richardson (2004) reported that poultry feed samples, meal and pellets, contaminated with *Salmonella* contained significantly higher *Enterobacteriaceae* counts. A study by Veldman et al. (1995), isolated predominantly thermotrophic *Enterobacteriaceae* from feedstuffs and found them to be useful markers of the rate of contamination with *salmonellae* and of the efficiency of decontamination of the feedstuffs by pelletisation. Whereas a study by Cox et al. (1983) showed no correlation between *Enterobacteriaceae* and *Salmonella*. Further studies showing the benefit of using as a hygiene indicator in feed therefore would be of benefit.

Therefore, the objective of this study was to assess the occurrence and characteristics of *Salmonella* in a range of feed ingredients and compound feeds sampled from feed mills supplying high *Salmonella* sero-prevalent pig farms in the Republic of Ireland, where on-farm bacteriology had confirmed *Salmonella* presence in both pigs and feed (Burns et al. 2013). *Enterobacteriaceae* counts were also performed and these may provide valuable data that could be used as a baseline for assessment of the hygienic standard of feed, which is currently rare in other studies.

3.3 Material and Methods

3.3.1 Sample Collection

Samples of feed ingredients and compound pig feed were collected monthly from five commercial feed mills (Mills A-D & F) and one home compounder (Mill E). All mills were operating under hazard analysis and critical control points (HACCP) quality assurance schemes and were all producing both meal and pelleted feed from a wide variety of ingredients. In all mills, pelleting was preceded by a steam conditioning step, whereas no heat treatment was applied to meal feed. Samples from each feed mill were taken over a 6 month period between November 2012 and September 2013. A total of 338 raw ingredients and 317 compound feed samples were obtained. The feed ingredients included cereals, vegetable protein ingredients and by-products of oil extraction and ethanol production and were the ingredients used in pig diet formulation at the time of the study. Compound feeds included meal and pelleted feed for all stages of pig production. For pelleted feed, pelleting was preceded by a steam conditioning step, whereas no heat treatment whatsoever was applied to meal feed. Feed ingredients were sampled at mill intakes from every ingredient load and finished feeds were sampled from every batch (from storage bins at the feed mills). All samples were composite samples taken by mill personnel in accordance with Commission Regulation (EC) No 152/2009. Sub samples (~150 g) were taken aseptically into sterile containers and submitted to the laboratory on a monthly basis, where they were refrigerated until analysis (within 24 h).

3.3.2 Salmonella isolation

The presence/absence of *Salmonella* in 10 g samples taken from the composite feed samples (150 g) was determined according to standard selective enrichment procedures (EN ISO 6579:2002/Cor 1:2004) with modified brilliant green agar (BGA; Oxoid, Basingstoke, Hampshire, UK) used for additional selective plating. Presumptive *Salmonella* isolates (identified based on the results of biochemical tests) were tested using a *Salmonella* latex agglutination kit (Oxoid, Basingstoke, Hampshire, UK).

3.3.*3 Confirmation of Salmonella isolates by Real-Time Polymerase Chain Reaction (PCR)*

DNA was extracted from isolates identified as *Salmonella* by the latex agglutination kit using a DNAeasy Tissue Kit for Gram-negative bacteria (Qiagen, Crawley, UK) according to manufacturer's instructions. The primer and *Taq*Man probe sequences used for the confirmation of *Salmonella* spp. were based on those of McCabe et al. (2011). The primer and probe sequences and labels for the *hil*A and pUC19 were as described by Fricker et al. (2007). All primers and probes were manufactured by Sigma-Aldrich (St. Louis, USA). Internal Amplification Control (IAC) template DNA was isolated from *E. coli* using a QIAprep Spin Miniprep kit (Qiagen) according to manufacturer's instructions. PCR amplification and detection were performed using a Roche LightCycler 480 (Roche Diagnostics Limited, Burgess Hill, West Sussex, United Kingdom) using LC480 probe master mix kit (Roche Diagnostics Limited). Isolates confirmed as *Salmonella* were grown on standard plate count agar (Oxoid) overnight at 37°C and a loopful of colonies was suspended in 0.5 ml 80 % glycerol, added to cryoprotectant beads and stored at -80°C .

3.3.4 Serotyping and Antimicrobial susceptibility testing of Salmonella isolates

All confirmed *Salmonella* isolates were serotyped according to the White-Kauffmann-Le Minor classification scheme (Grimont et al. 2007). *Salmonella enterica* subsp. *enterica* serovar (4,[5],12:i:-) that lack expression of the second-phase flagellar antigens were designated as monophasic variants of *S.* Typhimurium. Antimicrobial susceptibility testing was performed according to the broth dilution method of the Clinical and Laboratory Standards Institute (formerly NCCLS) (CLSI, 2008) using a broth microdilution assay (Sensititre, TREK Diagnostic Systems Inc., Sussex, England). The panel of antimicrobials included Azithromycin (Azt), Ampicillin (A), Amoxicillin/Clavulanic acid (Am), Ceftriaxone (Ax), Chloramphenicol (C), Ciprofloxacin (Cp), Trimethoprim/Sulfamethoxazole (Tm), Cefoxitin (F), Gentamicin (Gm), Kanamycin (Km), Nalidixic acid (Na), Sulfisoxazole (Su), Streptomycin (S), Tetracycline (T) and Ceftiofur (Ce). The cut-off values (mg/l) were as specified in EU Commission Decision 2007/407. *Escherichia coli* ATCC 25922 was used as a control. Isolates were either designated as "fully susceptible", "intermediate" or if resistance to any antimicrobial was found, this was indicated by using the abbreviation for the antimicrobial to which the strain was resistant.

3.3.5 Confirmation of Monophasic Salmonella Typhimurium by Multiplex Real-Time PCR

All isolates identified phenotypically as monophasic *S. enterica* subsp. *enterica* serovar Typhimurium 4,[5],12:i:− were tested by multiplex PCR with oligonucleotides specific to the antigenic genes *fliC* (H:i antigen), *fljB* (H:1,2

antigen) and *fljB*/IS200 as recommended by EFSA (2010). The primers used were those outlined by Prendergast et al. (2013) and were obtained from Eurofins MWG Operon, Dublin, Ireland. The probes were *Taq*man hydrolysis probes (TIB MolBiol Berlin, Germany), also as outlined by Prendergast et al. (2013) except that different fluorescent dyes were used. The primer sequences, probes and target genes are listed in Table 1. PCR amplification was performed in a final volume of 10 µl containing 9 µl master mix and 1 µl template DNA. The master mix consisted of 2× Lightcycler 480 probe master, PCR-grade water and optimized concentrations of the primers and probes. PCR amplification was performed in a Roche Lightcycler 480 96-well instrument with an initial denaturation step of 95 °C for 10 min followed by 30 cycles of 95 °C for 10 s, 60 °C for 1 min, 72 °C for 1 sec, followed by an extension step of 72 °C for 10 min. Fluorescence signals were detected in 6FAM (fliB/IS200), LC670 (fliC) and YAK (fljB.1, 2) channels. The reaction was considered positive when Ct values were ≤30.

3.3.6 Enumeration of Salmonella in Salmonella-positive samples by most probable number procedure

Salmonella enumeration was conducted on *Salmonella*-positive feed and ingredient samples using a three-tube most probable number (MPN) technique based on standard methods (ISO 6579:2002/Cor 1:2004 and ISO 7218-2007). Testing was performed on 10 g samples using buffered peptone water (BPW; Oxoid) as a pre-enrichment broth, followed by Rappaport-Vassiliadis broth **(**RVS; Oxoid) for selective enrichment and BGA and xylose lysine deoxycholate agar (XLD; Oxoid) as the selective media. The limit of detection of this assay, based on the dilutions used, was 0.30 MPN *Salmonella*/g.

3.3.7 Multilocus Variable Number Tandem Repeat Analysis (MLVA)

All *Salmonella* isolates confirmed by serotyping and multiplex PCR as *S.* Typhimurium and/ or monophasic variants of *S.* Typhimurium (1,4[5], 12:i) were subjected to multilocus variable number tandem repeat analysis (MLVA) using capillary electrophoresis methods as described previously by Prendergast et al. (2011). The variable number tandem repeat (VNTR) loci selected, along with the primers and dyes used, were as outlined by Lindstedt et al. (2004) and are listed in Table 2. Essentially, the loci were amplified in separate PCRs by using fluorescent primers. Raw fragment lengths for each locus were manually discarded using a minimum threshold of ± 3 bp to distinguish alleles. *S.* Typhimurium LT2 ATCC 29946 was used as a positive control during the analysis of each batch of samples on the ABI 3500 genetic analyser (Applied Biosystems, Foster City, California, US). Each peak was identified according to colour and size using GeneMapper (version 4.1) software (Applied Biosystems) and a set of 5 alleles for each isolate was defined as the MLVA profile. MLVA profiles were assigned based on the fragment size amplified from each locus, with 'NA' used to denote a locus not present.

3.3.8 Enterobacteriaceae counts in feed and ingredients

Enterobacteriaceae were enumerated in feed and feed ingredient samples using a plate count method according to standard microbiological procedures (EN ISO 7218-3:2007) which were modified to enhance the recovery of injured or stressed cells. Buffered peptone water (90 ml) was added to 10 g of the sample and mixed thoroughly until evenly suspended and incubated at 37°C for 1 h. To aid the emulsification of oily or waxy feed ingredient samples, Tween 80 (Sigma-Aldrich, Ireland) was added to the BPW at a rate of 1g/l. Tenfold serial dilutions were then

performed in maximum recovery diluent (Oxoid) and 1 ml of relevant dilutions was pour-plated in duplicate on violet red bile glucose agar (VRBGA; Oxoid). Each plate was then overlaid with VRBGA. Plates were incubated at 37°C for 24±2h.

3.3.9 Statistical analysis

Enterobacteriaceae counts were log-transformed to approximate normality prior to statistical analysis. They were analysed using the mixed procedure of the Statistical Analysis System version 9.3 (SAS Institute Inc., Cary, NC, USA). When analysing the effect of sample type (feed ingredient, compound feed) and feed mill on *Enterobacteriaceae* count, sample type, mill and their interaction were included in the model as fixed effects. When analysing the effect of diet form (meal, pellet) and mill on *Enterobacteriaceae* count, Mill E was omitted from the dataset as only one form of diet (meal) was produced in that mill. Diet form, feed mill and their interaction were included in the model as fixed effects. When analysing the effect of diet type (dry sow, lactation, finisher, and weaner) and mill on *Enterobacteriaceae* count, diet type, feed mill and their interaction were included in the model as fixed effects. Means are reported as least squares means ± their standard errors (SE) and the slice option was used to determine significance for simple main effects. Significance was reported for $P < 0.05$ and tendencies towards significance were reported for $0.05 < P < 0.10$. The individual compound diet / feed ingredient sample was considered the experimental unit in all cases

3.4. Results

As can be seen in Table 3, two of the six feed mills were found to be exposed to *Salmonella*-contaminated feed ingredients. Another two mills produced *Salmonella*-contaminated feed (including pelleted feed which undergoes steam treatment prior to pelleting). The prevalence of *Salmonella* was 0.6% (2/338) and 0.95% (3/317) in feed ingredients and compound feed, respectively. *Salmonella* contamination was found in two feed ingredients; wheat (n=1, Mill B) and soybean meal (n=1, Mill E). It was also found in compounded dry sow meal (n=1, Mill D), compounded dry sow pellets (n=1, Mill D) and in a compounded finisher meal used to balance liquid whey (Mill C; Table 3). The proportion of compound meal feed samples contaminated with *Salmonella* was 1.59% whereas only 0.54% of pelleted diets were contaminated. All of the *Salmonella*-positive samples (both feed and ingredients) had an MPN/g of <0.30 i.e. levels of contamination were below the limit of quantification (lowest quantity that can be distinguished within a 95% confidence limit).

All five *Salmonella* isolates recovered from the feed ingredient and compound feed samples were identified as monophasic variants of Typhimurium (4, [5] 12: i :-) by serotyping and multiplex PCR. All isolates were susceptible to azithromycin, amoxicillin/clavulanic acid, cefoxitin, ceftriaxone, kanamycin, and nalidixic acid. However, the isolates exhibited resistance to between two and seven antimicrobials with most frequent resistance to tetracycline (5/5 isolates), streptomycin (4/5), gentamicin (3/5), ampicillin (3/5), chloramphenicol (3/5), sulfisoxazole (3/5), trimethoprim/sulfamethoxazole (2/5), ciprofloxacin (2/5) and ceftiofur (2/5) (Table 3).

Five VNTR loci were used to genotype the five feed- and feed ingredient-derived *Salmonella* isolates. Two different MLVA profiles were identified – allele strings 3-13-16-NA-0211, designated Type A and 3-11-9-NA-0211, designated Type B (Table 3). The VNTR loci STTR-9 and STTR-3 were very conserved showing no degree of diversity. Likewise, the locus STTR10 located on the *Salmonella* virulence plasmid was absent in all five isolates, indicating possible monophasic variants of the virulence plasmid. The most variation was noted in loci STTR5 (two different alleles) and STTR6 (two different alleles). The most prominent combination of alleles was allele string 3-13-16-NA-0211. Interestingly, *Salmonella* isolates with the same MLVA profile were recovered from ingredients and finished feed i.e. Type A was recovered from wheat as well as sow meal and pellets and Type B from soybean meal and finisher meal (Table 3).

When the feed ingredient samples were categorised according to *Enterobacteriaceae* counts (Table 4), the samples that harboured the highest counts included pollard and malt sprouts, with the majority of these samples in the >10,000 cfu/g category. These ingredients, together with barley, wheat and Lactofeed were also most frequently contaminated with *Enterobacteriaceae* (*Enterobacteriaceae* were detected in 100, 77, 83, 68 and 67% of malt sprout, pollard, barley, wheat and Lactofeed samples, respectively; Table 4). The majority of compound meal feed samples harboured counts in excess of 10,000 cfu/g of *Enterobacteriaceae*, regardless of the production stage, whereas *Enterobacteriaceae* were non-detectable in the majority of the pelleted feed samples (Table 4). In general, *Enterobacteriaceae* were detected in 92% of meal samples, while only 29% of pelleted feed harboured

Enterobacteriaceae (Table 4). Overall, 27% of all compound pig feed samples analysed fell into the >10,000 cfu/g *Enterobacteriaceae* category. However, a large proportion of the feed ingredients and compound feeds (46% of each) from which *Enterobacteriaceae* were recovered had contamination levels below 10 cfu/g.

The effect of diet form (meal or pellet) on *Enterobacteriaceae* counts in samples from the five commercial feed mills is shown in Table 5. There was a mill x feed form interaction ($P<0.01$). When samples from all of the mills were compared, the mean *Enterobacteriaceae* count was lower in pelleted diets than in meal diets ($P<0.001$), in agreement with the data shown in Table 4. However, it was also found that the mill from which samples were taken influenced the *Enterobacteriaceae* counts in compound diets ($P<0.001$).

The effect of mill and compound diet type (dry sow, lactation, finisher, weaner) on *Enterobacteriaceae* counts and their interaction is shown in Table 6. There was no mill x compound diet type interaction ($P>0.05$). *Enterobacteriaceae* counts were similar for all diet types. However, the mill from which samples were taken was once again found to influence *Enterobacteriaceae* counts in compound diets ($P<0.05$).

3.5. Discussion

Salmonella contamination was found in two feed ingredients (wheat and soya) and in compound dry sow meal and pellets and in compound finisher meal. Soya, as a by-product of oil extraction, is particularly prone to contamination by salmonellae that are endemic in processing plants (Morita et al. 2006). A study by Papadopoulou et al. (2009) showed that *Salmonella* was more commonly isolated from wheat than from any other feed ingredient. The overall recovery rate of *Salmonella* in the compound feed samples analysed in the present study was 0.95% which is much lower than reported in other studies i.e. 2.8% (Harris et al. 1997), 3.6%, (Molla et al. 2010), 23.5% (Fedorka-Cray et al. 1997), 43.0% (Hacking et al. 1978), 42.0% (Isa et al. 1963) and 2.3-58.8% (Davies et al. 1997). However, it should be noted that the samples tested by Harris et al. (1997) and Molla et al. (2010) were taken on-farm and from feed trucks supplying the mills.

As the critical control points would have varied for each feed manufacturing plant, the testing procedures applied in this study focused on the contamination status of composite/pooled (150 g) samples of incoming feed ingredients and outgoing compound feed. It could be argued that this study has some limitations with regard to its use of a 10 g sample instead of the standard 25 g sample (EN ISO 6579:2002/Cor 1:2004). However, studies by Funk et al. (2000) and Arnold et al. (2005) illustrate (using faecal samples) that the use of a pooled sample may be a valid approach for measuring the occurrence of *Salmonella* in feed ingredients and compound feed. For example, Funk et al. found that using a pooled faecal sample of 10 g in comparison to 25 g yielded only a 5% drop in relative sensitivity.

In a previous study of compound feed for pigs, *S.* Agona, *S.* Livingstone, *S.* Senftenberg and *S.* Anatum were found to be the most commonly detected serovars (EFSA, 2010a). It is of interest that all isolates recovered in the present study were monophasic Typhimurium. In the EU the occurrence of monophasic variant human cases has increased from 360 in 2007 to 5932 in 2012, making it the third most common serovar isolated from humans in Europe (EFSA, 2014). Furthermore, in previous studies, Typhimurium DT104 was found to be the dominant serovar in Irish pig herds (Duggan et al. 2010; McCarthy et al. 2013; Rowe et al. 2003). However, in a short period of time the penta-resistant DT104 has been replaced by the emerging monophasic variant 4,[5],12:i:- with resistance to ampicillin, streptomycin, sulphonamides and tetracyclines (National Salmonella, Shigella & Listeria reference laboratory, 2013; EFSA, 2010). Interestingly, this tetra-resistance pattern was observed in one compound feed isolate obtained in the present study and two other isolates (one each from a feed ingredient and compound feed) also had this profile together with resistance to three additional antibiotics. Various European studies have also documented this ASSuT profile from human, pig and pig meat isolates (Arguello et al. 2014; Hopkins et al. 2010; Prendergast et al. 2013). The increased antimicrobial resistance and higher than anticipated occurrence of monophasic variants observed in the present study and other European studies highlights its importance as an emerging feed and foodborne pathogen.

While monophasic variants have been found in animal feed recently (Bugarel et al. 2012; Wasyl and Hoszowski, 2012), they were only the sixth most common *Salmonella* serotype found in 2002–2009 in animal feed and feed ingredient samples analysed under U.S. Food and Drug Administration (FDA) *Salmonella* surveillance

programmes (Li et al. 2012). In general, the serotypes obtained from feed prior to its arrival on-farm do not usually correspond to those most frequently found in humans and animals (Li et al. 2012; Lo Fo Wong, 2001). Moreover, the MLVA profile of the monophasic variant found in feed and feed ingredients sampled at feed mills in this study was identical to that of the dominant serovar recovered from the pig herds supplied by these mills (Burns et al. 2013). Urgent control measures are therefore needed to reduce the spread of infection to humans via the food chain to prevent the possible pandemic spread of serovar 4,[5],12:i:- as occurred with *S.* Typhimurium DT104 during the 1990s.

Enterobacteriaceae are enteric bacteria that are used as indicators of faecal contamination. In the case of food and animal feed, their presence indicates poor hygiene, inadequate processing or post-process contamination. Studies have shown that *Enterobacteriaceae* counts tend to be higher in feed samples positive for *Salmonella* than in *Salmonella*-negative samples and suggest that *Enterobacteriaceae* counts may be a useful indicator to assess the likelihood of *Salmonella* contamination in feed (Jones and Richardson, 2004). This was not always the case in the present study, as only two of the five *Salmonella*-positive samples had high *Enterobacteriaceae* counts (4.59 and 4.62 Log_{10}cfu/g).

Despite this lack of an observed correlation between *Salmonella* and *Enterobacteriaceae* this study also set out to monitor *Enterobacteriaceae* counts as an indirect indicator of hygiene which has been proposed as a useful tool in feed mill production systems as part of a HACCP systems. (EFSA, 2008, Jones and Richardson, 2004, Gradel et al. 2003).

The results showed that *Enterobacteriaceae* counts did not differ for dry sow, lactation, finisher and weaner compound diets in the present study. This is perhaps not surprising since the feed ingredients used to formulate these diets are similar. However, *Enterobacteriaceae* counts were significantly lower in pelleted diets than in meal. This is to be expected, as the pelleting process employed in the study feed mills involved temperatures of 80-90°C and *Salmonella* and other *Enterobacteriaceae* should be completely eliminated by pelleting at temperatures exceeding 83°C (Hald et al. 2012). Maciorowski et al. (2004) previously observed 99% (2 log) reductions in *Salmonella* at this temperature and others have achieved 4 log reductions at 85°C for 90s at 15% moisture (Himathongkham et al. 1996). In general, pelleting systems have been reported to reduce *Salmonella* isolation rates from between 50 to 93% (Jones, 2011). Our data are in agreement with the findings of other studies; for example, Threlfall et al. (2003) found that 8.8% of meal feed samples but only 4.2% of pelleted feed samples were contaminated with *Salmonella*.

Salmonella isolates with the same MLVA profile (A) were recovered from ingredients and finished feed although the feed and ingredients did not originate in the same feed mill and this may be indicative of a previously acquired contamination from the same source, e.g. both mills probably imported soybean meal from the same source. From an epidemiological perspective a time factor must also be considered. The same MLVA type was recovered from both meal and pelleted feed from Mill D, indicating either that pelleting did not eliminate *Salmonella* or it may be indicative that post-process contamination occurred in the mill i.e. between different production

lines (pelleted/heat treated and meal/ non heat treated feed), especially as no *Salmonella* was found in their feed ingredients.

Successful reductions of *Salmonella* during pelleting are dependent on many factors i.e. the time of heat exposure, temperature obtained, and the moisture content (a_w) of the feed at pelleting. In addition, the conditions required to eliminate *Salmonella* are not always achieved in practice due to the high energy cost involved, the heat damage to vitamins and other nutrients and the adverse effect on the integrity of the pellets (De Busser et al. 2013b; Jones et al. 1995) and even when achieved may not be sufficient for ingredients with high contamination levels (Fedorka-Cray et al. 1997). The presence of *Salmonella* and *Enterobactericeae* in pelleted feed in the present study may also be due to post-process contamination. Wierup and Häggblom (2010) demonstrated that heat-treated feed may become re-contaminated at different points along the production line. Pelleting systems rely on steam addition to eliminate any pathogens present which adds moisture to pelleted feeds. This moisture is removed via pellet coolers; however, malfunctions in these systems may cause condensation to occur on the interior surfaces of the coolers (Jones, 2011). This increase in moisture within the pellet cooler may provide an environment capable of supporting the growth of *Enterobacteriaceae*, in particular *Salmonella*.

Contamination of feed at the feed mill is also associated with other factors, such as cross-contamination by dust, presence of vectors and poor hygiene conditions (EFSA, 2008). Contamination may also occur during storage at the feed mill (Davies and Wales, 2010). These factors are likely to have had an influence in the present study considering that *Enterobacteriaceae* counts and *Salmonella* prevalence were

higher in compound feed than in feed ingredients (even in meal feed, which is not heat-treated), indicating post-process contamination and/or proliferation of surviving cells. Furthermore, it was also interesting that the mill had a significant effect on *Enterobacteriaceae* counts, with the home compounder (Mill E) having particularly high *Enterobacteriaceae* counts and one of the commercial mills (Mill F) having particularly low counts. Lower *Enterobacteriaceae* counts are most likely attributed to better management practises, as similar treatment methods were used across all feed mills. Therefore, a further study is required to obtain information on specific practices at each feed mill. Environmental samples taken from various surfaces within the feed mills may also help to assess the origins of contamination.

3.5 Conclusions

Salmonella contamination of feed ingredients and compound feeds was observed in this comprehensive Irish feed mill study, albeit at a low prevalence. All of the *Salmonella* isolates recovered were monophasic variants of the serovar Typhimurium, confirming the occurrence of this serotype in new geographical settings. All exhibited some degree of antibiotic resistance, with some multi-resistant isolates found. The data from this study also indicate that a large proportion of the raw materials used for pig feed manufacture are contaminated with high levels of *Enterobacteriaceae* ($>10^4$ cfu/g). Thus, raw materials should be viewed as a critical control point for the entry of pathogenic bacteria into the feed and food chains. While pelleting reduced *Salmonella* prevalence and *Enterobacteriaceae* counts in compound feed, it did not completely eliminate contamination. This, together with the fact that compound feed often had higher *Enterobacteriaceae* counts than ingredients, suggests post-process contamination within the feed mills. Despite a low prevalence of *Salmonella* in pig feed and feed ingredients in the present study still has the potential to affect many herds and may subsequently cause human infection via consumption of contaminated pork. Furthermore, the recovery of an emergent serotype and multi-resistant isolates is a potential cause for concern. In addition, the *Enterobacteriaceae* data generated in the present study show their relevance for the assessment of hygienic standards of feed.

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3.9 Tables and Figures

Table 3.1: Oligonucleotide and *Taq*man probe sequences used in the multiplex real-time PCR used for confirmation of monophasic *Salmonella* Typhimurium (4,[5],12:i:-)

Target Locus	Description	Sequence (5′–3′)	Dye Emission Wavelength (nm)	Reference
*fli*C	Forward	ccc cgc tta		O' Regan et al. 2008
	Reverse	agc ggg ttt		
	*Taq*Man	LC670-taa	~670	
*flj*B 1,2	Forward	tgt tac tat		Muñoz et al. 2010
	Reverse	cag cag gca		
	*Taq*Man	YAK-cgc	~550	
*flj*B/IS200	Forward	gat ctg tcg		Prendergast et al. 2013
	Reverse	aac gct tgt		
	*Taq*Man	6FAM- tcg	~515	Prendergast et al. 2013

Table 3.2: PCR primers used for *S.* Typhimurium VNTR loci for MLVA typing.

Target Locus	Primer Name	Dye	Sequence (5′–3′)
STTR3	STTR3-F[a]	HEX	ccc cct aag ccc gat aat gg
	STTR3-R[b]	-	tga cgc cgt tgc tga agg taa taa
STTR5	STTR5-F	HEX	atg gcg agg cga gca gca gt
	STTR5-R	-	ggt cag gcc gaa tag cag gat
STTR6	STTR6-F	6FAM	tcg ggc atg cgt tga aa
	STTR6-R	-	ctg gtg ggg aga atg act gg
STTR9	STTR9-F	6FAM	aga ggc gct gcg att gac gat a
	STTR9-R	-	cat ttt cca cag cgg cag ttt ttc
STTR10pl	STTR10-F	TAM	cgg gcg cgg ctg gag tat ttg
	STTR10-R	-	gaa ggg gcc ggg cag aga cag c

[a]F = forward primer, which was fluorescently labelled with the dye indicated

[b]R = reverse primer, which was unlabelled

Table 3.3: Serotypes, antibiotic resistance profiles, and MLVA profiles of *Salmonella* isolated from pig feed and pig feed ingredients sampled at commercial feed mills (Mills A-D & F) and one home compounder (Mill E). *Enterobacteriaceae* counts (Log_{10}cfu/g) are also shown.

				MLVA No. of Repeats						
Sample Type	**Feed Mill [a]**	***Salmonella* Serotype [b]**	**Antibiotic Resistance Profile [c]**	**STTR9**	**STTR5**	**STTR6**	**STTR10**	**STTR3**	**MLVA Type**	***Enterobacteriaceae* Count (Log_{10}cfu/g)**
Feed Ingredients										
Wheat	B	4, 12:i:-	ACSSuTTmGm	3	13	16	NA	0211	A	<1.0
Soybean meal	E	4, 12:i:-	TGm	3	11	9	NA	0211	B	<1.0
Compound feed										
Finisher meal	C	4,[5],12:i	ASSuT	3	11	9	NA	0211	B	4.59
Dry sow meal	D	4, 12:i:-	ACSSuTTmGm	3	13	16	NA	0211	A	4.62
Dry sow pellets	D	4, 12:i:-	ACSTCpCe	3	13	16	NA	0211	A	<1.0

[a] Mills A and F are not shown as *Salmonella* was not isolated from these mills.

[b] Only one strain of *Salmonella* was isolated from each feed type.

[c] Ampicillin (A), Chloramphenicol (C), Ciprofloxacin (Cp), Trimethoprim/Sulfamethoxazole (Tm), Gentamicin (Gm), Sulfisoxazole (Su), Streptomycin (S), Tetracycline (T) and Ceftiofur (Ce).

Table 3.4: Number (%) of feed ingredients and compound pig feed samples with different ranges of *Enterobacteriaceae* counts and number of samples in which *Enterobacteriaceae* were detected.

Sample Type	Non-Detectable (<10 cfu/g)	10-100 cfu/g	100-1000 cfu/g	1000-10000 cfu/g	>10000 cfu/g	Total no. of samples in which *Enterobacteriaceae* were detected (%)
Ingredients [a]						
Soya Products	59 (72.8)	8 (9.9)	7 (8.6)	3 (3.7)	4 (4.9)	22/81(27.2)
Maize	27 (45.0)	10 (16.7)	15 (25.0)	5 (8.3)	3 (5.0)	33/60 (55.0)
Wheat	16 (32.0)	5 (10.0)	11 (22.0)	6 (12.0)	12 (24.0)	34/50 (68.0)
Barley	8 (16.7)	1 (2.1)	4 (8.3)	18 (37.5)	17 (35.4)	40/48 (83.3)
Rapeseed	19 (65.5)	3 (10.4)	5 (17.2)	1 (3.5)	1 (3.5)	20/29 (69.0)
Pollard	3 (23.1)	1 (7.7)	1 (7.7)	1 (7.7)	7 (53.9)	10/13 (76.9)
Golden Distiller's Grain	10 (66.7)	3 (20.0)	2 (13.3)	0	0	5/15 (33.3)
Palm Kernel	11 (84.6)	0	2 (15.4)	0	0	2/13 (15.4)
Malt Sprouts	0	0	0	2 (40.0)	3 (60.0)	5/5 (100.0)
Sunflower	3 (75.0)	0	0	0	1 (25.0)	1/4 (25.0)
Lactofeed [b]	1 (33.3)	2 (66.7)	0	0	0	2/3 (66.7)
Compound feed						
Meal	12 (9.5)	1 (0.08)	12 (9.5)	23 (18.3)	78 (61.9)	114/126 (90.5)
Dry Sow	3 (12.5)	0	0	3 (12.5)	18 (75.0)	21/24 (87.5)
Lactating Sow	3 (12.5)	0	2 (8.3)	6 (25.0)	13 (54.2)	21/24 (87.5)
Weaners	1 (3.9)	1 (3.9)	2 (7.7)	5 (19.2)	17 (65.4)	25/26 (96.2)
Fatteners	5 (9.8)	0	8 (15.7)	8 (15.7)	30 (58.8)	46/51 (90.2)
Others [c]	0	0	0	1 (100.0)	0	1/1 (100.0)
Pelleted	133 (71.1)	20 (10.7)	16 (8.6)	11 (5.9)	7 (3.7)	54/187 (28.9)
Dry Sow	17 (70.8)	4 (16.7)	3 (12.5)	0	0	7/24 (29.2)
Lactating Sow	15 (51.7)	3 (10.4)	4 (13.8)	5 (17.2)	2 (6.9)	14/29 (48.3)
Weaners	30 (76.9)	1 (2.6)	4 (10.3)	3 (7.7)	1 (2.6)	9/39 (23.1)
Fatteners	62 (75.6)	9 (11.0)	4 (4.9)	3 (3.7)	4 (4.9)	20/82 (24.4)
Others [c]	9 (69.2)	3 (23.1)	1 (7.7)	0	0	4/13 (30.8)

[a] Ingredients in which *Enterobacteriaceae* were never detected were not tabulated. These include citrus pulp (n=6), beet pulp (n=5), whey syrup (n=3), tallow (n=1), lysine (n=1), milk powder (n=1), wheat/pollard (n=2).

[b] Lactofeed is a lactose source.

[c] Feeds in which production stage was not specified.

Table 3.5: Effect of diet form (meal, pellet) on mean *Enterobacteriaceae* counts (Log_{10} cfu/g) in samples from the five commercial feed mills (Mills A-D & F) tested

Mill [a]	Form of Compound Feed		Mean	S.E. [b]	P-values [c]		
	Meal	Pellet			Form	Mill	Form x Mill
A	4.85	1.72	3.29	0.143	0.001		
B	3.97	1.06	2.52	0.231	0.001		
C	3.05	1.19	2.12	0.210	0.001		
D	4.18	1.33	2.76	0.149	0.001		
F	3.05	1.07	2.06	0.267	0.001		
Mean	3.82	1.27		0.093	0.001	0.001	0.01

[a] Mill E (home compounder) was omitted from the dataset as all feed was produced as meal in this mill.

[b] S.E.; Standard Error

[c] P-values ≤ 0.05 are statistically significant.

Table 3.6: Effect of compound diet type (dry sow, finisher, lactation, weaner) on mean *Enterobacteriaceae* counts (log_{10} cfu/g) in samples from the five commercial feed mills (Mills A-D & F) and one home compounder (Mill E) tested

Mill	Compound Diet Type (Log_{10} CFU/g)				Mean	S.E. [a]	P-values [b]		
	Dry	Finisher	Lactation	Weaner			Type	Mill	Type x Mill
A	2.93	2.56	2.63	2.62	2.69	0.26	0.90		
C	1.98	2.17	3.34	1.95	2.36	0.42	0.35		
D	2.76	1.98	2.89	3.25	2.72	0.42	0.22		
E	2.61	4.15	3.35	3.57	3.42	0.49	0.38		
F	1.75	1.79	2.03	2.12	1.92	0.56	0.97		
Mean	2.41	2.53	2.85	2.70		0.28	0.76	0.02	0.54

[a] S.E.; Standard Error

[b] P-values ≤ 0.05 are statistically significant.

Chapter 4

Survival characteristics of monophasic *Salmonella* Typhimurium 4,[5],12:i:- derived from pig feed ingredients and compound feed

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4.1 Abstract

The presence of *Salmonella* in animal feed or feed ingredients at the feed mill or on-farm is a cause for concern, as it may lead to *Salmonella* transmission to food-producing animals and subsequently to humans. The objective of this study was to determine the survival characteristics of monophasic variant strains of *Salmonella* Typhimurium (n=5) recovered from pig feed ingredients and compound pig feed sampled from commercial feed mills. The first part of the study investigated the thermal inactivation of these strains using an immersed heating coil apparatus. A Weibull model provided a good fit, with low RMSE values (0.04-0.43) and high R^2 values (0.93-0.99) obtained. There was considerable inter-strain variation in heat resistance, with D-values ranging from 397.83 to 689 sec at 55ºC, 11.35 to 260.95 sec at 60ºC and 1.12 to 6.81 at 65ºC. Likewise, z-values ranged from 2.95 to 5.44°C. One strain (2278) demonstrated a significantly higher thermal tolerance, even though it had been isolated from a meal feed. However, overall the strains investigated do not appear to be that much more heat resistant than *Salmonella* previously studied. The second part of this study involved assessing the ability of the five *Salmonella* strains to survive during storage over a 28-day period in pelleted weaner pig feed treated with 0.3% sodium butyrate and stored under environmental conditions similar to those used on-farm. While a mean reduction in the *Salmonella* count of 0.79 $\log_{10}$ CFU was seen in the treated feed during the storage period, a reduction (albeit only 0.49 $\log_{10}$ CFU) was also observed in the control feed. Although there was no overall effect of treatment, sodium butyrate resulted in reductions in *Salmonella* counts of 0.75 and 0.22 $\log_{10}$ CFU at days 14 and 24 of feed storage, respectively but at the end of the 28-day storage period counts were 0.25 $\log_{10}$ CFU higher in the treated feed. Therefore, the sodium butyrate used appears unsuitable as

an agent for feed treatment and this lack of efficacy may be due to the fact that the particular feed additive used has a protective coating. Overall, the results of this study enhance knowledge about the behaviour and survival characteristics of monophasic variants of *S.* Typhimurium (4,[5],12:i:-) in animal feed and may assist the feed industry and pig producers in implementing effective intervention strategies for their control.

4.2 Introduction

Salmonella is a leading cause of gastroenteritis in humans and continues to be of significant public health concern. Animal feed is a well-documented vector for the entry of *Salmonella* into the food chain and contaminated animal feed can be an indirect cause of infection for people consuming foods of animal origin (EFSA, 2008; Crump et al. 2002). Recovery of *Salmonella* from animal feed and ingredients is not uncommon in the EU. In 2008, prevalence in compound feed was reported to range from 0 to 6% (EFSA, 2008). More recent studies have found similar contamination rates i.e. 3.2% in a Spanish study of different animal feeds (Torres et al. 2011), 1.5% in a UK poultry feed study (Davies and Wales, 2010) and 0.95% in a survey of pig feed conducted by our group in Ireland (Burns et al. in press). The latter study isolated monophasic variants of *Salmonella* Typhimurium (4,[5],12:i:-) (i.e. those that lack the expression of flagellar Phase 2 antigens) from feed ingredients and compound feed (Burns et al. in press). This is a cause for concern considering that the occurrence of monophasic variants in human cases of illness in the EU has increased rapidly from 360 in 2007 to 5932 in 2012, along with the number of countries reporting this serotype (EFSA, 2014). It is also worrying that this serotype has been isolated from a wide range of animals and foods of animal origin, with pigs/pig meat appearing to be a common reservoir of infection (Hopkins et al. 2010).

The physiology of *Salmonella* lends itself well to survival on a wide range of feeds and feed ingredients (Maciorowski, et al. 2007), as it has developed diverse mechanisms to survive at low water activity (a_w) and at low concentrations of available carbon, nitrogen, and phosphorus, the latter by means of a starvation stress

response (Spector, 1998). Transient low pH levels are tolerated by means of a stationary phase acid tolerance response (Lee et al. 2 1994). One study reported *Salmonella* survival for 26 months in poultry feed (Davies and Wray, 1997), while another demonstrated survival times of up to 3 years in pig and poultry feeds (D'Aoust and Sewell, 1986). Survival may be influenced by factors such as the strain of *Salmonella*, growth phase of cells, presence of antimicrobials, a_w, feed structure, acidity and storage temperature (Andino and Hanning, 2015). Certain *Salmonella* serotypes are isolated more often from feed and feed mills, as a result of their physiology and in particular their ability to survive in dry environments (Binter et al., 2011). However, due to the recent emergence of monophasic variants of *S.* Typhimurium, only a few studies to date have investigated their phenotypic traits (Mandilara, et al, 2013; Bugarel et al., 2012;Seixas, et al. , 2014). As a result, only limited information is available on their survival characteristics, with no data available for survival in animal feed.

Control of *Salmonella* spp in animal feed may require multiple interventions. Some that have been proposed and applied include heat treatment, irradiation and chemical treatment with organic acids (usually formic or propionic) and their salts, formaldehyde and bacterial membrane disruptors such as terpenes and essential oils (Himathongkham et al. 1996; Koyuncu et al., 2013; Wales et al. 2010 and Jones, 2011). The use of heat treatment to accomplish microbial population reductions is the most common and is based on the destructive effects of appropriate time-temperature combinations. In Ireland, any feed intended for poultry must be subjected to heat treatment to produce a minimum temperature of 75˚C at the core for 1 min as specified by S.I. No. 364/1991. A guidance note for the control of

Salmonella in pigs issued by the Department of Agriculture, Food and the Marine (2007) also specifies a similar heat treatment for pig feed, although this is not a legal requirement. Excessive heating during processing, however, can lead to destruction of essential amino acids. With the exception of *S.* Senftenberg and some other heat resistant serotypes, D-values (decimal reduction times i.e. the time taken at a given temperature to produce a 10-fold reduction in viable cell numbers) for *Salmonella* are typically 0.18-10 min at 60˚C and < 1 min at 70˚C (Scientific Committee on Veterinary Measures Related to Public Health, 2003). Typical z-values (change in temperature neces10-fold reduction in the D-value) range from 4 to 5˚C (Scientific Committee on Veterinary Measures Related to Public Health, 2003). However, factors such as a_w, fat, carbohydrate and protein content, presence of salts, pH, number of organisms, inhibitory compounds, temperature and duration of heating may all influence the effectiveness of heat treatments (Olsen and Nottingham, 1980). The advantage of chemical treatment is that residual effects contribute to the control of *Salmonella* on stored feed and limit the potential for survival of the pathogen if re-contamination occurs during storage (Koyuncu et al. 2010). There may also be residual anti-*Salmonella* effects in the gastrointestinal tract of the animal post-consumption (Berge and Wierup, 2012). However, the use of chemical treatments (e.g. organic acids and formaldehyde) should be approached with caution, as recent research suggests that they interfere with *Salmonella* detection rather than killing the organism (Carrique-Mas et al. 2007).

The objective of this study was to examine the survival of monophasic variants of *S.* Typhimurium recently isolated from feed ingredients and finished feed sampled at

Irish feed mills in terms of their thermal tolerance and ability to persist on stored feed treated with a sodium butyrate feed additive.

4.3 Material and Methods

4.3.1 Bacterial strains, culture conditions and preparation of inocula

The five monophasic *S.* Typhimurium isolates used in this study were isolated from pig feed ingredients and compound pig feed (meal and pellets) sampled from five Irish commercial feed mills and one home miller (Burns et al., in press). Details of these five isolates are listed in Table 1. All *Salmonella* isolates were maintained on Protect™ cryoprotectant beads (Technical Service Consultants Limited, Lancashire, UK) at -80°C. Each was resuscitated by streaking a Protect™ bead onto tryptone soya agar (TSA; Oxoid, Basingstoke, UK) and incubating at 37°C for 22 ± 2 h. A single colony was then inoculated into 25mL tryptone soya broth (TSB; Oxoid) and incubated at 37°C for 18 ± 2h. These cultures were centrifuged at 10,000g for 10min at 4°C and the supernatant discarded. The pellet was then re-suspended in 25mL TSB, creating an inoculum containing ~8 $\log_{10}$ CFU/mL. Inocula were stored at 4°C for a maximum of 1h prior to use.

4.3.2 Thermal inactivation experiments

Thermal inactivation experiments were carried out for each of the monophasic *S.* Typhimurium isolates using an immersed heating coil apparatus (Sherwood instruments, Lynnwood, MA, USA). This apparatus, originally designed by Cole and Jones (1990), has a narrow bore stainless steel coil fully submerged in a thermostatically controlled water bath. The three treatment temperatures used were 55, 60 and 65°C. The apparatus was adjusted to the target temperature and allowed to equilibrate for at least 2h prior to commencement of a run. In line with the manufacturer's recommendations, 10mL of *Salmonella* inoculum (prepared as

outlined in Section 2.1) was injected into the coil apparatus using a disposable syringe, and treated aliquots (400µL) were dispensed automatically at pre-determined time intervals into sterile glass vials on a revolving carousel. The collection vials were pre-filled with 1.6mL of cooled TSB to aid dilution. A flushing step was used between samples to remove sample which may have remained at the tip of the coil tubing. An unheated aliquot of TSB was also collected before commencement of each thermal inactivation cycle to serve as the T0 sample. Samples were collected every 480s at 55°C, every 30s at 60°C and every 3s at 65°C and immediately cooled on ice. *Salmonella* was enumerated by preparing a 10-fold dilution series in maximum recovery diluent (MRD; Oxoid) and spread-plating 100µL aliquots of appropriate dilutions in duplicate onto a selective agar, xylose lysine deoxycholate agar (XLD; Oxoid). Aliquots were also spread-plated onto non-selective TSA plates to allow recovery of injured cells. Both XLD and TSA plates were incubated at 37°C for 22 ± 2 h. Following incubation, colonies were counted to obtain the number of surviving *Salmonella* cells at each time point for each temperature. Three replicates were performed for each *Salmonella* isolate at each temperature.

4.3.3 Survival of *Salmonella* in pig feed during storage

Commercially produced first stage weaner pig feed which had been finely ground and subsequently pelleted to a diameter of 3mm was used. The feed was produced with and without supplementation with 0.3% sodium butyrate (Adimix®, Nutriad, Kasterlee, Belgium). Prior to use, 90g samples of each feed were taken and from these 25g was confirmed as *Salmonella*-negative by analysing for the presence of *Salmonella* spp. according to standard microbiological procedures (EN ISO

6579:2002/Cor 1:2004) with modified brilliant green agar (Oxoid) used for additional selective plating. Control and sodium butyrate-treated feed was then inoculated with each of the five monophasic *S.* Typhimurium isolates in triplicate as follows. Feed (2kg) was transferred to sterile 10L stainless steel containers and 4.5mL of *Salmonella* inoculum [prepared as outlined in Section 2.1, except that isolates were resuscitated from frozen stocks on plate count agar (Oxoid)] was added in order to give a final inoculum of ~9 $\log_{10}$ CFU/g feed. This was done using an atomizer followed by immediate mixing and a ~4h post-inoculation drying period at room temperature (~20°C). Each stainless steel container was then stored at 10°C (average temperature for Ireland over the last three years as calculated from Met Eireann data recorded at Dublin airport) in order to simulate environmental conditions for handling and storage of pig feed on Irish commercial pig farms. Since pig feeds are generally stored for less than 1 month from production to consumption, a period of 28 days was chosen over which to evaluate the survival of *Salmonella*. Duplicate 25g samples of inoculated feed were sampled on day 0 and thereafter intermittently over the 28-day storage period. The samples were homogenized for 90sec with 225mL of buffered peptone water (Oxoid) in a stomacher at normal speed. *Salmonella* was enumerated in these samples by making 10-fold serial dilutions of the suspension in sterile tubes containing 9mL of MRD. Aliquots (0.1mL) from each dilution (10^{-1} to 10^{-3}) were spread-plated on XLD agar. After incubating plates at 37°C for 24 ± 2h, presumptive *Salmonella* colonies were enumerated. At each time point, up to 5 colonies per XLD plate were confirmed as *Salmonella* using a *Salmonella* latex agglutination kit (Oxoid). The water activity (a_w) values of all samples were measured using an Aqualab model CX-2 water activity meter (Labcell, Alton, UK), calibrated daily using distilled water (a_w =1.000

± 0.003) and a saturated solution of sodium chloride (a_w =0.755 ± 0.001 at 20˚C). The pH of the buffered peptone water homogenate of all samples (prepared as outlined above) was measured using an Orion ROSS™ epoxy body, flat surface, combination pH electrode (Thermo Scientific, Beverly, USA).

4.3.4 Statistical Analysis

The thermal inactivation kinetics of the *S.* Typhimurium 4,[5],12:i:- strains were determined using a regression analysis of the microbial inactivation data. Microsoft Excel Addin tool, the GInaFiT was employed to obtain a D-value and shape factor (β) (Geeraerd et al. 2005) by fitting microbial inactivation data to the Weibull model [Eq-1]

$$\log(Nt) = \log(No) - \left[\frac{t}{D}\right]^{\beta} \quad [1]$$

Where N_t (CFU/mL) was the number of microorganisms at time t (min), N_0 (CFU/mL) the initial number of microorganisms, D (min) the time for the first decimal reduction and β [-] the scale and shape of the inactivation curve. For evaluation of the fitting capacity of the models the statistical criterion of the adjusted coefficient of multiple determination R^2_{adj} and the root mean squared error (*RMSE)* was used.

$$R^2_{adj} = 1 - \left[\frac{nt-1}{nt-np}\right] \cdot \frac{SSE}{SSTO} \quad [2]$$

Herein, *SSTO* is the total sum of squared errors $\sum(yi - \bar{y})^2$and SSE the sum of squared errors $\sum(yexp(ti) - y(ti, pls))$.

$$RMSE = \sqrt{\frac{\sum_{i=1}^{nt}(yexp(ti) - y(ti,pls))^2}{nt - np}} \quad [3]$$

Where $y_{exp}(t_i)$ denoted the experimental observations, $y(t_i,p_{ls})$ the predicted values, n_t the total number of data points, n_p the number of estimated model parameters.

The z-value was calculated by using the equation [4].

$$z\ value = \frac{T2 - T1}{\log D1 - \log D2} \quad [4]$$

Where D1 and D2 are decimal reduction time (min) at temperature T1 and T2 (^{o}C), respectively.

For the survival of *Salmonella* in pig feed, the data were analysed as a three-way factorial combination of treatment, strain and day. The variables tested were strains (2278, 2888, 3836, 3844 and 3845), feed treated with and without sodium butyrate and number of days (0-28). The analysis was fitted using the mixed procedure in SAS statistical software package (SAS Institute Inc., 2011). Means comparisons were carried out to describe significant effects and a Tukey adjustment was used for multiple comparisons. Residual checks were made to ensure that the assumptions of the analysis were met and, where appropriate, the response was log-transformed.

4.4 Results

4.4.1 Thermal inactivation of monophasic *S.* Typhimurium

The thermal inactivation curves for five feed- and feed ingredient-derived strains of *S.* Typhimurium 4,[5],12:i:- at temperatures of 55, 60 and 65°C following recovery on XLD and TSA are shown in Fig. 1-3. The Weibull model was shown to be a good fit to the survivor curves, with R^2 values ranging from 0.92 to 0.99 and small RMSE values i.e. ranging from 0.04 to 0.43 at all temperatures (Table 2). There was considerable variation noted in the shape of the survivor curves at each temperature, as described by the shape factor (β). When $\beta = 1$ it indicates a linear curve, when $\beta > 1$ the curves have a concave, downward shape, indicating the presence of shoulders (population surviving longer at the start of heating) and when β is < 1 the survivor curves have an upward concavity, indicating a tailing or resistant population at the end of thermal treatment. In general, shoulder populations ($\beta > 1$) were more common at 55 and 60˚C and tailing ($\beta < 1$) was more common at 65˚C (Table 2). However, there was inter-strain variation in the β values obtained at each temperature; for example, when recovered on TSA, strain 3845 had a higher β value than three of the other strains ($P<0.05$) at 55˚C whereas at 60 and 65˚C strain 2278 had a higher β value than all other strains ($P<0.05$) (Table 2). Strain 2278 in particular showed considerable tailing at 55˚C and shoulders at 60˚C (Fig. 1 and 2). For some strains there were significant differences in the β values obtained when XLD was used as recovery medium versus TSA and, in general, the β values were lower for XLD than TSA at 60 and 65°C ($P<0.05$).

The decimal reduction (D) values calculated by the Weibull model for the five *Salmonella* strains at the three heating temperatures are presented in Table 2. As the

heating temperature increased, the D-values decreased. The results show that the recovery method had a significant impact, especially at the higher temperatures, with higher D values reported from TSA than XLD for one strain at 55°C, three strains at 60°C and all but one strain at 65°C ($P<0.05$). There was considerable inter-strain variation in D-values at all heating temperatures; at 55°C the value obtained for strain 3845 was higher than that obtained for all except one other strain using both recovery media ($P<0.05$). While at 60°C strain 2278 was the most heat resistant, with higher D-values than all but one other strain using TSA and all other strains using XLD ($P<0.05$). The same was found at 65°C when TSA was used, with 2278's D-value higher than that of all other strains ($P<0.05$); however, using XLD its D-value was higher than that of only one other strain ($P<0.05$).

Based on the z-values (change in temperature required for one $\log_{10}$ reduction in the D-value), strain 2278 was more thermotolerant than the other four strains based on the TSA recovery method ($P<0.001$; Table 3), whereas strain 3845 was the least resistant, with a lower z-value than all but one other strain ($P<0.001$; Table 3). Using the XLD recovery method the most thermotolerant strain was 2888, with a higher z-value than two other strains ($P<0.05$; Table 3), and the least was strain 3836, but its z-value was only lower than that of strain 2278 ($P<0.05$; Table 3).

4.4.2 Survival of monophasic *S.* Typhimurium in stored feed treated with sodium butyrate

Mean data for the survival of the five monophasic *S.* Typhimurium isolates used to inoculate weaner pig feed, either treated with 3% sodium butyrate or untreated over a 28-day storage period are presented in Tables 4 and 5. There were no strain x treatment x time or strain x treatment interactions and for this reason these data are not shown. However, there was a treatment x time interaction ($P<0.001$), with *Salmonella* counts increasing for the control feed at day 14 but reducing for the sodium butyrate-treated feed at day 14 (Table 4). There was also a strain x time interaction ($P<0.001$; Table 5). In terms of the overall effect of treatment, there were no differences in mean *Salmonella* counts between the treated and the untreated feed over the 28-day storage period ($P>0.05$; Table 4); however, treatment was shown to be significant on days 14, 24 and 28, with the sodium butyrate-treated feed having lower counts on days 14 and 24 ($P<0.01$) but higher on day 28 ($P<0.01$). There was an overall effect of time, with mean *Salmonella* counts in both the sodium butyrate-treated and control feed declining during storage ($P<0.001$; Table 4). The first reduction in *Salmonella* counts in the control and treated feed was seen at day 7 of storage and no further reductions compared to this were seen up to day 28 of storage ($P<0.05$). There was no overall strain effect ($P>0.05$); however, there were strain effects seen at every time point during feed storage ($P<0.05$). Immediately after inoculation, counts of strains 3844, 3845 and 2278 were higher than those of strains 2888 and 3836 and this was also the case at day 28 of storage (Table 5; $P<0.05$).

No differences in pH were observed between the control and sodium butyrate-treated feed samples ($P>0.05$), with pH values ranging from 6.78-7.03 (data not shown).

With respect to a_w, there was a strain x treatment ($P<0.001$; Table 6) and a strain x time ($P<0.001$; data not shown) interaction and there was a tendency for a treatment x time interaction ($P=0.08$; data not shown). There was also an effect of strain ($P<0.001$; Table 6) and time ($P<0.001$; data not shown). With respect to the effect of treatment, the a_w was higher in the treatment than the control overall and for each of the strains ($P<0.001$; Table 6). In addition, the a_w was higher for the sodium butyrate-treated feed in comparison to the control feed immediately following inoculation ($P<0.001$), and remained so for the duration of storage (data not shown).

4.5 Discussion

Monophasic variants of *S.* Typhimurium have recently emerged as one of the main causes of human salmonellosis (EFSA, 2014). However, studies on their survival characteristics are limited and these are needed in order to ensure that adequate controls are put in place in the agri-food sector. The behaviour of the strains investigated was of particular interest, as they were recently isolated from feed ingredients and compound pig feed sampled from Irish feed mills supplying pig farms on which *Salmonella* was isolated from both pigs and feed (Burns et al. in press).

A key intervention in the control of *Salmonella* is the use of thermal treatment, which may be implemented during feed production or later in the food processing chain. In this study a submerged heating coil apparatus was used to study thermal inactivation kinetics, as it has previously been shown to be accurate and to reduce experimental error (Coles and Jones, 1990; Duffy et al. 1995; Loss and Hotchkiss, 2004). The effectiveness of the coil was demonstrated by Duffy et al. (1995), with a considerably lower D-value (2.09 min) obtained at 55˚C for *Salmonella* than that obtained using an open test tube system (6.62 min) which has been reported to give non-uniform heating. The only disadvantage of the apparatus is that it cannot be used for solid samples; hence, our data are for survival in broth rather than animal feed.

The Weibull model has previously been employed by others to describe microbial, enzymatic and chemical degradation kinetics (Cunha et al 1998) and was shown to be a good fit for the *Salmonella* thermal inactivation data in the present study. The Weibull model applied was flexible due to the inclusion of a shape constant in

addition to the rate constant. Other studies have shown the Weibull model to be the best choice for describing *Salmonella* survival/inactivation kinetics (Farakos et al. 2013; Ma et al. 2009). For example, Ma et al. (2009) showed that a thermal treatment of 0.26, 9, 42, and 120 min would be needed to reduce *Salmonella* in peanut butter by 1, 3, 5 and 7 log units, respectively, according to the Weibull model, whereas 13.4, 40, 67 and 94 min, respectively were predicted using a first order kinetics model. These differences could have resulted in either over-processing if the reduction target was below 5 log units and in under-processing if the target was above 5 logs.

The shape of the survivor curves showed that some of the monophasic *S.* Typhimurium strains had either shoulder or tailing populations at the start or end of the heating period, respectively. The fact that shoulders were mainly seen at 55 and 60˚C and tailing populations at 65˚C is in contrast to the findings of Humpheson et al. (1998) who found that inactivation of *Salmonella* at temperatures of 55-60°C at 1°C intervals gave rise to tailed survivor curves in all cases. Likewise, deviations from first order kinetics were seen by Juneja et al. (2001) using a cocktail of *S.* Typhimurium DT104 isolates heated to 58-62ºC in beef, but, similar to the findings of the present study, the survivor curves exhibited shoulders. The presence of shoulders may be caused by microbial populations that consist of several sub-populations, each with its own inactivation kinetics, clumping of cells, poor heat transfer or multiple targets within a cell (Awuah et al. 2007). Tailing, on the other hand, has been shown to occur after initial inactivation of the more sensitive members of the bacterial population leaves behind a significant portion of progressively more resistant microorganisms (Bermúdez-Aguirre and Corradini,

2012), which have acquired resistance most likely as a result of synthesis of heat shock proteins (Humpheson et al. 1998). This indicates the presence of subpopulations with different death mechanisms or different sensitivities to heat (Amado et al. 2014).

In agreement with the findings of Aljarallah and Adams (2007), the D-values for most of the *Salmonella* strains at the higher temperatures were increased when TSA was used as the recovery medium compared to XLD. This is because TSA, being non-selective, supports the growth of injured cells and thus gives a more accurate reflection of the total number of surviving cells, while XLD is selective and therefore not conducive to the recovery of heat-injured cells. These data also indicate that, following treatment at the higher temperatures, the outer membrane of the *Salmonella* cells had been damaged (Aljarallah and Adams, 2007).

Despite the fact that all of the *Salmonella* strains evaluated were monophasic variants of Typhimurium, the D-values at all three heating temperatures showed considerable inter-strain variation. Together with the Z-values, they showed that one strain (2278) was more thermotolerant. This was most pronounced at 60°C when 4.4 min was required to kill 90% of the population, whereas it took only 0.2-0.5 min to achieve the same reduction in the other strains. This may indicate that this strain was producing higher levels of heat shock proteins or that it was capable of biofilm formation. The latter has previously been demonstrated for monophasic variants of *S.* Typhimurium and may explain their ability to survive in adverse environments (Seixas et al. 2014). However, the 2278 strain was isolated from a meal feed (Burns et al. in press) which would not have been subjected to the high temperatures

experienced during steam conditioning prior to pelleting. Nonetheless, as animal feed is at the beginning of the food chain in the 'farm-to-fork' model, the presence of such a heat resistant strain with an ASSuT antibiotic resistance profile is a real concern in terms of its transmission though the food chain to the consumer, as heat treatment is one of the most effective means of ensuring the microbial safety of feeds.

It is well documented that D-values can vary between different strains of the same microorganism (Lianou and Koutsoumanis, 2013; Doyle et al. 2001) and indeed there is substantial variation in the D-values published for different strains of *Salmonella*. Values of 0.18-10 min at 60˚C and <1 min at 70˚C have been reported (Scientific Committee on Veterinary Measures Related to Public Health, 2003) with the exception of the notoriously heat-resistant *S.* Senftenberg 775W (Doyle and Mazzotta, 2000; Aljarallah and Adams, 2007; Sörqvist, 2003). Although the monophasic *S.* Typhimurium variants are of increasing importance as a cause of foodborne disease in many EU countries, to our knowledge no other studies have investigated their thermal resistance properties (Mandilara et al. 2013). The D-values obtained at 60ºC in the present study fall within the ranges outlined above for *Salmonella* (Scientific Committee on Veterinary Measures Related to Public Health, 2003). They also compare well with those obtained in different matrices for *S.* Typhimurium (the serotype of which the monophasic strains are variants) (Gabriel, 2007; Doyle and Mazzotta, 2000). However, mean values obtained at 55 and 65ºC (8.59 and 0.04 min, respectively) were higher and lower, respectively than those reported by Amado et al. (2014) for untyped *S. enterica* isolated from vegetable cattle feed ingredients (5.70 min and 0.22 min, respectively). These differences could be due to variations in experimental conditions, as, apart from being

influenced by the strain, the D-value may also be affected by factors such as the stage of microbial growth, homogeneity of the bacterial population and the heating medium and apparatus used (Doyle et al. 2001; Bacon et al. 2003).

Overall, the results from the present study indicate that the monophasic variant strains of *S.* Typhimurium studied are likely to survive heat processing during feed manufacture, especially as the D-values obtained are for broth and are likely to be higher in feed, as *Salmonella* may be protected by feed constituents. For this reason, and because feed may become contaminated post-manufacture, the second part of the study investigated the efficacy of sodium butyrate in reducing *Salmonella* in pig feed during storage under conditions typical of those used on-farm. Although *Salmonella* counts in both the sodium butyrate-treated and control feed declined during the 28-day storage period, it survived well, with mean reductions of only 0.79 and 0.49 $\log_{10}$ CFU observed, respectively, which equate to average reductions of 0.03 $\log_{10}$ and 0.02 $\log_{10}$ CFU/day. Interestingly, strain 2278, which was shown to be particularly heat tolerant, survived well on both feeds, with mean reductions of only $\log_{10}$ 0.61 CFU/g observed (only one other strain demonstrated less of a reduction). Inter-strain variation has previously been seen for survival of *S.* Typhimurium in poultry feed (Andino et al., 2014), but it was more pronounced than that observed in the present study. Greater reductions in *Salmonella* occurred, with the largest (1.9 and 1.6 $\log_{10}$ CFU/g) observed after 4 h and 4 days, respectively. In comparison, no reductions were seen until day 7 in the present study.

The use of sodium butyrate as a feed additive has been proven to reduce intestinal colonization and faecal shedding of *Salmonella* in pigs (Boyen et al. 2008).

However, the present study is the first to evaluate it as a treatment for feed. Although there were no overall treatment effects, sodium butyrate resulted in reductions in *Salmonella* counts of 0.75 and 0.22 $\log_{10}$ CFU at days 14 and 24 of feed storage, respectively but at the end of the 28-day storage period counts were 0.25 $\log_{10}$ CFU higher in the treated feed. However, the main mode of action of sodium butyrate is down-regulation of expression of invasion genes in *Salmonella* which reduces the ability of the bacteria to invade host intestinal epithelial cells (Boyen et al. 2008). In addition, the sodium butyrate used in the present study had a protective coating which facilitates targeted release in the lower intestinal tract, which may have prevented contact between it and the *Salmonella* inoculum. For these reasons, the poor efficacy of this sodium butyrate additive as an anti-*Salmonella* agent for feed may be attributed to the fact that effects are only possible *in vivo* i.e. when it arrives in the lower intestinal tract of the animal. In contrast, a study by Koyuncu et al. (2013) found a 2.5 $\log_{10}$ reduction in *Salmonella* counts after 5 days in pelleted or meal feed treated with formic acid or a formic acid/propionic acid blend. However, the acids were added to the feed following *Salmonella* inoculation whereas in the present study acid addition was during feed manufacture, which is more realistic.

The pH of both the control and sodium butyrate-treated feeds was similar, remaining around neutral (6.78-7.03) throughout the study and this pH would not have had any anti-*Salmonella* effect. At formulation, the control and treatment feeds both had a mean a_w of 0.49 but after inoculation, the aw of the treatment feed increased to 0.72 and remained around this for the duration of the study. The reason for this difference is not known but increases in a_w have previously been seen in feed post-inoculation (Andino et al. 2014). The fact that microbial growth and survival is generally poorer

at low a_w might explain the lower count in the control than the treatment feed at day 28. However, previous studies suggest that *Salmonella* survival is higher in foods with an a_w of 0.43-0.55 than 0.75 (Juven et al. 1984; Petkar et al. 2011) and this may help to explain the lower counts in the acid-treated feed at days 14 and 24.

4.6 Conclusions

Recent data from our group suggests that monophasic variants of *S.* Typhimurium, which are becomingly increasingly important as a cause of foodborne disease, are harboured in animal feed and feed ingredients. Therefore, studies investigating their survival characteristics are important in order to allow the implementation of appropriate intervention strategies so as to reduce transmission via the food chain. All of the monophasic *S.* Typhimurium strains tested in the present study were isolated from pig feed and feed ingredients sampled from commercial feed mills in Ireland. The thermotolerance of some of these strains is of concern, in particular one which was isolated from a compound finisher feed, albeit a meal feed which would not have been subjected to steam conditioning. The heat resistance of this strain may be as a result of survival adaptions such as biofilm formation but this would require further investigation. Overall, the results from the present study indicate that the monophasic variant strains of *S.* Typhimurium studied, while not appearing to be much more thermotolerant than other *Salmonella* previously studied, are likely to survive heat processing during feed manufacture and indeed one strain did, as it had been isolated from a pelleted feed. For this reason, and because feed may become contaminated post-manufacture, the second part of the study investigated the efficacy of sodium butyrate in reducing *Salmonella* in pig feed during storage. While reductions in counts of the monophasic variant strains of *S.* Typhimurium were seen in the treated feed, they were minimal, also observed in the control feed (although to a lesser extent) and only observed at certain time points during storage. Moreover, at the end of the 28-day storage period, counts were in fact higher in the treated feed. This lack of efficacy may be due to the fact that the sodium butyrate used has a protective coating which facilitates targeted release in the intestine. Furthermore, its

main mode of action is to prevent *Salmonella* invasion of host intestinal cells. Anti-*Salmonella* effects would perhaps therefore only be expected *in vivo* and consequently, the particular feed additive chosen appears unsuitable as an agent for feed treatment. Taken together, the results of this research provide an understanding of the behaviour of monophasic variants of *S.* Typhimurium in feed and feed ingredients and provide important baseline data which may assist the feed industry and pig producers in implementing effective intervention strategies for their control.

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4.9 Tables and Figures

Table 4.1. Characteristics of monophasic *Salmonella* Typhimurium strains used in the present study.

Teagasc strain ID	Origin	Serotype	Antibiotic resistance profile [a]
2278	Finisher meal feed	4,5,12:i	ASSuT
2888	Soybean meal	4, 12:i:-	TGm
3836	Wheat	4, 12:i:-	ACSSuTTmGm
3844	Dry sow meal feed	4, 12:i:-	ACSSuTTmGm
3845	Dry sow pelleted feed	4, 12:i:-	ACSTCpCe

[a]Ampicillin (A), Chloramphenicol (C), Trimethoprim/Sulfamethoxazole (Tm), Gentamicin (Gm), Nalidixic acid (Na), Sulfisoxazole (Su), Ciprofloxacin (Cp), Streptomycin (S), Tetracycline (T) and Ceftiofur (Ce).

Table 4.2. Weibull model parameters and statistical parameters for the survival of monophasic *Salmonella* Typhimurium 4,[5],12:i- strains following thermal inactivation using TSA (tryptone soya agar) and XLD (xylose lysine deoxycholate agar) as recovery media.

	TSA					XLD				
Strain	Log_{10} CFU/mL (N_o)[1]	D-value (sec)[2]	β [3]	RMSE [4]	R^2 [5]	Log_{10} CFU/mL (N_o)	D-value (sec)	β	RMSE	R^2
	Temperature of 55 °C									
2278	8.88 (0.19)	456.8 (168.99)^{b}	0.73 (0.19)^{y}	0.04	0.95	8.84 (0.15)	397.22 (83.02)^{bc}	0.85 (0.05)^{xy}	0.12	0.93
2888	9.30 (0.11)	397.83 (65.57)^{bA}	1.02 (0.05)^{xX}	0.08	0.98	9.44 (0.06)	74.71 (11.05)^{dB}	0.68 (0.01)^{xY}	0.99	0.98
3836	8.88 (0.18)	544.05 (54.66)^{ab}	1.15 (0.01)^{wx}	0.07	0.98	8.67 (0.13)	565.83 (131.42)^{ab}	1.24 (0.18)^{w}	0.14	0.98
3844	8.88 (0.19)	488.63 (70.10)^{b}	1.08 (0.04)^{x}	0.08	0.97	8.75 (0.12)	332.45 (162.05)^{c}	1.00 (0.20)^{x}	0.11	0.99
3845	8.92 (0.22)	689.0 (147.96)^{a}	1.29 (0.06)^{wX}	0.07	0.98	8.64 (0.07)	603.7 (48.79)^{a}	1.36 (0.09)^{wW}	0.15	0.98
	Temperature of 60 °C									
2278	8.60 (0.20)	260.95 (25.53)^{aA}	1.67 (0.12)^{wW}	0.16	0.97	8.82 (0.08)	66.79 (21.77)^{aB}	1.03 (0.15)^{wxX}	0.12	0.99
2888	9.18 (0.16)	27.35 (8.61)^{aB}	1.12 (0.13)^{xW}	0.09	0.99	9.25 (0.25)	8.64 (2.99)^{bB}	0.91 (0.14)^{xX}	0.34	0.98
3836	8.97 (0.05)	17.16 (2.98)^{b}	1.00 (0.09)^{y}	0.21	0.98	8.98 (0.40)	21.49 (9.29)^{b}	1.19 (0.22)^{w}	0.21	0.99
3844	8.81 (0.07)	11.35 (2.99)^{b}	0.79 (0.16)^{z}	0.14	0.93	8.96 (0.17)	9.02 (1.61)^{b}	0.90 (0.01)^{x}	0.20	0.99
3845	8.86 (0.12)	28.02 (1.99)^{bA}	1.10 (0.08)^{wW}	0.13	0.97	9.04 (0.08)	6.31 (1.60)^{bB}	0.81 (0.11)^{xX}	0.50	0.99
	Temperature of 65 °C									
2278	8.74 (0.25)	6.81 (3.10)^{a}	1.50 (0.37)^{wW}	0.12	0.96	8.90 (0.21)	0.82 (0.38)^{a}	0.76 (0.10)^{wxX}	0.17	0.97
2888	9.54 (0.12)	1.12 (0.47)^{bA}	0.72 (0.09)^{x}	0.21	0.96	9.23 (0.25)	0.60 (0.53)^{abB}	0.78 (0.28)^{wx}	1.29	0.92
3836	9.12 (0.17)	1.96 (0.53)^{bA}	1.04 (0.16)^{xW}	0.43	0.98	9.75 (0.03)	0.25 (0.12)^{bB}	0.58 (0.06)^{xX}	0.19	0.98
3844	9.12 (0.07)	1.39 (0.26)^{bA}	0.91 (0.07)^{x}	0.15	0.98	9.14 (0.07)	0.60 (0.27)^{abB}	0.83 (0.17)^{w}	0.51	0.98
3845	9.01 (0.35)	1.18 (0.30)^{bA}	0.87 (0.10)^{x}	0.40	0.97	9.36 (0.35)	0.59 (0.28)^{abB}	0.80 (0.21)^{wx}	0.84	0.98

[1]Log_{10} CFU/mL (N_o): Log value of initial count. Values are the means of three replicate experiments and values in parentheses are standard deviations.

[2]D-values: decimal reduction times i.e. the time taken at a given temperature to produce a 10-fold reduction in viable cell numbers. Values are the means of three replicate experiments and values in parentheses are standard deviations.

[3]RMSE: root mean square error

[4]β: shape factor. Values are the means of three replicate experiments and values in parentheses are standard deviations.

[5]R^2: regression coefficient

[abcd, wxyz] For each temperature, values within a column that share a common superscript are not significantly different at $P<0.05$. Where no letters appear within a column there were no significant differences.

[ABCD,WXYZ] Within rows, values that share a common superscript are not significantly different at $P<0.05$. Where no letters appear within a row there were no significant differences.

Table 4.3. Z-values[1] [change in temperature (°C) required for one $\log_{10}$ reduction in the D-value] for monophasic *Salmonella* Typhimurium 4,[5],12:i- strains heated in tryptone soya broth at 55, 60 and 65°C and enumerated on TSA (tryptone soya agar) and XLD (xylose lysine deoxycholate agar).

Medium	**Strain**						
	2278	**2888**	**3836**	**3844**	**3845**	**SE**	**P value**
TSA	5.44^{a}	3.89^{c}	4.08^{b}	3.92^{b}	3.61^{c}	0.058	<0.001
XLD	3.69^{ab}	4.50^{a}	2.95^{b}	3.65^{ab}	3.30^{b}	0.174	0.020

[1]Values are the mean of 3 replicates.

[abcd] Values within a row that share a common superscript are not significantly different at P<0.05.

Table 4.4 Effect of treatment (sodium butyrate) over time on the mean viable numbers of five strains of monophasic *Salmonella* Typhimurium 4,[5],12:i- (log_{10} CFU/g) in weaner pig feed during storage at 10 ºC for 28 days.

Storage time (Days)				P value		
	Control	Treatment	SED[1]	Treatment[1]	Time	Treatment x Time
1	3.78^{a}	3.73^{a}	0.109	0.69		
4	3.51^{abc}	3.63^{ab}	0.087	0.19		
7	3.22^{def}	3.22^{def}	0.079	0.96		
11	3.33^{bcde}	3.35^{bcd}	0.079	0.72		
14	3.82^{bcde}	3.07^{ef}	0.079	0.002		
17	3.26^{cdef}	3.16^{def}	0.081	0.21		
21	3.27^{cdef}	3.29^{cde}	0.083	0.79		
24	3.39^{bcd}	3.17^{def}	0.079	0.006		
28	2.99^{f}	3.24^{cdef}	0.079	0.002		
Mean	3.40	3.32	0.084	0.418	0.001	0.001

^{abcdef}Mean values (n=3) within a column that share a common superscript are not significantly different at $P<0.05$ using Tukey's adjustment of least square means.

[1]Standard errors of difference and treatment P values refer to simple effect tests at each time.

Table 4.5. Effect of strain type (2278, 2888, 3836, 3844 and 3845) on survival of monophasic *Salmonella* Typhimurium 4,[5],12:i- (log_{10} CFU/g) in weaner pig feed [mean of control (untreated) and sodium butyrate-treated samples] during storage at 10 ºC for 28 days.

Storage time (Days)	Strain					SED	P value		
							Strain[1]	Time	Strain x Time
	2278	**2888**	**3836**	**3844**	**3845**				
1	3.93^{a}	3.45^{b}	3.51^{b}	3.95^{a}	3.93^{a}	0.171	0.001		
4	3.33^{c}	3.39^{bc}	3.63^{ab}	3.74^{a}	3.76^{a}	0.137	0.002		
7	2.88^{c}	3.36^{ab}	3.58^{a}	3.16^{b}	3.11^{bc}	0.125	0.001		
11	3.46^{ab}	3.02^{c}	3.25^{bc}	3.43^{ab}	3.54^{a}	0.125	0.001		
14	3.12^{ab}	2.95^{b}	3.30^{a}	3.27^{a}	3.33^{a}	0.125	0.014		
17	2.91^{c}	2.87^{c}	3.24^{b}	3.45^{ab}	3.56^{a}	0.128	0.001		
21	3.26^{bc}	2.90^{d}	3.03^{cd}	3.86^{a}	3.36^{b}	0.132	0.001		
24	3.43^{a}	2.94^{b}	3.10^{b}	3.37^{a}	3.57^{a}	0.125	0.001		
28	3.32^{a}	2.91^{b}	2.84^{b}	3.25^{a}	3.27^{a}	0.125	0.001		
Mean	3.29	3.09	3.28	3.50	3.89	0.132	0.142	0.001	0.001

^{abcd} Mean values (n=3) within a row that share a common superscript are not significantly different at P<0.05 using Tukey's adjustment of least square means.

[1]Standard errors of difference and strain P values refer to simple effect tests at each time.

Table 4.6. Effect of treatment (sodium butyrate) on the water activity of weaner pig feed samples over a 28-day storage period following inoculation with monophasic *Salmonella* Typhimurium 4,[5],12:i- strains 2278, 2888, 3836, 3844 and 3845

				P Value		
Strain	**Control**	**Treatment**	**SED[1]**	**Treatment**	**Strain**	**Strain x Treatment**
2278	0.477[c]	0.737[a]	0.0022	<0.001		
2888	0.539[b]	0.725[b]	0.0022	<0.001		
3836	0.546[a]	0.728[b]	0.0022	<0.001		
3844	0.455[b]	0.713[c]	0.0022	<0.001		
3845	0.462[d]	0.727[b]	0.0022	<0.001		
Mean	0.496	0.726		<0.001	<0.001	<0.001

[abcde]Mean values (n=3) within a column that share a common superscript are not significantly different at P<0.05 using Tukey's adjustment of least square means.

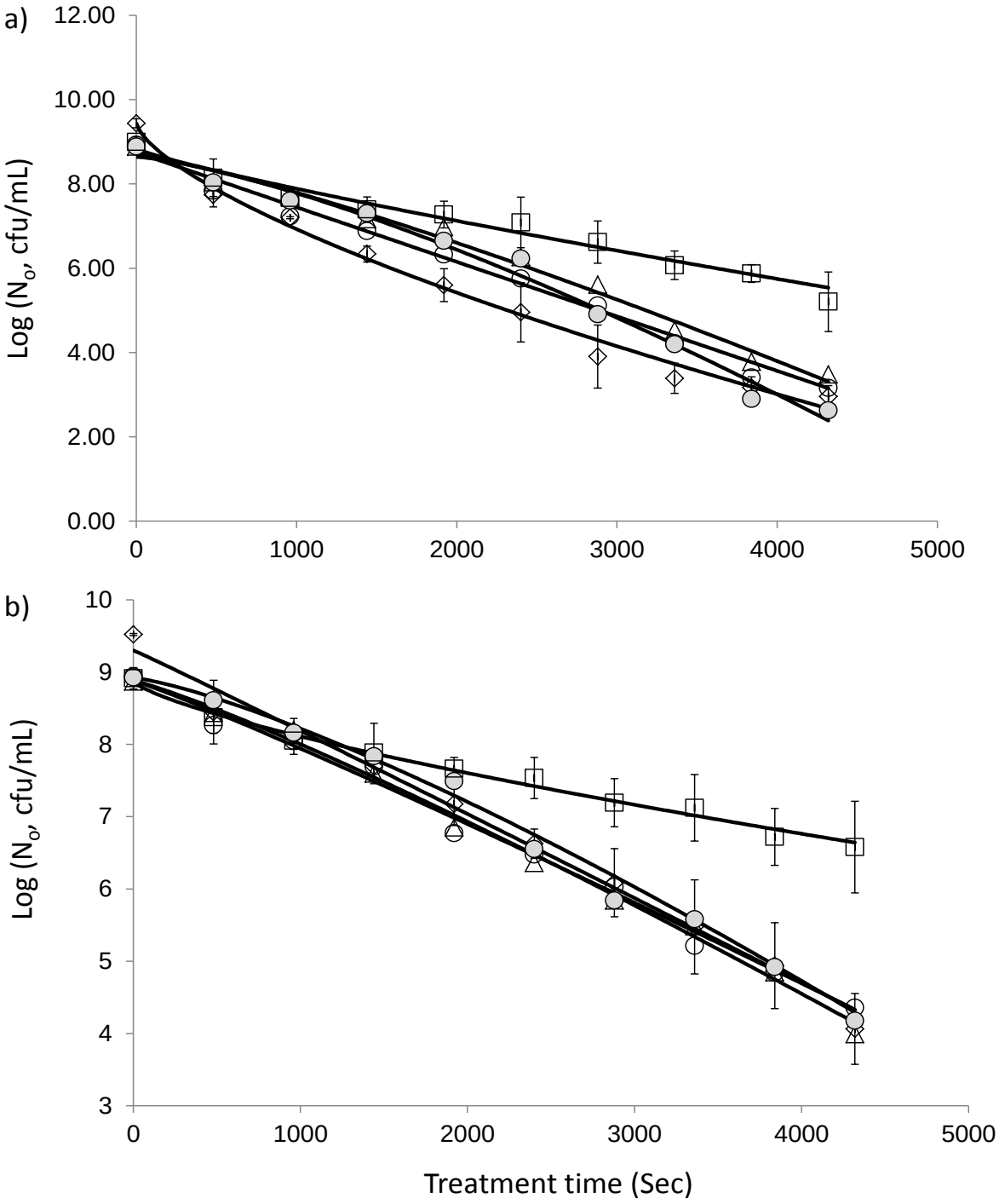

Fig. 4.1. Thermal inactivation curves at 55^{o}C for monophasic *Salmonella* Typhimurium 4,[5],12:i- strains [(□) 2278; (◊) 2888; (Δ) 3836; (○) 3844, (●) 3845] plated on XLD (a) and TSA (b) fitted to Weibull model. Values are the mean of 3 replicates, with SE indicated by error bars.

Log ($N_{o,}$ CFU/mL): Log value of initial count.

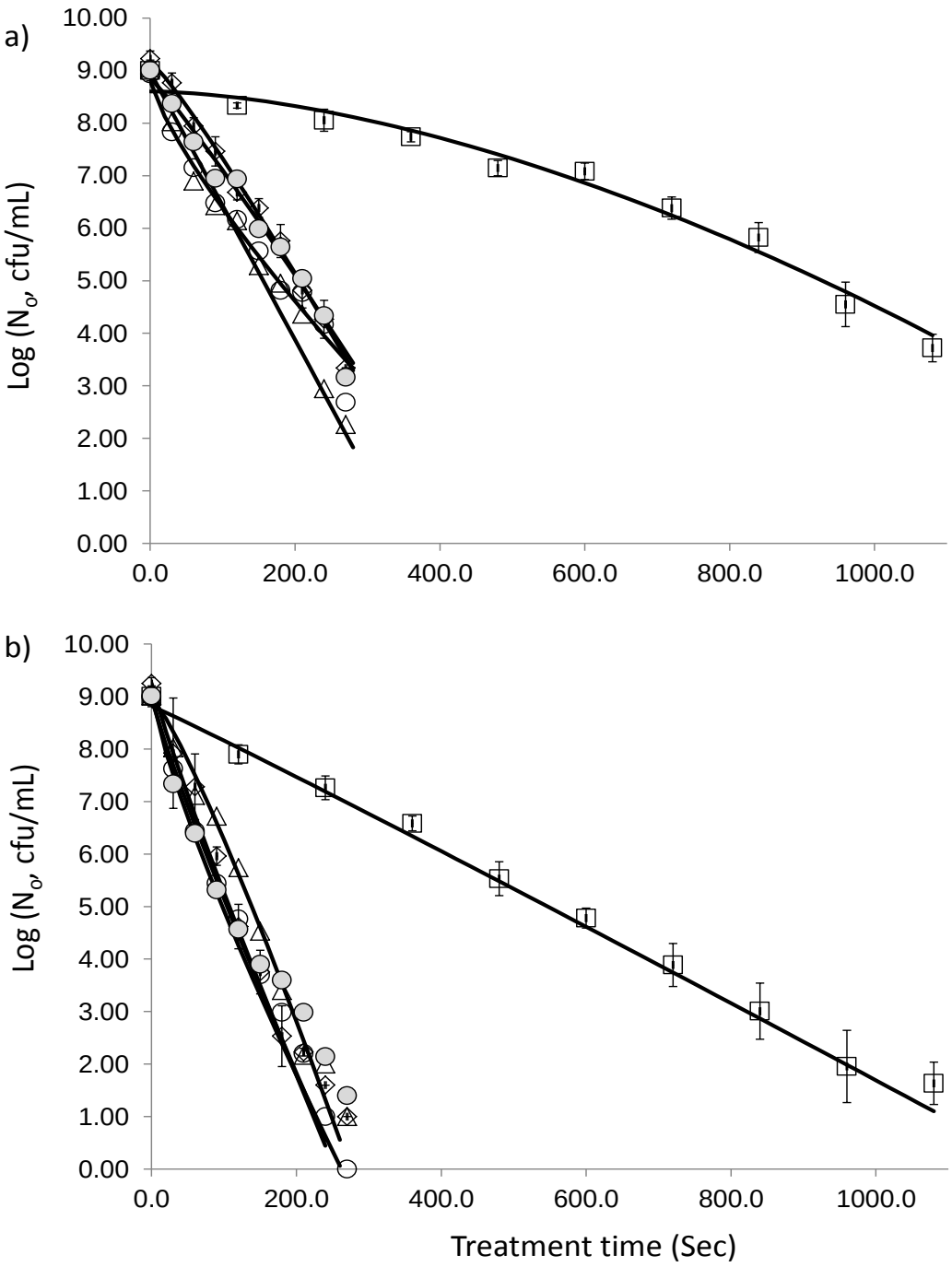

Fig. 4.2. Thermal inactivation curves at 60^{o}C for monophasic *Salmonella* Typhimurium strains [(□) 2278; (◊) 2888; (Δ) 3836; (○) 3844, (●) 3845] plated on XLD (a) and TSA (b) fitted to Weibull model. Values are the mean of 3 replicates, with SE indicated by error bars.

Log ($N_{o,}$ CFU/mL): Log value of initial count.

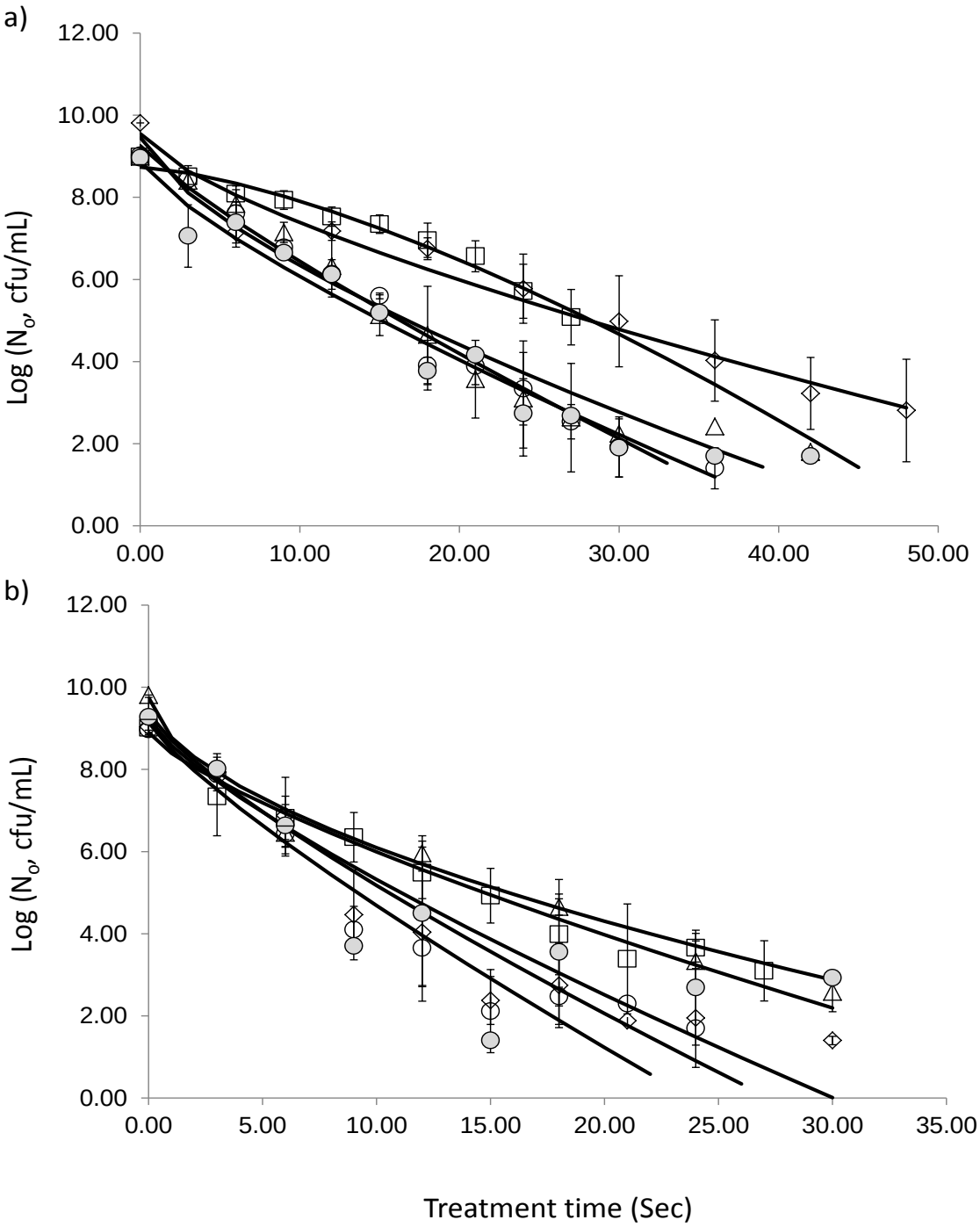

Fig.4.3. Thermal inactivation curves at 65^{o}C for strains [(□) 2278; (◊) 2888; (Δ) 3836; (○) 3844, (●) 3845] plated on XLD (a) and TSA (b) fitted to Weibull model. Values are the mean of 3 replicates, with SE indicated by error bars. Log ($N_{o,}$ CFU/mL):

Log value of initial count.

5. General Discussion

The basis of this study arose from public concern surrounding highly publicised foodborne scares over the last three decades (*Salmonella* contamination of eggs in the 1990s, BSE infected beef carcasses and more recently dioxin contamination of pork in 2008) all which have contributed to the loss of consumer confidence in the food supply chain, thereby creating an awareness of the importance of food safety. This is particularly so in the Republic of Ireland which exports approximately 60% of pig-meat produced and so its ability to capture and retain an export market is likely to be influenced by the quality and food safety standards of the end-product. In order to boost consumer confidence in the safety and integrity of the pork supply chain, it is essential to be able to provide full traceability from the point of origin to the point of consumption.

The European Food Safety Authority noted in a scientific opinion that feed is a risk factor for *Salmonella* prevalence in pigs (EFSA, 2008). Following a "farm-to-fork" model, animal feed is at the beginning of the food safety chain. In countries with low on-farm *Salmonella* prevalence, human infection linked to pork consumption has been traced back to *Salmonella*-contaminated feedstuffs (Hald et al. 2006, Wierup et al. 2010; Crump et al. 2002). The objective of this study was to determine the relatedness of *Salmonella* isolates recovered from feed, pig faeces and the environment on 10 Irish commercial pig farms identified as having a high *Salmonella* sero-prevalence by the Irish National Pig *Salmonella* Control Programme, in order to assess the role, if any, of feed in the transmission of *Salmonella* to pigs.

Large numbers of positive faecal samples were detected in gilts (19.1% prevalence), weaners (21.5%) and finishers (19.5%). High carriage rates are commonly seen in weaners and finishers (Davies et al. 1999); however, few studies have investigated *Salmonella* carriage

from farrow to finish. The high prevalence within the replacement breeding stock (gilts) indicates that these animals may be an important source of on-farm *Salmonella* infection and this could be of particular importance on farms where replacement breeding stock are purchased onto the unit.

A *Salmonella* Control Programme (SCP) for pigs has existed in Ireland (with some revisions) since 1997. The programme is enforced under national law (S.I. 521/2009: S.I. 522/2009) and is based on an ELISA test of meat juice samples taken in the slaughterhouse, which is used to estimate the exposure rate of pigs to a range of *Salmonella* serovars on the farm of origin. Herds are categorised according to the prevalence of ELISA-positive test results in meat juice samples. Since complete eradication is unrealistic, the National Pig *Salmonella* Control Programme is aimed at reducing the prevalence of *Salmonella* in slaughtered pigs to a level that is no longer a major threat to human health, with the intention of focusing on the entire food chain, comprising of pre-harvest (farm and transport) and post-harvest, (lairage, slaughter and processing) components. The farms used in this study were all identified from the >50% sero-prevalence category from the National *Salmonella* Control Programme. However, none of the farms were found to have a prevalence within this range. Four farms were found to have facecal bacteriological prevalence's >20%, whereas the remaining farms were found to have prevalence's ranging from 0-17.5%. If there had been a correlation between herd serology and *Salmonella*-positive faecal samples, it would have suggested that reducing the average prevalence obtained from the meat juice serology would also reduce the prevalence of *Salmonella* infection in pigs at slaughter. Unfortunately, testing for serologically-positive pigs, rather than for *Salmonella* infection, does not seem to provide an indication of the situation on the farm during the entire rearing period of the tested pigs. Each farm tested in this study had a different pattern of infection which means, to be effective, the

control programme must be specific for each farm. The cost of using both serological and bacteriological sampling is high; nevertheless, the use of both methods may provide the most comprehensive picture of the infection cycle on a farm.

Since the introduction of the revised *Salmonella* Control programme (in December 2010), the programme has failed to show any effect with the number of herds with a *Salmonella* prevalence >50% having increased from 11 % to 20%, i.e. an increase of over 100%. One reason that may explain the increase in *Salmonella* prevalence is that there are no implications for herd owners with chronically high *Salmonella* prevalences bar exclusion from the Bord Bia scheme, so therefore without a bonus–malus system for many there is no incentitive for improvement. It is perhaps not surprising that the majority of member states do not have such a defined programme.

Interestingly one farm (E) had notably higher faecal prevalence than the other farms (37.2%), which correlates with the fact that it had the second highest sero-prevalence (46.9%), as determined by the National *Salmonella* Control programme. This farm also had the highest number of *Salmonella*-positive environmental and water samples (20 and 28.3% samples *Salmonella*-positive, respectively) but no *Salmonella*-positive feed samples. Like other epidemiological data on *Salmonella* infection on pig farms there appears to be numerous sources of infection and possible modes of transmission and a combination of factors may result in an increased probability of infection. Therefore, any singular pre-harvest interventions that may be introduced on a pig farm may not yield any measureable improvements in *Salmonella* prevalence or level of environmental contamination, with multiple simultaneous interventions being required. There is strong evidence to suggest that adherence to particular principles of biosecurity (e.g. premises access of machinery and visitors, bought-in stock, rodent and wild bird control, sourcing and treatment of feed and

water) and management practises (work flow, pig flow, disinfection) are of significant benefit to control *Salmonella* as well as prevent the introduction of other pig diseases e.g. Trichinella, PRRS.

To enable effective control of *Salmonella* it is necessary to obtain detailed information about *Salmonella* transmission and the relationship between farm conditions and prevalence. A relatively high contamination rate (9.5%) was found in the study for water samples; however, the majority of these samples were collected from troughs and nipple drinkers from within pens so this may be the result of secondary contamination. However, the possibility of delivery of contaminated water into the farms cannot be excluded. The contamination level in the environment is probably the most important factor affecting the spread of *Salmonella* in a pen. *Salmonella*-positive environmental samples were found in > 50% of the farms, and the characteristics of these *Salmonella* strains supported the idea of pigs as a major source of *Salmonella* contamination of the farm environment. Strict hygienic measures should be considered in areas of high prevalence of infection to lessen the load of environmental contamination.

Difficulties associated with the detection of *Salmonella* in feed, due to the fact that the organism is usually present in low numbers and unevenly distributed, may explain the low prevalence of *Salmonella* found in on-farm and feed mill samples in this study. Contrary to other epidemiological investigations that showed benefits in herds using liquid feed in comparison to herds using dry feed, the farms using liquid feed in this study showed prevalence's of 10.4-28.3% therefore putting two of the farms in the upper end of the scale for farm prevalence's in this study. High prevalence herds can reduce the prevalence of *Salmonella* by a combination of feed-related interventions and improved management and

hygiene. Feed interventions such as feed form or the use of organic acids in feed have been shown to reduce the prevalence in high prevalence herds.

Salmonella was detected in 138/926 (14.9%) pig faecal samples across all production stages on 9 farms with an overall prevalence of 48.1% (138/287). Faecal samples had the highest *Salmonella* prevalence (14.9%), followed by water (9.5), environmental (9.0%) and feed (2.4%). Large numbers of *Salmonella*-positive faecal samples were detected in gilts, weaners and finishers. The high prevalence within the replacement breeding stock (gilts) in the present study indicates that these animals may be an important source of on-farm *Salmonella* infection and this could be of particular importance on farms where replacement breeding stock are purchased onto the unit. This is backed up by the PFGE data which showed that *Salmonella* strains recovered from breeding stock were often found in weaners and finishers on the same farm, indicating that certain strains may be endemic on particular farms. This was reflected in the environmental samples taken from these stages but not in the feed samples, as the highest prevalence was found in dry sow feed.

A total of 287 *Salmonella* isolates were isolated in the entire study; these were recovered from faeces (n=138), environmental swabs (n=92), feed (n=14) and water samples (n=43). Eleven *Salmonella* serotypes were recovered, with a monophasic variant of Typhimurium (4,[5],12:i:) predominating. *Salmonella* was detected in 14 feed samples that originated from 6 herds, with a prevalence of 2.4% (14/585 samples). Six of these *Salmonella*-positive feed samples originated on farms using liquid feed (farms A, B and J). The feed isolates were identified as 4,[5],12:I, Typhimurium, Typhimurium Copenhagen, Derby and Tennessee. In general, the serotypes obtained from feed prior to its arrival on-farm do not usually correspond to those most frequently found in humans and animals (Li et al. 2012; Lo Fo

Wong, 2001). Moreover, the MLVA profile of the monophasic variant found in feed in this study was identical to that of the dominant serovar recovered from the pig herds.

The isolates were tested and categorized based on serogrouping, antimicrobial susceptibility testing, MLVA and PFGE fingerprinting. PFGE results demonstrated five genotypic clusters of highly similar isolates whereas the MLVA profiles classified the isolates into 12 main clusters, of which 7 clusters consisted of monophasic *Salmonella* isolates. Six of these clusters showed a genotypic relationship between the isolates of feed origin and those of faecal origin. The five clusters identified by MLVA with feed and faecal isolate relationships were cluster B with ASSuTTm resistance, cluster E with ASSuT resistance, cluster J with ACSSuT resistance, cluster K with susceptible resistance and cluster L with ASuT resistance. The one cluster identified by PFGE was C with ASSuT resistance. Various European studies have also documented this ASSuT profile within human, pig and pig meat isolates (Arguello et al. 2014; Hopkins et al. 2010; Prendergast et al. 2013). The significance of finding genotypically related and, in some cases, clonal strains, including multidrug-resistant isolates in commercially produced feed and faecal samples, suggests an epidemiological connection between the isolates and indicates commercial feed as a potential vehicle of *Salmonella* transmission. The increased antimicrobial resistance and higher than anticipated occurrence of monophasic variants observed in the present study and other European studies highlights its importance as an emerging feed and foodborne pathogen.

The purpose of Chapter 3 was to assess the occurrence of non-typhoidal *Salmonellae* and *Enterobacteriaceae* counts in feed ingredients and compound feeds sampled from feed mills manufacturing pig diets. *Salmonella* was recovered from 2/338 (0.6%) ingredients (wheat and soybean meal), at two of the six mills. *Salmonella* was also detected in 3/317 (0.95%)

compound feeds including pelleted feed which undergoes heat treatment. Despite the low prevalence of *Salmonella* in pig feed and feed ingredients observed in the present study, feed still has the potential to affect many herds and subsequently cause human infection via consumption of contaminated pork. Feed as a potential source of *Salmonella* is however more important, relatively speaking, in lower prevalence countries but less-so in countries in countries with higher prevalence, such as Ireland. Like the majority of the feed isolates recovered from Chapter 2 the feed ingredient and compound feed isolates were verified as monophasic variants of *Salmonella* Typhimurium (4,[5],12:i:-) which exhibited multi drug resistance.

Two distinct MLVA profiles were observed amongst isolates recovered from the feed mill study, with the same profile recovered from both feed and ingredients, although these did not originate at the same mill. In general, the serotypes obtained from feed prior to its arrival on-farm do not usually correspond to those most frequently found in humans and animals (Li et al. 2012; Lo Fo Wong, 2001). However, a direct association was seen between the contaminated feed/ingredients found at the mills in Chapter 3 that were supplying the high *Salmonella* sero-prevalence farms studied in Chapter 2. The MLVA profile of the monophasic variant found in feed and feed ingredients sampled at feed mills in Chapter 3 was found to be identical to that of the dominant serovar recovered from the pig herds supplied by these mills.

The data from this study indicate that a large proportion of the raw materials used for pig feed manufacture are contaminated with high levels of *Enterobacteriaceae* ($>10^4$ cfu/g). Studies have shown that *Enterobacteriaceae* counts tend to be higher in feed samples positive for *Salmonella* than in *Salmonella*-negative samples and suggest that *Enterobacteriaceae* counts

may be a useful indicator to assess the likelihood of *Salmonella* contamination in feed (Jones and Richardson, 2004). There was no relationship; however, found in Chapter 3 between the occurrence of *Salmonella* in feed and feed ingredients and high *Enterobacteriaceae* counts, as only two of the five *Salmonella*-positive samples had high *Enterobacteriaceae* counts (4.59 and 4.62 Log_{10}cfu/g). However, it was shown that *Enterobacteriaceae* counts were significantly lower in pelleted feed (heat treated) than in meal (no heat treatment) and that *Enterobacteriaceae* counts would be a very useful indicator in a HACPP programme.

Chapter 4 examined the survival of monophasic variants of *S.* Typhimurium isolated from feed ingredients and finished feed sampled at Irish feed mills in terms of their thermal tolerance and ability to persist on stored feed treated with a sodium butyrate feed additive. Studies on thee survival characteristics of monophasic variants of *S.* Typhimurium are limited and these are needed in order to ensure that adequate controls are put in place in the agri-food sector. The first part of the study investigated the thermal inactivation of these strains using an immersed heating coil apparatus. A Weibull model provided a good fit, with low RMSE values (0.04-0.43) and high R^2 values (0.93-0.99) obtained. There was considerable inter-strain variation in heat resistance, with D-values ranging from 397.83 to 689 sec at 55ºC, 11.35 to 260.95 sec at 60ºC and 1.12 to 6.81 at 65ºC. Likewise, z-values ranged from 2.95 to 5.44°C. One strain (2278) demonstrated a significantly higher thermal tolerance, even though it had been isolated from a meal feed. The presence of such a heat resistant strain with an ASSuT antibiotic resistance profile is a real concern in terms of its transmission though the food chain to the consumer, as heat treatment is one of the most effective means of ensuring the microbial safety of feeds. However, overall, the strains investigated do not appear to be that much more heat resistant than *Salmonella* previously studied.

The second part of this Chapter 4 involved assessing the ability of the five *Salmonella* strains to survive during storage over a 28-day period in pelleted weaner pig feed treated with 0.3% sodium butyrate and stored under environmental conditions similar to those used on-farm. While a mean reduction in the *Salmonella* count of 0.79 $\log_{10}$ CFU was seen in the treated feed during the storage period, a reduction (albeit only 0.49 $\log_{10}$ CFU) was also observed in the control feed. Although there was no overall effect of treatment, sodium butyrate resulted in reductions in *Salmonella* counts of 0.75 and 0.22 $\log_{10}$ CFU at days 14 and 24 of feed storage, respectively but at the end of the 28-day storage period counts were 0.25 $\log_{10}$ CFU higher in the treated feed. Therefore, the sodium butyrate used appears unsuitable as an agent for feed treatment and this lack of efficacy may be due to the fact that the particular feed additive used has a protective coating.

There is limited scientific literature on the differing types of pig feed and the merits and demerits of their role in control or reduction of the prevalence of *Salmonella* spp in pigs. Although some studies have taken place on acidification of feed, coarse ration introduction and wet feeding resulting in considerable evidence to suggest that manipulation of the pH of the feed, on farm or in the stomach of the animal can impact the introduction of *Salmonella* to the herd. These possible benefits for farmers in adjusting their feed regime need to be further examined, so that herdowners can make informed judgements on how to mitigate the *Salmonella* spp risk to their herd. The work presented in this thesis enhances knowledge about the behaviour and survival characteristics of monophasic variants of *S.* Typhimurium (4,[5],12:i:) in animal feed and may assist the feed industry and pig producers in implementing effective intervention strategies for their control.

6. Further research

This research has highlighted many areas that would benefit from further investigation. It would have been of interest to investigate other areas surrounding this project such as:

- Further studies of the antimicrobial-resistant genes in the *Salmonella* isolates recovered, including determination of the mode of gene acquisition and the relation to DT104 strain and the monophasic variants of other possible *Salmonella* serovars.
- To investigate the plasmids and mobile elements carrying antimicrobial resistance and virulence genes within the monophasic S. Typhimurium isolates.
- To investigate the monophasic S. Typhimurium isolates pathogenicity and whether they pose any greater of risk of persistence in the pork chain than other serovars.
- Whole genome sequencing to enable Irish monophasic *S.* Typhimurium isolates to be placed within the phylogenetic context of other European isolates (UK, Spain, Portugal, Italy, etc.)
- Phenotypic studies to assess the ability of porcine and feed isolates to form biofilms and persist in the pork chain environment.
- Murine colitis model or other suitable models to allow the assessment of human pathogenicity of monophasic S. Typhimurium isolates.
- Antimicrobial resistance to heavy metals i.e. Cu, and Zn, which are used as micronutrients or antimicrobials in pig feed to investigate the hypothesis that acquisition of such resistance may have created a selective pressure for the emergence of this serovar.

7. **Appendices**

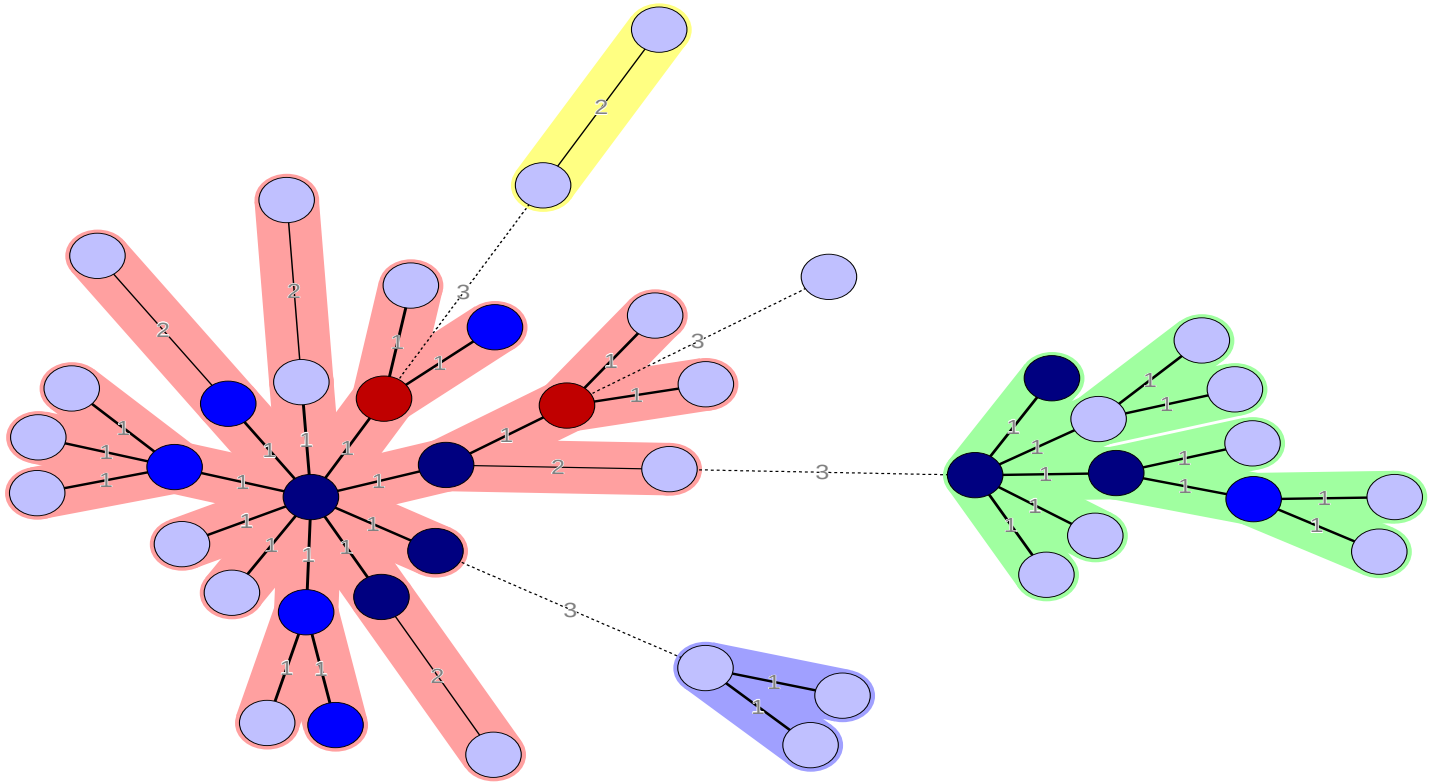

Figure A1: Minimum-spanning tree of multilocus variable number tandem repeat analysis (MLVA) of *Salmonella enterica* serotype *S.* Typhimurium, *S.* Typhimurium Copenhagen and/ or monophasic variants of *S.* Typhimurium (1,4,[5],12:i) strains isolated from farms and mills. Each MLVA profile is indicated by one node or branch tip, displayed as circles that are connected by branches on a minimum-spanning tree. The length and the colour of the branches represent genetic distances (changes in loci) between two neighboring types. The sizes of the different colour circles depend on their population size.

Table A1: Multilocus variable number tandem repeat analysis (MLVA) clusters for *Typhimurium* and *Salmonella enterica* serotype 1,4,[5],12:– strains isolated from farms and mills.

Isolate	Genus	Farm[a]	Stage[a]	Sample Type	Visit[a]	Antibiotic Profile[b]	MLVA Profile[c]
660A	Typhimurium	D	Finishers	Pen	1	ACSSuT	3-15-7-12-0311
694E	4,5,12:i	D	Farrowing	Trough	1	CSuT	3-15-7-12-0311
2455C	Typhimurium	D	Farrowing	Feed	2	ACSSuT	3-15-6-12-0311
598B	4:12:i	D	1st Stage Weaners	Water	1	ACSuT	3-15-7-12-NA
2977A	Typhimurium	G	Finishers	Pen	2	Susceptible	4-11-17-8-0111
2978A	Typhimurium	G	Finishers	Faecal	2	Susceptible	4-11-17-8-0111
3003A	Typhimurium	G	Gilts	Pen	2	Susceptible	4-11-17-8-0111
3005A	Typhimurium	G	Gilts	Water	2	Susceptible	4-11-17-8-0111
3006A	Typhimurium	G	Gilts	Water Drinker	2	Susceptible	4-11-17-8-0111
3009A	Typhimurium	G	Gilts	Pen	2	Susceptible	4-11-17-8-0111
1086C§	Typhimurium Copenhagen	G	Finishers	Pen	1	ASuT	4-11-14-8-0111
1092A§	Typhimurium Copenhagen	G	Gilts	Feed	1	Tazt	4-11-14-8-0111
1089D§	Typhimurium	G	Gilts	Faecal	1	Susceptible	4-12-17-8-0111
1098A§	Typhimurium	G	Gilts	Feed	1	Susceptible	4-12-17-8-0111
1323B	Typhimurium Copenhagen	I	2nd Stage Weaners	Trough	1	A(C)TAmFAzt	4-13-17-8-0111
1367A	Typhimurium Copenhagen	I	Gilts	Water Drinker	1	T	4-13-17-8-0111
1376A	Typhimurium Copenhagen	I	Gilts	Pen	1	T	4-13-17-8-0111
3338A	Typhimurium	I	Finishers	Water	2	T	4-13-17-8-0111
3341A	Typhimurium	I	Finishers	Trough	2	T	4-13-17-8-0111
3356A	Typhimurium	I	Finishers	Water	2	T	4-13-17-8-0111
3379A	Typhimurium	I	Gilts	Trough	2	T	4-13-17-8-0111
2936A	Typhimurium	G	1st Stage Weaners	Pen	2	ASSuT	4-11-17-9-0111
1048A	Typhimurium	G	2nd Stage Weaners	Pen	1	Susceptible	4-11-16-8-0111
1060C	Typhimurium	G	2nd Stage Weaners	Pen	1	Susceptible	4-11-16-8-0111
1067B	Typhimurium	G	Finishers	Trough	1	Tm	4-11-16-8-0111
1068A	Typhimurium	G	Finishers	Pen	1	Susceptible	4-11-16-8-0111
1106A	Typhimurium	G	Gilts	Pen	1	Su	4-11-16-8-0111
2954A	Typhimurium	G	2nd Stage Weaners	Water Drinker	2	ASSuTTm	4-11-16-8-0111
2956A	Typhimurium	G	2nd Stage Weaners	Trough	2	Susceptible	4-11-16-8-0111
2957A	Typhimurium	G	2nd Stage Weaners	Pen	2	Susceptible	4-11-16-8-0111
2971A	Typhimurium	G	Finishers	Pen	2	Susceptible	4-11-16-8-0111
2976B	Typhimurium	G	Finishers	Trough	2	Susceptible	4-11-16-8-0111
2906A	Typhimurium	G	Dry Sow	Water	2	ASSuT	4-11-18-9-0111
2963A	Typhimurium	G	2nd Stage Weaners	Pen	2	ASSuT	4-11-18-9-0111
2930A	Typhimurium	G	1st Stage Weaners	Pen	2	ASSuT	5-11-17-9-0111
3339A	Typhimurium	I	Finishers	Water Drinker	2	T	4-13-20-8-0111
3391A	Typhimurium	I	Gilts	Trough	2	T	4-13-20-8-0111

Table A1 contd.: Multilocus variable number tandem repeat analysis (MLVA) clusters for *Typhimurium* and *Salmonella enterica* serotype 1,4,[5],12:– strains isolated from farms and mills.

Isolate	Genus	Farm[a]	Stage[a]	Sample Type	Visit[a]	Antibiotic Profile[b]	MLVA Profile[c]
1338A	Typhimurium Copenhagen	I	Finishers	Pen	1	T	4-13-18-8-0111
1339A	Typhimurium Copenhagen	I	Finishers	Faecal	1	T(Ce)	4-13-18-8-0111
1344A	4,5,12:i	I	Finishers	Pen	1	T	4-13-18-8-0111
1337C	Typhimurium Copenhagen	I	Finishers	Trough	1	ST	4-13-18-8-NA
2911A	Typhimurium	I	Dry Sow	Faecal	2	Susceptible	4-12-18-9-0111
911A	Typhimurium	F	2nd Stage Weaners	Pen	1	ASSuTNa(Am)	6-17-9-13-0211
678A	4:12:i	D	Gilts	Feed	1	ACSuT	
3494A	4:12:i	J	1st Stage Weaners	Feed Bin	2	Su	
3088A	Typhimurium	H	1st Stage Weaners	Water	2	ASSuT	0-14-NA-NA-0012
343A	4:12:i	B	2nd Stage Weaners	Water Drinker	1	ASSuT	3-14-10-NA-NA
1950A	4,5,12:i	B	1st Stage Weaners	Pen	2	ASSuT	3-14-9-NA-0211
3083C	4,5,12:i	H	1st Stage Weaners	Water Drinker	2	ASSuTAzt(F)	3-14-9-NA-0211
3094A	Typhimurium	H	2nd Stage Weaners	Water	2	ASSuAzt	3-14-9-NA-0211
309C§	4:12:i	B	1st Stage Weaners	Faecal	1	ASSuTTm	3-14-10-NA-0211
314A§	4:12:i	B	1st Stage Weaners	Pen	1	ASSuTTm	3-14-10-NA-0211
315D§	4:12:i	B	1st Stage Weaners	Faecal	1	ACSSuTTmGmAzt	3-14-10-NA-0211
322D§	4:12:i	B	1st Stage Weaners	Water Drinker	1	ASSuTTm	3-14-10-NA-0211
323A§	4:12:i	B	1st Stage Weaners	Water Drinker	1	ASSuTTmCp	3-14-10-NA-0211
327B§	4:12:i	B	1st Stage Weaners	Faecal	1	A(C)SSuTTm(F)	3-14-10-NA-0211
329B§	4:12:i	B	1st Stage Weaners	Water Drinker	1	ASSuTTm	3-14-10-NA-0211
342E§	4:12:i	B	2nd Stage Weaners	Water	1	ASSuT	3-14-10-NA-0211
364A§	4:12:i	B	Finishers	Feed	1	ASSuT	3-14-10-NA-0211
1939A§	4:12:i	B	1st Stage Weaners	Faecal	2	ASSuTTm	3-14-10-NA-0211
1940A§	4:12:i	B	1st Stage Weaners	Water	2	ASSuTTm	3-14-10-NA-0211
1941A§	4:12:i	B	1st Stage Weaners	Water Drinker	2	ASSuTTm	3-14-10-NA-0211
1943A§	4:12:i	B	1st Stage Weaners	Trough	2	ASSuTTm	3-14-10-NA-0211
1944A§	4:12:i	B	1st Stage Weaners	Pen	2	ASSuTTm	3-14-10-NA-0211
1948B§	4:12:i	B	1st Stage Weaners	Feed	2	Ce	3-14-10-NA-0211
1951A§	4:12:i	B	1st Stage Weaners	Faecal	2	ASSuTTm	3-14-10-NA-0211
1956A§	4:12:i	B	1st Stage Weaners	Pen	2	ASSuTTm	3-14-10-NA-0211
1958A§	4:12:i	B	1st Stage Weaners	Water	2	ASSuTTm	3-14-10-NA-0211
1959A§	4:12:i	B	1st Stage Weaners	Water Drinker	2	ASSuTTm	3-14-10-NA-0211
1962A§	4:12:i	B	1st Stage Weaners	Pen	2	ASSuTTm	3-14-10-NA-0211
1969A§	4:12:i	B	2nd Stage Weaners	Faecal	2	ASSuTTmF	3-14-10-NA-0211

Table A1 contd.: Multilocus variable number tandem repeat analysis (MLVA) clusters for *Typhimurium* and *Salmonella enterica* serotype 1,4,[5],12:– strains isolated from farms and mills.

Isolate	Genus	Farm[a]	Stage[a]	Sample Type	Visit[a]	Antibiotic Profile[b]	MLVA Profile[c]
1971A§	4:12:i	B	2nd Stage Weaners	Water Drinker	2	ASSuTTm	3-14-10-NA-0211
1973A§	4:12:i	B	2nd Stage Weaners	Trough	2	ASSuTTm	3-14-10-NA-0211
1974A§	4:12:i	B	2nd Stage Weaners	Pen	2	ASSuTTm	3-14-10-NA-0211
1976A§	4:12:i	B	2nd Stage Weaners	Water	2	ASSuTTm	3-14-10-NA-0211
1977A§	4:12:i	B	2nd Stage Weaners	Water Drinker	2	ASSuTTm	3-14-10-NA-0211
1979A§	4:12:i	B	2nd Stage Weaners	Trough	2		3-14-10-NA-0211
1980A§	4:12:i	B	2nd Stage Weaners	Pen	2	ASSuTTm	3-14-10-NA-0211
1981A	4:12:i	B	2nd Stage Weaners	Faecal	2	ASSuTTm	3-14-10-NA-0211
1989B	4:12:i	B	2nd Stage Weaners	Water Drinker	2	Ssu	3-14-10-NA-0211
1757A	4:12:i	A	2nd Stage Weaners	Feed	2	ASSuTTm	3-9-10-NA-0211
1985A	Stanley	B	2nd Stage Weaners	Trough	2		1-9-10-NA-0013
3476A‡	4,5,12:i	J	1st Stage Weaners	Faecal	2	ACSSuTTmGm	3-13-16-NA-0211
3836A‡	4,5,12:i	Mill	Wheat			ACSSuTTmGm	3-13-16-NA-0211
3844A‡	4,5,12:i	Mill	Dry Sow Meal	Meal		ACSSuTTmGm	3-13-16-NA-0211
3845A‡	4,5,12:i	Mill	Dry Sow Pellets	Pellets		ACSTCpCe	3-13-16-NA-0211
1054D	Typhimurium	G	2nd Stage Weaners	Pen	1	ACSSuTTm(Am)	3-13-16-19-0311
3520A	4,5,12:i	J	2nd Stage Weaners	Faecal	2	ASSuT	3-16-10-NA-0211
3524A	4,5,12:i	J	2nd Stage Weaners	Trough	2	ASSuT	3-16-10-NA-0211
3525A	4,5,12:i	J	2nd Stage Weaners	Pen	2	ASSuT(F)	3-16-10-NA-0211
3529A	4,5,12:i	J	Gilts	Water	2	ASSuTAzt	3-16-12-NA-0211
3521A	4,5,12:i	J	2nd Stage Weaners	Water	2	ASSuT	3-16-10-NA-NA
3528A	4,5,12:i	J	Gilts	Faecal	2	ASSuTGm(Am)	3-16-11-NA-0211
3500B	4,5,12:i	J	2nd Stage Weaners	Trough	2	ACSSuTTmGm	3-13-10-NA-NA
3508A	4,5,12:i	J	2nd Stage Weaners	Faecal	2	ACSSuTTmGm	3-13-10-NA-NA
2024A	4:12:i	B	Finishers	Pen	2	ASSuTTm	3-13-9-NA-0211
171B	4:12:i	A	1st Stage Weaners	Faecal	1	ASSuTTm	3-12-10-NA-0211
179B	4:12:i	A	2nd Stage Weaners	Faecal	1	ASSuTTm	3-12-10-NA-0211
286B	4:12:i	A	Farrowing	Pen	1	ASSuTTm	3-12-10-NA-0211
316E	4:12:i	B	1st Stage Weaners	Water	1	ASSuTTm	3-12-10-NA-0211
1446A	4:12:i	J	1st Stage Weaners	Faecal	1	ASSuT(Am)	3-12-14-NA-0211
211A§	4:12:i	A	Finishers	Faecal	1	ASSuTTm	3-12-9-NA-0211
234B§	4,5,12:i	A	Finishers	Pen	1	ASSuTTm	3-12-9-NA-0211
248A§	4:12:i	A	Gilts	Pen	1	ASSuTTm	3-12-9-NA-0211
287A§	4,5,12:i	A	Farrowing	Feed Bin	1	ASSuTTmKm	3-12-9-NA-0211
1448A	4:12:i	J	1st Stage Weaners	Water Drinker	1	ASSuT	3-13-11-NA-0211

Table A1 contd.: Multilocus variable number tandem repeat analysis (MLVA) clusters for *Typhimurium* and *Salmonella enterica* serotype 1,4,[5],12:– strains isolated from farms and mills.

Isolate	Genus	Farm[a]	Stage[a]	Sample Type	Visit[a]	Antibiotic Profile[b]	MLVA Profile[c]
1451A	4:12:i	J	1st Stage Weaners	Pen	1	ASSuT	3-13-11-NA-0211
1495C	4:12:i	J	2nd Stage Weaners	Pen	1	ACSSuTTmGmFKm	3-13-11-NA-0211
1620C	4,5,12:i	J	Farrowing	Trough	1	ACSSuTTmGm	3-13-11-NA-0211
2207A	4,5,12:i	C	Finishers	Pen	2	ASSuT	3-13-11-NA-0211
2213A	4,5,12:i	C	Finishers	Pen	2	ASSuT	3-13-11-NA-0211
3541A	Typhimurium	J	Gilts	Water	2	ACSSuTTm	5-19-11-NA-0211
3542B	Typhimurium	J	Gilts	Water Drinker	2	ACSSuTTm	5-19-11-NA-0211
335B	4:12:i	B	2nd Stage Weaners	Faecal	1	ASSuTTm	3-15-10-NA-0211
337D	4:12:i	B	2nd Stage Weaners	Water Drinker	1	ASSuTTm	3-15-10-NA-0211
384C	4:12:i	B	Finishers	Pen	1	ASSuT	3-15-10-NA-0211
2002A	4:12:i	B	Finishers	Water	2	ASSuTTm	3-15-10-NA-0211
2003A	4:12:i	B	Finishers	Water Drinker	2	ASSuTm	3-15-10-NA-0211
2005A	4:12:i	B	Finishers	Trough	2	ASSuTTm	3-15-10-NA-0211
167D§	4:12:i	A	1st Stage Weaners	Water Drinker	1	ASSuTTm	3-11-10-NA-0211
180B§	4:12:i	A	2nd Stage Weaners	Water	1	ASSuT	3-11-10-NA-0211
182B§	4:12:i	A	2nd Stage Weaners	Feed	1	ASSuTTm	3-11-10-NA-0211
197B§	4:12:i	A	2nd Stage Weaners	Faecal	1	ASSuTTm	3-11-10-NA-0211
229A§	4:12:i	A	Finishers	Faecal	1	ASSuTTm(F)	3-11-10-NA-0211
1733C§	4:12:i	A	1st Stage Weaners	Pen	2	ASSuTTm	3-11-10-NA-0211
1759B§	4,5,12:i	A	2nd Stage Weaners	Pen	2	ASSuT	3-11-10-NA-0211
153D‡	4:12:i	A	1st Stage Weaners	Faecal	1	ASSuT	3-11-9-NA-0211
155A‡	4:12:i	A	1st Stage Weaners	Water Drinker	1	ASSuT	3-11-9-NA-0211
158A‡	4:12:i	A	1st Stage Weaners	Pen	1	ASSuT	3-11-9-NA-0211
160A‡	4:12:i	A	1st Stage Weaners	Water	1	ASSuT	3-11-9-NA-0211
181A‡	4:12:i	A	2nd Stage Weaners	Water Drinker	1	ASSuT	3-11-9-NA-0211
185A‡	4:12:i	A	2nd Stage Weaners	Faecal	1	AT	3-11-9-NA-0211
187A‡	4:12:i	A	2nd Stage Weaners	Water Drinker	1	ASSuTTm	3-11-9-NA-0211
191B‡	4:12:i	A	2nd Stage Weaners	Faecal	1	ASSuT	3-11-9-NA-0211
216A‡	4:12:i	A	Finishers	Pen	1	ASSuT	3-11-9-NA-0211
1728A‡	4:12:i	A	1st Stage Weaners	Faecal	2	ASSuT	3-11-9-NA-0211
1740A‡	4:12:i	A	1st Stage Weaners	Faecal	2	ASSuT	3-11-9-NA-0211
1746A‡	4:12:i	A	1st Stage Weaners	Faecal	2	ASSuTF	3-11-9-NA-0211
1754C‡	4:12:i	A	2nd Stage Weaners	Faecal	2	SuTTmGmCe	3-11-9-NA-0211
1760A‡	4:12:i	A	2nd Stage Weaners	Faecal	2	ASSuT	3-11-9-NA-0211
1764C‡	4:12:i	A	2nd Stage Weaners	Trough	2	ASSuT	3-11-9-NA-0211

Table A1 contd.: Multilocus variable number tandem repeat analysis (MLVA) clusters for *Typhimurium* and *Salmonella enterica* serotype 1,4,[5],12:– strains isolated from farms and mills.

Isolate	Genus	Farm[a]	Stage[a]	Sample Type	Visit[a]	Antibiotic Profile[b]	MLVA Profile[c]
1765A‡	4,5,12:i	A	2nd Stage Weaners	Pen	2	ASSuT	3-11-9-NA-0211
1769B‡	4:12:i	A	2nd Stage Weaners	Feed	2	ASSuTTmNa	3-11-9-NA-0211
1770A‡	4:12:i	A	2nd Stage Weaners	Trough	2	ASSuT	3-11-9-NA-0211
1772B‡	4:12:i	A	2nd Stage Weaners	Faecal	2	ASSuT	3-11-9-NA-0211
1774A‡	4,5,12:i	A	2nd Stage Weaners	Water Drinker	2	ASSuT	3-11-9-NA-0211
1776A‡	4,5,12:i	A	2nd Stage Weaners	Trough	2	ASSuT	3-11-9-NA-0211
1777A‡	4,5,12:i	A	2nd Stage Weaners	Pen	2	ASuT	3-11-9-NA-0211
1792A‡	4:12:i	A	Finishers	Pen	2	ASSuT	3-11-9-NA-0211
1870C‡	4,5,12:i	A	Finishers	Water Drinker	2	ASSuT	3-11-9-NA-0211
1872A‡	4,5,12:i	A	Finishers	Trough	2	ASSuT	3-11-9-NA-0211
1873A‡	4,5,12:i	A	Finishers	Pen	2	ASSuT	3-11-9-NA-0211
1882A‡	4:12:i	A	Finishers	Water Drinker	2	ASSuT	3-11-9-NA-0211
1884A‡	4:12:i	A	Finishers	Trough	2	ASSuT	3-11-9-NA-0211
1885A‡	4:12:i	A	Finishers	Pen	2	ASSuT	3-11-9-NA-0211
2278A‡	4,5,12:i	Mill	Whey Grower Feed	Meal	2	ASSuT	3-11-9-NA-0211
2888B‡	4:12:i	Mill	Soya		2	TGm	3-11-9-NA-0211
2985A‡	4,5,12:i	G	Finishers	Water	2	T	3-11-9-NA-0211
2994A‡	4,5,12:i	G	Gilts	Water Drinker	2	T	3-11-9-NA-0211
2999A‡	4,5,12:i	G	Gilts	Water	2	T	3-11-9-NA-0211
217A	4,5,12:i	A	Finishers	Pen	1	T	3-11-9-NA-0212
591C	4,5,12:i	D	Dry Sow	Water Drinker	1	T	3-11-9-NA-NA
3593A	Typhimurium	J	Finishers	Water Drinker	2	ACSSUTTmGm	3-11-13-8-0211
1465A	4:12:i	J	1st Stage Weaners	Water	1	ACSSuTTmGm	3-13-10-NA-0211
1468A	4:12:i	J	1st Stage Weaners	Trough	1	ACSSuTTmGm	3-13-10-NA-0211
1469C	4:12:i	J	1st Stage Weaners	Pen	1	ACSSuTTmGmF	3-13-10-NA-0211
3496A	4,5,12:i	J	2nd Stage Weaners	Faecal	2	ACSSuTTmGm	3-13-10-NA-0211
3501A	4,5,12:i	J	2nd Stage Weaners	Pen	2	TAmAzt	3-13-10-NA-0211
3519A	4,5,12:i	J	2nd Stage Weaners	Pen	2	ACSSuTTmGm	3-13-10-NA-0211

[a] Source i.e. farm and stage of production from where the isolate was isolated from, isolates were isolated from farms (A-J) with a history of high (>50%) *Salmonella* seroprevalence. Farms were sampled on one occasion between March-August 2012 and again between December-June 2013.

[b] Antibiotic profile Azithromycin (Azt), Ampicillin (A), Amoxicillin/Clavulinic acid (Am), Chloramphenicol (C), Ciprofloxacin (Cp), Trimethoprim/Sulfamethoxazole (Tm), Cefoxitin (F), Gentamicin (Gm), Kanamycin (Km), Nalidixic acid (Na), Sulfisoxazole (Su), Streptomycin (S), Tetracycline (T) and Ceftiofur (Ce).

[c] The MLVA profile using the five loci ST9-ST5-ST6-STTR10-ST3 (Lindestadt et al., 2004) was used for cluster analysis using BioNumerics v6.0 software.

[§] These feed isolates had the same MLVA profile as isolates isolated from the farms.

[‡] These feed isolates isolated from the feed mills had the same MLVA profile as isolates isolated from the farms.

Table A2: Multilocus variable number tandem repeat analysis (MLVA) clusters and grouping for *Typhimurium* and *Salmonella enterica* serotype 1,4,[5],12:– strains isolated from farms and mills; isolates were grouped in larger clusters and were considered related if the difference in the MLVA profile in the number of repeats at the contingency loci was observed directly to be minimal.

Isolate	Genus	Farm[a]	Stage[a]	Sample Type	Antibiotic Profile[b]	MLVA Profile[c]	Group/No. Isolates*
660A	Typhimurium	D	Finishers	Pen	ACSSuT	3-15-7-12-0311	1(4)
694E	4,5,12:i	D	Farrowing	Trough	CSuT	3-15-7-12-0311	
2455C	Typhimurium	D	Farrowing	Feed	ACSSuT	3-15-6-12-0311	
598B	4:12:i	D	1st Stage Weaners	Water	ACSuT	3-15-7-12-NA	
2977A	Typhimurium	G	Finishers	Pen	Susceptible	4-11-17-8-0111	2(6)
2978A	Typhimurium	G	Finishers	Faecal	Susceptible	4-11-17-8-0111	
3003A	Typhimurium	G	Gilts	Pen	Susceptible	4-11-17-8-0111	
3005A	Typhimurium	G	Gilts	Water	Susceptible	4-11-17-8-0111	
3006A	Typhimurium	G	Gilts	Water Drinker	Susceptible	4-11-17-8-0111	
3009A	Typhimurium	G	Gilts	Pen	Susceptible	4-11-17-8-0111	
1086C§	Typhimurium Copenhagen	G	Finishers	Pen	ASuT	4-11-14-8-0111	3(2)
1092A§	Typhimurium Copenhagen	G	Gilts	Feed	Tazt	4-11-14-8-0111	
1089D§	Typhimurium	G	Gilts	Faecal	Susceptible	4-12-17-8-0111	4(9)
1098A§	Typhimurium	G	Gilts	Feed	Susceptible	4-12-17-8-0111	
1323B	Typhimurium Copenhagen	I	2nd Stage Weaners	Trough	A(C)TAmFAzt	4-13-17-8-0111	
1367A	Typhimurium Copenhagen	I	Gilts	Water Drinker	T	4-13-17-8-0111	
1376A	Typhimurium Copenhagen	I	Gilts	Pen	T	4-13-17-8-0111	
3338A	Typhimurium	I	Finishers	Water	T	4-13-17-8-0111	
3341A	Typhimurium	I	Finishers	Trough	T	4-13-17-8-0111	
3356A	Typhimurium	I	Finishers	Water	T	4-13-17-8-0111	
3379A	Typhimurium	I	Gilts	Trough	T	4-13-17-8-0111	
2936A	Typhimurium	G	1st Stage Weaners	Pen	ASSuT	4-11-17-9-0111	5(11)
1048A	Typhimurium	G	2nd Stage Weaners	Pen	Susceptible	4-11-16-8-0111	
1060C	Typhimurium	G	2nd Stage Weaners	Pen	Susceptible	4-11-16-8-0111	
1067B	Typhimurium	G	Finishers	Trough	Tm	4-11-16-8-0111	
1068A	Typhimurium	G	Finishers	Pen	Susceptible	4-11-16-8-0111	
1106A	Typhimurium	G	Gilts	Pen	Su	4-11-16-8-0111	
2954A	Typhimurium	G	2nd Stage Weaners	Water Drinker	ASSuTTm	4-11-16-8-0111	
2956A	Typhimurium	G	2nd Stage Weaners	Trough	Susceptible	4-11-16-8-0111	
2957A	Typhimurium	G	2nd Stage Weaners	Pen	Susceptible	4-11-16-8-0111	
2971A	Typhimurium	G	Finishers	Pen	Susceptible	4-11-16-8-0111	
2976B	Typhimurium	G	Finishers	Trough	Susceptible	4-11-16-8-0111	

Table A2 contd.: MLVA clusters and grouping for *Typhimurium* and *Salmonella enterica* serotype 1,4,[5],12:– strains isolated from farms and mills

Isolate	Genus	Farm[a]	Stage[a]	Sample Type	Antibiotic Profile[b]	MLVA Profile[c]	Group/No. Isolates*
3339A	Typhimurium	I	Finishers	Water Drinker	T	4-13-20-8-0111	6(7)
3391A	Typhimurium	I	Gilts	Trough	T	4-13-20-8-0111	
1338A	Typhimurium Copenhagen	I	Finishers	Pen	T	4-13-18-8-0111	
1339A	Typhimurium Copenhagen	I	Finishers	Faecal	T(Ce)	4-13-18-8-0111	
1344A	4,5,12:i	I	Finishers	Pen	T	4-13-18-8-0111	
1337C	Typhimurium Copenhagen	I	Finishers	Trough	ST	4-13-18-8-NA	
2911A	Typhimurium	I	Dry Sow	Faecal	Susceptible	4-12-18-9-0111	
678A	4:12:i	D	Gilts	Feed	ACSuT		7(2)
3494A	4:12:i	J	1st Stage Weaners	Feed Bin	Su		
343A	4:12:i	B	2nd Stage Weaners	Water Drinker	ASSuT	3-14-10-NA-NA	8(25)
1950A	4,5,12:i	B	1st Stage Weaners	Pen	ASSuT	3-14-9-NA-0211	
3083C	4,5,12:i	H	1st Stage Weaners	Water Drinker	ASSuTAzt(F)	3-14-9-NA-0211	
3094A	Typhimurium	H	2nd Stage Weaners	Water	ASSuAzt	3-14-9-NA-0211	
309C§	4:12:i	B	1st Stage Weaners	Faecal	ASSuTTm	3-14-10-NA-0211	
314A§	4:12:i	B	1st Stage Weaners	Pen	ASSuTTm	3-14-10-NA-0211	
315D§	4:12:i	B	1st Stage Weaners	Faecal	ACSSuTTmGmAzt	3-14-10-NA-0211	
322D§	4:12:i	B	1st Stage Weaners	Water Drinker	ASSuTTm	3-14-10-NA-0211	
323A§	4:12:i	B	1st Stage Weaners	Water Drinker	ASSuTTmCp	3-14-10-NA-0211	
327B§	4:12:i	B	1st Stage Weaners	Faecal	A(C)SSuTTm(F)	3-14-10-NA-0211	
329B§	4:12:i	B	1st Stage Weaners	Water Drinker	ASSuTTm	3-14-10-NA-0211	
342E§	4:12:i	B	2nd Stage Weaners	Water	ASSuT	3-14-10-NA-0211	
364A§	4:12:i	B	Finishers	Feed	ASSuT	3-14-10-NA-0211	
1939A§	4:12:i	B	1st Stage Weaners	Faecal	ASSuTTm	3-14-10-NA-0211	
1940A§	4:12:i	B	1st Stage Weaners	Water	ASSuTTm	3-14-10-NA-0211	
1941A§	4:12:i	B	1st Stage Weaners	Water Drinker	ASSuTTm	3-14-10-NA-0211	
1943A§	4:12:i	B	1st Stage Weaners	Trough	ASSuTTm	3-14-10-NA-0211	
1944A§	4:12:i	B	1st Stage Weaners	Pen	ASSuTTm	3-14-10-NA-0211	
1948B§	4:12:i	B	1st Stage Weaners	Feed	Ce	3-14-10-NA-0211	
1951A§	4:12:i	B	1st Stage Weaners	Faecal	ASSuTTm	3-14-10-NA-0211	
1956A§	4:12:i	B	1st Stage Weaners	Pen	ASSuTTm	3-14-10-NA-0211	
1958A§	4:12:i	B	1st Stage Weaners	Water	ASSuTTm	3-14-10-NA-0211	
1959A§	4:12:i	B	1st Stage Weaners	Water Drinker	ASSuTTm	3-14-10-NA-0211	
1962A§	4:12:i	B	1st Stage Weaners	Pen	ASSuTTm	3-14-10-NA-0211	
1969A§	4:12:i	B	2nd Stage Weaners	Faecal	ASSuTTmF	3-14-10-NA-0211	

Table A2 contd.: MLVA clusters and grouping for *Typhimurium* and *Salmonella enterica* serotype 1,4,[5],12:– strains isolated from farms and mills

Isolate	Genus	Farm[a]	Stage[a]	Sample Type	Antibiotic Profile[b]	MLVA Profile[c]	Group/No. Isolates*
1765A‡	4,5,12:i	A	2nd Stage Weaners	Pen	ASSuT	3-11-9-NA-0211	9(20)
1769B‡	4:12:i	A	2nd Stage Weaners	Feed	ASSuTTmNa	3-11-9-NA-0211	
1770A‡	4:12:i	A	2nd Stage Weaners	Trough	ASSuT	3-11-9-NA-0211	
1772B‡	4:12:i	A	2nd Stage Weaners	Faecal	ASSuT	3-11-9-NA-0211	
1774A‡	4,5,12:i	A	2nd Stage Weaners	Water Drinker	ASSuT	3-11-9-NA-0211	
1776A‡	4,5,12:i	A	2nd Stage Weaners	Trough	ASSuT	3-11-9-NA-0211	
1777A‡	4,5,12:i	A	2nd Stage Weaners	Pen	ASuT	3-11-9-NA-0211	
1792A‡	4:12:i	A	Finishers	Pen	ASSuT	3-11-9-NA-0211	
1870C‡	4,5,12:i	A	Finishers	Water Drinker	ASSuT	3-11-9-NA-0211	
1872A‡	4,5,12:i	A	Finishers	Trough	ASSuT	3-11-9-NA-0211	
1873A‡	4,5,12:i	A	Finishers	Pen	ASSuT	3-11-9-NA-0211	
1882A‡	4:12:i	A	Finishers	Water Drinker	ASSuT	3-11-9-NA-0211	
1884A‡	4:12:i	A	Finishers	Trough	ASSuT	3-11-9-NA-0211	
1885A‡	4:12:i	A	Finishers	Pen	ASSuT	3-11-9-NA-0211	
2278A‡	4,5,12:i	Mill	Whey Grower Feed	Meal	ASSuT	3-11-9-NA-0211	
2888B‡	4:12:i	Mill	Soya		TGm	3-11-9-NA-0211	
2985A‡	4,5,12:i	G	Finishers	Water	T	3-11-9-NA-0211	
2994A‡	4,5,12:i	G	Gilts	Water Drinker	T	3-11-9-NA-0211	
2999A‡	4,5,12:i	G	Gilts	Water	T	3-11-9-NA-0211	
217A	4,5,12:i	A	Finishers	Pen	T	3-11-9-NA-0212	
1465A	4:12:i	J	1st Stage Weaners	Water	ACSSuTTmGm	3-13-10-NA-0211	10(6)
1468A	4:12:i	J	1st Stage Weaners	Trough	ACSSuTTmGm	3-13-10-NA-0211	
1469C	4:12:i	J	1st Stage Weaners	Pen	ACSSuTTmGmF	3-13-10-NA-0211	
3496A	4,5,12:i	J	2nd Stage Weaners	Faecal	ACSSuTTmGm	3-13-10-NA-0211	
3501A	4,5,12:i	J	2nd Stage Weaners	Pen	TAmAzt	3-13-10-NA-0211	
3519A	4,5,12:i	J	2nd Stage Weaners	Pen	ACSSuTTmGm	3-13-10-NA-0211	

[a] Source i.e. farm and stage of production from where the isolate was isolated from, isolates were isolated from farms (A-J) with a history of high (>50%) *Salmonella* seroprevalence.

[b] Antibiotic profile Azithromycin (Azt), Ampicillin (A), Amoxicillin/Clavulinic acid (Am), Chloramphenicol (C), Ciprofloxacin (Cp), Trimethoprim/Sulfamethoxazole (Tm), Cefoxitin (F), Gentamicin (Gm), Kanamycin (Km), Nalidixic acid (Na), Sulfisoxazole (Su), Streptomycin (S), Tetracycline (T) and Ceftiofur (Ce).

[c] The MLVA profile using the five loci ST9-ST5-ST6-STTR10-ST3 (Lindestadt et al., 2004) was used for cluster analysis using BioNumerics v6.0 software.

[§] These feed isolates had the same MLVA profile as isolates isolated from the farms.

[‡] These feed isolates isolated from the feed mills had the same MLVA profile as isolates isolated from the farms.

[*] When deviations from the MLVA profile were minimal a loss or gain in a repeat at the contingency locus was observed; losses and gain in one repeat at loci is more likely to occur in related isolates. Using this rule of thumb the isolates were broken down into 10 groups (a cluster is when five or more isolates had the same MLVA profile). Note feed samples were included even if not belonging to a group big enough to form a cluster.

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Salmonella occurrence and *Enterobacteriaceae* counts in pig feed ingredients and compound feed from feed mills in Ireland

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ABSTRACT

The purpose of this study was to assess the occurrence of non-typhoidal *Salmonellae* and Enterobacteriaceae counts in raw ingredients and compound feeds sampled from feed mills manufacturing pig diets. Between November 2012 and September 2013, feed ingredients ($n = 340$) and compound pig feed ($n = 313$) samples were collected from five commercial feed mills and one home compounder at various locations throughout Ireland. Feed ingredients included cereals, vegetable protein sources and by-products of oil extraction and ethanol production. The compound feeds included meal and pelleted feed for all stages of pig production. Samples were analysed for *Salmonella* using standard enrichment procedures. Recovered isolates were serotyped, characterised for antibiotic resistance and subtyped by multi locus variance analysis (MLVA). Total *Enterobacteriaceae* counts were also performed. *Salmonella* was recovered from 2/338 (0.6%) ingredients (wheat and soybean meal), at two of the six mills. *Salmonella* was also detected in 3/317 (0.95%) compound feeds including pelleted feed which undergoes heat treatment. All isolates recovered from feed ingredient and compound feed samples were verified as *Salmonella enterica* subsp. *enterica* serotype (4,[5],12:i:-) that lack the expression of flagellar Phase 2 antigens representing monophasic variants of *Salmonella* Typhimurium (4,[5],12:i:-). Isolates exhibited resistance to between two and seven antimicrobials. Two distinct MLVA profiles were observed, with the same profile recovered from both feed and ingredients, although these did not originate at the same mill. There was no relationship between the occurrence of *Salmonella* and a high *Enterobacteriaceae* counts but it was shown that *Enterobacteriaceae* counts were significantly lower in pelleted feed (heat treated) than in meal (no heat treatment) and that *Enterobacteriaceae* counts would be very useful indicator in HACPP programme. Overall, although the prevalence of *Salmonella* in pig feed and feed ingredients in the present study was low, even minor *Salmonella* contamination in feed has the potential to affect many herds and may subsequently cause human infection. Furthermore, the recovery of a recently emerged serovar with multi-antibiotic resistance is a potential cause for concern.

1. Introduction

Non-typhoidal *Salmonellae* can colonise a wide range of hosts including all the major livestock species (poultry, cattle, and pigs), often asymptomatically, potentially leading to contamination of meat and other food products (Stephens et al., 2009). Following a "farm-to-fork" model, animal feed is at the beginning of the food safety chain. Therefore, the presence of *Salmonella* in animal feed or feed ingredients at the feed mill or on-farm is a cause for public health concern. This is evidenced by a number of incidences where animal infection has been traced back to contaminated animal feed. For example, Österberg et al. (2006) established that contaminated feed was the cause of an outbreak of *Salmonella* Cubana on a number of Swedish pig farms. Furthermore, Molla et al. (2010) found genotypically related and in some cases clonal *Salmonella* strains in commercially processed pig feed and pig faecal samples.

Abbreviations: MLVA, multi locus variance analysis; EFSA, European Food Safety Authority; NSRL, National Salmonella Reference Laboratory; MPN, most probable number; BPW, buffered peptone water; PCR, polymerase chain reaction; VNTR, variable number tandem repeat.

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A number of different feed ingredients may potentially harbour pathogenic micro-organisms including non-typhoidal *Salmonellae*. Historically, a number of studies have shown the presence of *Salmonella* in feed ingredients of animal origin (e.g. rendered animal by-products) (Clise and Swecker, 1965; Franco, 2005); however, such ingredients are no longer an issue following their ban in animal feed in the European Union (EU) in 2001 in the aftermath of the Bovine Spongiform Encephalopathy (BSE) crisis (Commission Regulation (EC) No 163, 2009). Exceptions have been made for the use of certain animal protein sources including fish meal, milk powders, certain blood products and dicalcium phosphate by-products (e.g. from the production of gelatin) as feed for monogastric animals (Commission Regulation (EC) No 1292, 2005). However, these ingredients are not without risk, as evidenced by the reported introduction of *S.* Agona to the United States (US) food chain via imported Peruvian fish meal, as quoted by Clark et al., 1973.

However, the risk of *Salmonella* contamination of pig feed from ingredients of animal origin may not be an issue, as the protein-rich ingredients currently used to formulate pig diets are principally of vegetable origin. Any ingredient of vegetable origin may become contaminated with *Salmonella* from contact with infected or carrier wildlife or production animals during storage or transit and/or from the use of manure or sludge as fertilizers on the growing crop. However, the risk is greater with imported ingredients as they may originate in countries with different regulations and there is an opportunity for contamination during transit. The Republic of Ireland relies on importing a much higher proportion of its animal feed requirement compared to other EU countries. In 2014, Ireland was importing 65% of its requirements, with ~3 million tonnes of cereals being imported annually, ~55% of which comes from countries outside the EU (DAFM, 2015). The EU in 2014 was 35% deficient in its requirement for protein for animal feed, so third-country imports are unavoidable (Popp et al., 2013; DAFM, 2015). In the EU, these are largely imported in the form of soybean from North and South America (de Visser et al., 2014). The contamination of cereals with *Salmonella* was estimated to range between 0.2 and 0.6% in 2012 in a study by the European Food Safety Authority (EFSA, 2014). This is much lower than for feed ingredients such as soybean meal (3.2–6.7%) and rapeseed (6.8%) which are by-products from other processing operations (EFSA, 2008). In one surveillance study, *Salmonella* was isolated from 14.6% of soybean meal consignments and 10% of rapeseed meal samples (Wierup and Haggblom, 2010).

The reported incidence of *Salmonella* in compound animal feed is generally low and when present, prevalence ranges on average from 0.6 to 1.7% (EFSA, 2008). It is also considered that the reported incidence in both feed ingredients and compound feed is probably lower than the true incidence due to under-reporting, sub optimal sampling procedures and for other reasons such as *Salmonella* detection methods may not offer all *Salmonella* serotypes an equal chance of isolation (Jones, 2011), especially in samples where multiple serotypes are present (De Busser et al., 2013a). A comprehensive sampling plan is therefore required for the monitoring of *Salmonella* in animal feed, as *Salmonella,* when present, is usually in low numbers and unevenly distributed. However, even low numbers of *Salmonella* may be sufficient to cause infection (Finn et al., 2013). This is particularly true for feeds of high fat content in which *Salmonella* can be protected from host gastric defence mechanisms (Jones et al., 1982). *Salmonella,* if present in the feed, also has the potential to multiply in warm, moist conditions, either at the feed mill or on the farm (Davies and Hinton, 2000; Hilbert et al., 2012).

As food-producing animals are the primary source of *Salmonella* infections in humans (Forshell and Wierup, 2006), it follows that contamination of animal feed with this pathogen should not be overlooked as an important origin of foodborne illness and outbreaks. The same *Salmonella* serotypes have been recovered from commercial pig feed and pigs sampled on the same farm (Burns et al., 2013). However, it remains unclear whether the feed contamination arose on-farm or whether the commercial feed introduced onto the farm was already contaminated.

The total number of *Enterobacteriaceae* can serve as a hygiene indicator in food and feed. *Enterobacteriaceae* have the advantage of being enumerated inexpensively and easily and are useful for quantifying the hygienic performance of a production process, when particular pathogens or spoilage organisms might be difficult to detect (Jordan et al., 2007). In the EU there is legislation (Commission Regulation (EC) No 2073, 2005) setting microbial process hygiene criteria for *Enterobacteriaceae* counts on foods including carcasses, milk and dairy products, and eggs. Equally, the determination of *Enterobacteriaceae* counts could be used to assess and subsequently improve mill hygiene and the quality of animal feeds (Jones and Richardson, 2004, Veldman et al., 1995). However the relevance of *Enterobacteriaceae* in feed should, however, be assessed and interpreted carefully and recognition given that there is conflicting studies on the correlation between *Enterobacteriaceae* count and the presence of *Salmonella* in feed. Jones and Richardson (2004) reported that poultry feed samples, meal and pellets, contaminated with *Salmonella* contained significantly higher *Enterobacteriaceae* counts. A study by Veldman et al. (1995), isolated predominantly thermotrophic *Enterobacteriaceae* from feedstuffs and found them to be useful markers of the rate of contamination with *salmonellae* and of the efficiency of decontamination of the feedstuffs by pelletisation. Whereas a study by Cox et al. (1983) showed no correlation between *Enterobacteriaceae* and *Salmonella*. Further studies showing the benefit of using as a hygiene indicator in feed therefore would be of benefit.

Therefore, the objective of this study was to assess the occurrence and characteristics of *Salmonella* in a range of feed ingredients and compound feeds sampled from feed mills supplying high *Salmonella* sero-prevalent pig farms in the Republic of Ireland, where on-farm bacteriology had confirmed *Salmonella* presence in both pigs and feed (Burns et al., 2013). *Enterobacteriaceae* counts were also performed and these may provide valuable data that could be used as a baseline for assessment of the hygienic standard of feed, which is currently rare in other studies.

2. Material and methods

2.1. Sample collection

Samples of feed ingredients and compound pig feed were collected monthly from five commercial feed mills (Mills A–D and F) and one home compounder (Mill E). All mills were operating under hazard analysis and critical control points (HACCP) quality assurance schemes and were all producing both meal and pelleted feed from a wide variety of ingredients. In all mills, pelleting was preceded by a steam conditioning step, whereas no heat treatment was applied to meal feed. Samples from each feed mill were taken over a 6 month period between November 2012 and September 2013. A total of 338 raw ingredients and 317 compound feed samples were obtained. The feed ingredients included cereals, vegetable protein ingredients and by-products of oil extraction and ethanol production and were the ingredients used in pig diet formulation at the time of the study. Compound feeds included meal and pelleted feed for all stages of pig production. For pelleted feed, pelleting was preceded by a steam conditioning step, whereas no heat treatment whatsoever was applied to meal feed. Feed ingredients were sampled at mill intakes from every ingredient load and finished feeds were sampled from every batch (from storage bins at the feed mills). All samples were composite samples taken by mill personnel in accordance with Commission Regulation (EC) No 152 (2009). Sub

samples (~150 g) were taken aseptically into sterile containers and submitted to the laboratory on a monthly basis, where they were refrigerated until analysis (within 24 h).

2.2. Salmonella isolation

The presence/absence of *Salmonella* in 10 g samples taken from the composite feed samples (150 g) was determined according to standard selective enrichment procedures (EN ISO 6579:2002/Cor 1:2004) with modified brilliant green agar (BGA; Oxoid, Basingstoke, Hampshire, UK) used for additional selective plating. Presumptive *Salmonella* isolates (identified based on the results of biochemical tests) were tested using a *Salmonella* latex agglutination kit (Oxoid, Basingstoke, Hampshire, UK).

2.3. Confirmation of Salmonella isolates by real-time polymerase chain reaction (PCR)

DNA was extracted from isolates identified as *Salmonella* by the latex agglutination kit using a DNAeasy Tissue Kit for Gram-negative bacteria (Qiagen, Crawley, UK) according to manufacturer's instructions. The primer and *Taq*Man probe sequences used for the confirmation of *Salmonella* spp. were based on those of McCabe et al. (2011). The primer and probe sequences and labels for the *hil*A and pUC19 were as described by Fricker et al. (2007). All primers and probes were manufactured by Sigma–Aldrich (St. Louis, USA). Internal amplification control (IAC) template DNA was isolated from *Escherichia coli* using a QIAprep Spin Miniprep kit (Qiagen) according to manufacturer's instructions. PCR amplification and detection were performed using a Roche LightCycler 480 (Roche Diagnostics Limited, Burgess Hill, West Sussex, United Kingdom) using LC480 probe master mix kit (Roche Diagnostics Limited). Isolates confirmed as *Salmonella* were grown on standard plate count agar (Oxoid) overnight at 37 °C and a loopful of colonies was suspended in 0.5 ml 80% glycerol, added to cryoprotectant beads and stored at −80 °C.

2.4. Serotyping and Antimicrobial susceptibility testing of Salmonella isolates

All confirmed *Salmonella* isolates were serotyped according to the Kauffmann-White classification scheme (Grimont et al., 2007). *Salmonella enterica* subsp. *enterica* serovar (4,[5],12:i:-) that lack expression of the second-phase flagellar antigens were designated as monophasic variants of *S.* Typhimurium. Antimicrobial susceptibility testing was performed according to the broth dilution method of the Clinical and Laboratory Standards Institute (formerly NCCLS) (CLSI, 2008) using a broth microdilution assay (Sensititre, TREK Diagnostic Systems Inc., Sussex, England). The panel of antimicrobials included azithromycin (Azt), ampicillin (A), amoxicillin/clavulanic acid (Am), ceftriaxone (Ax), chloramphenicol (C), ciprofloxacin (Cp), trimethoprim/sulfamethoxazole (Tm), cefoxitin (F), gentamicin (Gm), kanamycin (Km), nalidixic acid (Na), sulfisoxazole (Su), streptomycin (S), tetracycline (T) and ceftiofur (Ce). The cut-off values (mg/l) were as specified in EU Commission Decision 407, (2007). *E. coli* ATCC 25922 was used as a control. Isolates were either designated as "fully susceptible", "intermediate" or if resistance to any antimicrobial was found, this was indicated by using the abbreviation for the antimicrobial to which the strain was resistant.

2.5. Confirmation of monophasic Salmonella Typhimurium by multiplex real-time PCR

All isolates identified phenotypically as monophasic *S. enterica* subsp. *enterica* serovar Typhimurium 4,[5],12:i:– were tested by multiplex PCR with oligonucleotides specific to the antigenic genes *fliC* (H:i antigen), *fljB* (H:1,2 antigen) and *fljB*/IS200 as recommended by EFSA (2010). The primers used were those outlined by Prendergast et al. (2013) and were obtained from Eurofins MWG Operon, Dublin, Ireland. The probes were *Taq*man hydrolysis probes (TIB MolBiol Berlin, Germany), also as outlined by Prendergast et al. (2013) except that different fluorescent dyes were used. The primer sequences, probes and target genes are listed in Table 1. PCR amplification was performed in a final volume of 10 μl containing 9 μl master mix and 1 μl template DNA. The master mix consisted of 2× Lightcycler 480 probe master, PCR-grade water and optimized concentrations of the primers and probes. PCR amplification was performed in a Roche Lightcycler 480 96-well instrument with an initial denaturation step of 95 °C for 10 min followed by 30 cycles of 95 °C for 10 s, 60 °C for 1 min, 72 °C for 1 s, followed by an extension step of 72 °C for 10 min. Fluorescence signals were detected in 6FAM (fliB/IS200), LC670 (fliC) and YAK (fljB.1, 2) channels. The reaction was considered positive when Ct values were ≤30.

2.6. Enumeration of Salmonella in Salmonella-positive samples by most probable number procedure

Salmonella enumeration was conducted on *Salmonella*-positive feed and ingredient samples using a three-tube most probable number (MPN) technique based on standard methods (ISO 6579:2002/Cor 1:2004 and ISO 7218-2007). Testing was performed on 10 g samples using buffered peptone water (BPW; Oxoid) as a pre-enrichment broth, followed by Rappaport-Vassiliadis broth (RVS; Oxoid) for selective enrichment and BGA and xylose lysine deoxycholate agar (XLD; Oxoid) as the selective media. The limit of detection of this assay, based on the dilutions used, was 0.30 MPN *Salmonella*/g.

2.7. Multilocus variable number tandem repeat analysis (MLVA)

All *Salmonella* isolates confirmed by serotyping and multiplex PCR as *S.* Typhimurium and/or monophasic variants of *S.* Typhimurium (1,4[5], 12:i) were subjected to multilocus variable number tandem repeat analysis (MLVA) using capillary electrophoresis methods as described previously by Prendergast et al. (2011). The variable number tandem repeat (VNTR) loci selected, along with the primers and dyes used, were as outlined by Lindstedt et al. (2004) and are listed in Table 2. Essentially, the loci were amplified in separate PCRs by using fluorescent primers. Raw fragment lengths for each locus were manually discarded using a minimum threshold of ±3 bp to distinguish alleles. *S.* Typhimurium LT2 ATCC 29946 was used as a positive control during the analysis of each batch of samples on the ABI 3500 genetic analyser (Applied Biosystems, Foster City, California, US). Each peak was identified according to colour and size using GeneMapper (version 4.1) software (Applied Biosystems) and a set of 5 alleles for each isolate was defined as the MLVA profile. MLVA profiles were assigned based on the fragment size amplified from each locus, with 'NA' used to denote a locus not present.

2.8. Enterobacteriaceae counts in feed and ingredients

Enterobacteriaceae were enumerated in feed and feed ingredient samples using a plate count method according to standard microbiological procedures (EN ISO 7218-3:2007) which were modified to enhance the recovery of injured or stressed cells. Buffered peptone water (90 ml) was added to 10 g of the sample and mixed thoroughly until evenly suspended and incubated at 37 °C for 1 h. To aid the emulsification of oily or waxy feed ingredient samples, Tween 80 (Sigma–Aldrich, Ireland) was added to the BPW at a rate of 1 g/l. Tenfold serial dilutions were then performed in maximum

Table 1
Oligonucleotide and Taqman probe sequences used in the multiplex real-time PCR used for confirmation of monophasic *Salmonella* Typhimurium (4,[5],12:i:-).

Target locus	Description	Sequence (5′–3′)	Dye emission wavelength (nm)	Reference
*fli*C	Forward primer	ccc cgc tta cag gtg gac tac		O'Regan et al., 2008
	Reverse primer	agc ggg ttt tcg gtg gtt gt		
	*Taq*Man hydrolysis probe	LC670-taa agc cgc att gac agc agc agg tg-BHQ2	~670	
*flj*B 1,2	Forward primer	tgt tac tat tgg tgg ctt tac tgg		Muñoz et al. (2010)
	Reverse primer	cag cag gca ttg tgg tct tag		
	*Taq*Man hydrolysis probe	YAK-cgc cag ccg caa ggg tta ctg tac-BBQ	~550	
*flj*B/IS200	Forward primer	gat ctg tcg atg att cat ctt ctg ac		Prendergast et al. (2013)
	Reverse primer	aac gct tgt ctt cgg tat ttg g		
	*Taq*Man hydrolysis probe	6FAM-tcg ggt gtg cgc taa gct ctt tt-BBQ	~515	Prendergast et al. (2013)

recovery diluent (Oxoid) and 1 ml of relevant dilutions was pour-plated in duplicate on violet red bile glucose agar (VRBGA; Oxoid). Each plate was then overlaid with VRBGA. Plates were incubated at 37 °C for 24 ± 2 h.

2.9. Statistical analysis

Enterobacteriaceae counts were log-transformed to approximate normality prior to statistical analysis. They were analysed using the mixed procedure of the Statistical Analysis System version 9.3 (SAS Institute Inc., Cary, NC, USA). When analysing the effect of sample type (feed ingredient, compound feed) and feed mill on *Enterobacteriaceae* count, sample type, mill and their interaction were included in the model as fixed effects. When analysing the effect of diet form (meal, pellet) and mill on *Enterobacteriaceae* count, Mill E was omitted from the dataset as only one form of diet (meal) was produced in that mill. Diet form, feed mill and their interaction were included in the model as fixed effects. When analysing the effect of diet type (dry sow, lactation, finisher, and weaner) and mill on *Enterobacteriaceae* count, diet type, feed mill and their interaction were included in the model as fixed effects. Means are reported as least squares means ± their standard errors (SE) and the slice option was used to determine significance for simple main effects. Significance was reported for $P < 0.05$ and tendencies towards significance were reported for $0.05 < P < 0.10$. The individual compound diet/feed ingredient sample was considered the experimental unit in all cases.

3. Results

As can be seen in Table 3, two of the six feed mills were found to be exposed to *Salmonella*-contaminated feed ingredients. Another two mills produced *Salmonella*-contaminated feed (including pelleted feed which undergoes steam treatment prior to pelleting). The prevalence of *Salmonella* was 0.6% (2/338) and 0.95% (3/317) in feed ingredients and compound feed, respectively. *Salmonella* contamination was found in two feed ingredients; wheat ($n = 1$, Mill B) and soybean meal ($n = 1$, Mill E). It was also found in compounded dry sow meal ($n = 1$, Mill D), compounded dry sow pellets ($n = 1$, Mill D) and in a compounded finisher meal used to balance liquid whey (Mill C; Table 3). The proportion of compound meal feed samples contaminated with *Salmonella* was 1.59% whereas only 0.54% of pelleted diets were contaminated. All of the *Salmonella*-positive samples (both feed and ingredients) had an MPN/g of <0.30 i.e. levels of contamination were below the limit of quantification (lowest quantity that can be distinguished within a 95% confidence limit).

All five *Salmonella* isolates recovered from the feed ingredient and compound feed samples were identified as monophasic variants of Typhimurium (4, [5] 12: i:-) by serotyping and multiplex PCR. All isolates were susceptible to azithromycin, amoxicillin/clavulanic acid, cefoxitin, ceftriaxone, kanamycin, and nalidixic acid. However, the isolates exhibited resistance to between two and seven antimicrobials with most frequent resistance to tetracycline (5/5 isolates), streptomycin (4/5), gentamicin (3/5), ampicillin (3/5), chloramphenicol (3/5), sulfisoxazole (3/5), trimethoprim/sulfamethoxazole (2/5), ciprofloxacin (2/5) and ceftiofur (2/5) (Table 3).

Five VNTR loci were used to genotype the five feed- and feed ingredient-derived *Salmonella* isolates. Two different MLVA profiles were identified – allele strings 3-13-16-NA-0211, designated Type A and 3-11-9-NA-0211, designated Type B (Table 3). The VNTR loci STTR-9 and STTR-3 were very conserved showing no degree of diversity. Likewise, the locus STTR10 located on the *Salmonella* virulence plasmid was absent in all five isolates, indicating possible monophasic variants of the virulence plasmid. The most variation was noted in loci STTR5 (two different alleles) and STTR6 (two different alleles). The most prominent combination of alleles was allele string 3-13-16-NA-0211. Interestingly, *Salmonella* isolates with the same MLVA profile were recovered from ingredients and finished feed i.e. Type A was recovered from wheat as well as sow meal and pellets and Type B from soybean meal and finisher meal (Table 3).

Table 2
PCR primers used for *S.* Typhimurium VNTR loci for MLVA typing.

Target locus	Primer name	Dye	Sequence (5′–3′)
STTR3	STTR3-F[a]	HEX	ccc cct aag ccc gat aat gg
	STTR3-R[b]	–	tga cgc cgt tgc tga agg taa taa
STTR5	STTR5-F	HEX	atg gcg agg cga gca gca gt
	STTR5-R	–	ggt cag gcc gaa tag cag gat
STTR6	STTR6-F	6FAM	tcg ggc atg cgt tga aa
	STTR6-R	–	ctg gtg ggg aga atg act gg
STTR9	STTR9-F	6FAM	aga ggc gct gcg att gac gat a
	STTR9-R	–	cat ttt cca cag cgg cag ttt ttc
STTR10pl	STTR10-F	TAM	cgg gcg cgg ctg gag tat ttg
	STTR10-R	–	gaa ggg gcc ggg cag aga cag c

[a] F: forward primer, which was fluorescently labelled with the dye indicated.
[b] R: reverse primer, which was unlabelled.

Table 3
Serotypes, antibiotic resistance profiles, and MLVA profiles of *Salmonella* isolated from pig feed and pig feed ingredients sampled at commercial feed mills (Mills A–D and F) and one home compounder (Mill E). *Enterobacteriaceae* counts (Log_{10} cfu/g) are also shown.

Sample type	Feed mill[a]	*Salmonella* Serotype[b]	Antibiotic resistance profile[c]	MLVA No. of repeats					MLVA type	*Enterobacteriaceae* count (Log_{10} cfu/g)
				STTR9	STTR5	STTR6	STTR10	STTR3		
Feed ingredients										
Wheat	B	4, 12:i:-	ACSSuTTmGm	3	13	16	NA	0211	A	<1.0
Soybean meal	E	4, 12:i:-	TGm	3	11	9	NA	0211	B	<1.0
Compound feed										
Finisher meal	C	4, (British Standards Institution, 2007),12:i	ASSuT	3	11	9	NA	0211	B	4.59
Dry sow meal	D	4, 12:i:-	ACSSuTTmGm	3	13	16	NA	0211	A	4.62
Dry sow pellets	D	4, 12:i:-	ACSTCpCe	3	13	16	NA	0211	A	<1.0

[a] Mills A and F are not shown as *Salmonella* was not isolated from these mills.
[b] Only one strain of *Salmonella* was isolated from each feed type.
[c] Ampicillin (A), chloramphenicol (C), ciprofloxacin (Cp), trimethoprim/sulfamethoxazole (Tm), gentamicin (Gm), sulfisoxazole (Su), streptomycin (S), tetracycline (T) and ceftiofur (Ce).

When the feed ingredient samples were categorised according to *Enterobacteriaceae* counts (Table 4), the samples that harboured the highest counts included pollard and malt sprouts, with the majority of these samples in the >10,000 cfu/g category. These ingredients, together with barley, wheat and Lactofeed were also most frequently contaminated with *Enterobacteriaceae* (*Enterobacteriaceae* were detected in 100, 77, 83, 68 and 67% of malt sprout, pollard, barley, wheat and Lactofeed samples, respectively; Table 4). The majority of compound meal feed samples harboured counts in excess of 10,000 cfu/g of *Enterobacteriaceae*, regardless of the production stage, whereas *Enterobacteriaceae* were non-detectable in the majority of the pelleted feed samples (Table 4). In general, *Enterobacteriaceae* were detected in 92% of meal samples, while only 29% of pelleted feed harboured *Enterobacteriaceae* (Table 4). Overall, 27% of all compound pig feed samples analysed fell into the >10,000 cfu/g *Enterobacteriaceae* category. However, a large proportion of the feed ingredients and compound feeds (46% of each) from which *Enterobacteriaceae* were recovered had contamination levels below 10 cfu/g.

The effect of diet form (meal or pellet) on *Enterobacteriaceae* counts in samples from the five commercial feed mills is shown in Table 5. There was a mill x feed form interaction ($P<0.01$). When samples from all of the mills were compared, the mean *Enterobacteriaceae* count was lower in pelleted diets than in meal diets ($P<0.001$), in agreement with the data shown in Table 4. However, it was also found that the mill from which samples were taken influenced the *Enterobacteriaceae* counts in compound diets ($P<0.001$).

Table 4
Number (%) of feed ingredients and compound pig feed samples with different ranges of *Enterobacteriaceae* counts and number of samples in which *Enterobacteriaceae* were detected.

Sample type	Non-detectable (<10 cfu/g)	10–100 cfu/g	100–1000 cfu/g	1000–10000 cfu/g	>10000 cfu/g	Total no. of samples in which *Enterobacteriaceae* were detected (%)
Ingredients[a]						
Soya products	59 (72.8)	8 (9.9)	7 (8.6)	3 (3.7)	4 (4.9)	22/81(27.2)
Maize	27 (45.0)	10 (16.7)	15 (25.0)	5 (8.3)	3 (5.0)	33/60 (55.0)
Wheat	16 (32.0)	5 (10.0)	11 (22.0)	6 (12.0)	12 (24.0)	34/50 (68.0)
Barley	8 (16.7)	1 (2.1)	4 (8.3)	18 (37.5)	17 (35.4)	40/48 (83.3)
Rapeseed	19 (65.5)	3 (10.4)	5 (17.2)	1 (3.5)	1 (3.5)	20/29 (69.0)
Pollard	3 (23.1)	1 (7.7)	1 (7.7)	1 (7.7)	7 (53.9)	10/13 (76.9)
Golden distiller's grain	10 (66.7)	3 (20.0)	2 (13.3)	0	0	5/15 (33.3)
Palm kernel	11 (84.6)	0	2 (15.4)	0	0	2/13 (15.4)
Malt sprouts	0	0	0	2 (40.0)	3 (60.0)	5/5 (100.0)
Sunflower	3 (75.0)	0	0	0	1 (25.0)	1/4 (25.0)
Lactofeed[b]	1 (33.3)	2 (66.7)	0	0	0	2/3 (66.7)
Compound feed						
Meal	12 (9.5)	1 (0.08)	12 (9.5)	23 (18.3)	78 (61.9)	114/126 (90.5)
Dry sow	3 (12.5)	0	0	3 (12.5)	18 (75.0)	21/24 (87.5)
Lactating sow	3 (12.5)	0	2 (8.3)	6 (25.0)	13 (54.2)	21/24 (87.5)
Weaners	1 (3.9)	1 (3.9)	2 (7.7)	5 (19.2)	17 (65.4)	25/26 (96.2)
Fatteners	5 (9.8)	0	8 (15.7)	8 (15.7)	30 (58.8)	46/51 (90.2)
Others[c]	0	0	0	1 (100.0)	0	1/1 (100.0)
Pelleted	133 (71.1)	20 (10.7)	16 (8.6)	11 (5.9)	7 (3.7)	54/187 (28.9)
Dry sow	17 (70.8)	4 (16.7)	3 (12.5)	0	0	7/24 (29.2)
Lactating sow	15 (51.7)	3 (10.4)	4 (13.8)	5 (17.2)	2 (6.9)	14/29 (48.3)
Weaners	30 (76.9)	1 (2.6)	4 (10.3)	3 (7.7)	1 (2.6)	9/39 (23.1)
Fatteners	62 (75.6)	9 (11.0)	4 (4.9)	3 (3.7)	4 (4.9)	20/82 (24.4)
Others[c]	9 (69.2)	3 (23.1)	1 (7.7)	0	0	4/13 (30.8)

[a] Ingredients in which *Enterobacteriaceae* were never detected were not tabulated. These include citrus pulp ($n=6$), beet pulp ($n=5$), whey syrup ($n=3$), tallow ($n=1$), lysine ($n=1$), milk powder ($n=1$), wheat/pollard ($n=2$).
[b] Lactofeed is a lactose source.
[c] Feeds in which production stage was not specified.

Table 5
Effect of diet form (meal, pellet) on mean *Enterobacteriaceae* counts (Log_{10} cfu/g) in samples from the five commercial feed mills (Mills A–D and F) tested.

Mill[a]	Form of Compound Feed		Mean	S.E.[b]	P-values[c]		
	Meal	Pellet			Form	Mill	Form × mill
A	4.85	1.72	3.29	0.143	0.001		
B	3.97	1.06	2.52	0.231	0.001		
C	3.05	1.19	2.12	0.210	0.001		
D	4.18	1.33	2.76	0.149	0.001		
F	3.05	1.07	2.06	0.267	0.001		
Mean	3.82	1.27		0.093	0.001	0.001	0.01

[a] Mill E (home compounder) was omitted from the dataset as all feed was produced as meal in this mill.
[b] *P*-values ≤ 0.05 are statistically significant.
[c] S.E.: Standard error.

Table 6
Effect of compound diet type (dry sow, finisher, lactation, weaner) on mean *Enterobacteriaceae* counts (log_{10} cfu/g) in samples from the five commercial feed mills (Mills A–D and F) and one home compounder (Mill E) tested.

Mill	Compound diet type				Mean	S.E.[a]	*P*-values[b]		
	Dry	Finisher	Lactation	Weaner			Type	Mill	Type × mill
A	2.93	2.56	2.63	2.62	2.69	0.260	0.90		
C	1.98	2.17	3.34	1.95	2.36	0.417	0.35		
D	2.76	1.98	2.89	3.25	2.72	0.417	0.22		
E	2.61	4.15	3.35	3.57	3.42	0.494	0.38		
F	1.75	1.79	2.03	2.12	1.92	0.561	0.97		
Mean	2.41	2.53	2.85	2.70		0.283	0.76	0.02	0.54

[a] S.E.: Standard error.
[b] *P*-values ≤ 0.05 are statistically significant.

The effect of mill and compound diet type (dry sow, lactation, finisher, weaner) on *Enterobacteriaceae* counts and their interaction is shown in Table 6. There was no mill x compound diet type interaction ($P > 0.05$). *Enterobacteriaceae* counts were similar for all diet types. However, the mill from which samples were taken was once again found to influence *Enterobacteriaceae* counts in compound diets ($P < 0.05$).

4. Discussion

Salmonella contamination was found in two feed ingredients (wheat and soya) and in compound dry sow meal and pellets and in compound finisher meal. Soya, as a by-product of oil extraction, is particularly prone to contamination by salmonellae that are endemic in processing plants (Morita et al., 2006). A study by Papadopoulou et al. (2009) showed that *Salmonella* was more commonly isolated from wheat than from any other feed ingredient. The overall recovery rate of *Salmonella* in the compound feed samples analysed in the present study was 0.95% which is much lower than reported in other studies i.e. 2.8% (Harris et al., 1997), 3.6%, (Molla et al., 2010), 23.5% (Fedorka-Cray et al., 1997), 43.0% (Hacking et al., 1978), 42.0% (Isa et al., 1963) and 2.3–58.8% (Davies et al., 1997). However, it should be noted that the samples tested by Harris et al. (1997) and Molla et al. (2010) were taken on-farm and from feed trucks supplying the mills.

As the critical control points would have varied for each feed manufacturing plant, the testing procedures applied in this study focused on the contamination status of composite/pooled (150 g) samples of incoming feed ingredients and outgoing compound feed. It could be argued that this study has some limitations with regard to its use of a 10 g sample instead of the standard 25 g sample (EN ISO 6579:2002/Cor 1:2004). However, studies by Funk et al. (2000) and Arnold et al. (2005) illustrate (using faecal samples) that the use of a pooled sample may be a valid approach for measuring the occurrence of *Salmonella* in feed ingredients and compound feed. For example, Funk et al. found that using a pooled faecal sample of 10 g in comparison to 25 g yielded only a 5% drop in relative sensitivity.

In a previous study of compound feed for pigs, *S.* Agona, *S.* Livingstone, *S.* Senftenberg and *S.* Anatum were found to be the most commonly detected serovars (EFSA, 2010a). It is of interest that all isolates recovered in the present study were monophasic Typhimurium. In the EU the occurrence of monophasic variant human cases has increased from 360 in 2007 to 5932 in 2012, making it the third most common serovar isolated from humans in Europe (EFSA, 2014). Furthermore, in previous studies, Typhimurium DT104 was found to be the dominant serovar in Irish pig herds (Duggan et al., 2010; McCarthy et al., 2013; Rowe et al., 2003). However, in a short period of time the penta-resistant DT104 has been replaced by the emerging monophasic variant 4,[5], 12:i:- with resistance to ampicillin, streptomycin, sulphonamides and tetracyclines (National Salmonella, Shigella & Listeria reference laboratory of Ireland, 2013; EFSA, 2010). Interestingly, this tetra-resistance pattern was observed in one compound feed isolate obtained in the present study and two other isolates (one each from a feed ingredient and compound feed) also had this profile together with resistance to three additional antibiotics. Various European studies have also documented this ASSuT profile from human, pig and pig meat isolates (Arguello et al., 2014; Hopkins et al., 2010; Prendergast et al., 2013). The increased antimicrobial resistance and higher than anticipated occurrence of monophasic variants observed in the present study and other European studies highlights its importance as an emerging feed and foodborne pathogen.

While monophasic variants have been found in animal feed recently (Bugarel et al., 2012; Wasyl and Hoszowski, 2012), they were only the sixth most common *Salmonella* serotype found in 2002–2009 in animal feed and feed ingredient samples analysed under U.S. Food and Drug Administration (FDA) *Salmonella* surveillance programmes (Li et al., 2012). In general, the serotypes obtained from feed prior to its arrival on-farm do not usually correspond to those most frequently found in humans and animals (Li et al., 2012; Lo Fo Wong, 2001). Moreover, the MLVA profile of the monophasic variant found in feed and feed ingredients sampled at feed mills in this study was identical to that of the dominant serovar recovered from the pig herds supplied by these mills (Burns et al.,

2013). Urgent control measures are therefore needed to reduce the spread of infection to humans via the food chain to prevent the possible pandemic spread of serovar 4,[5],12:i:- as occurred with *S.* Typhimurium DT104 during the 1990s.

Enterobacteriaceae are enteric bacteria that are used as indicators of faecal contamination. In the case of food and animal feed, their presence indicates poor hygiene, inadequate processing or post-process contamination. Studies have shown that *Enterobacteriaceae* counts tend to be higher in feed samples positive for *Salmonella* than in *Salmonella*-negative samples and suggest that *Enterobacteriaceae* counts may be a useful indicator to assess the likelihood of *Salmonella* contamination in feed (Jones and Richardson, 2004). This was not always the case in the present study, as only two of the five *Salmonella*-positive samples had high *Enterobacteriaceae* counts (4.59 and 4.62 Log_{10}cfu/g).

Despite this lack of an observed correlation between *Salmonella* and *Enterobacteriaceae* this study also set out to monitor *Enterobacteriaceae* counts as an indirect indicator of hygiene which has been proposed as a useful tool in feed mill production systems as part of a HACCP systems. (EFSA, 2008; Jones and Richardson, 2004; Gradel et al., 2003).

The results showed that *Enterobacteriaceae* counts did not differ for dry sow, lactation, finisher and weaner compound diets in the present study. This is perhaps not surprising since the feed ingredients used to formulate these diets are similar. However, *Enterobacteriaceae* counts were significantly lower in pelleted diets than in meal. This is to be expected, as the pelleting process employed in the study feed mills involved temperatures of 80–90 °C and *Salmonella* and other *Enterobacteriaceae* should be completely eliminated by pelleting at temperatures exceeding 83 °C (Hald et al., 2012). Maciorowski et al. (2004) previously observed 99% (2 log) reductions in *Salmonella* at this temperature and others have achieved 4 log reductions at 85 °C for 90 s at 15% moisture (Himathongkham et al., 1996). In general, pelleting systems have been reported to reduce *Salmonella* isolation rates from between 50 and 93% (Jones, 2011). Our data are in agreement with the findings of other studies; for example, Threlfall et al. (2003) found that 8.8% of meal feed samples but only 4.2% of pelleted feed samples were contaminated with *Salmonella*.

Salmonella isolates with the same MLVA profile (A) were recovered from ingredients and finished feed although the feed and ingredients did not originate in the same feed mill and this may be indicative of a previously acquired contamination from the same source, e.g. both mills probably imported soy bean meal from the same source. From an epidemiological perspective a time factor must also be considered. The same MLVA type was recovered from both meal and pelleted feed from Mill D, indicating either that pelleting did not eliminate *Salmonella* or it may be indicative that post-process contamination occurred in the mill i.e. between different production lines (pelleted/heat treated and meal/ non heat treated feed), especially as no *Salmonella* was found in their feed ingredients.

Successful reductions of *Salmonella* during pelleting are dependent on many factors i.e. the time of heat exposure, temperature obtained, and the moisture content (a_w) of the feed at pelleting. In addition, the conditions required to eliminate *Salmonella* are not always achieved in practice due to the high energy cost involved, the heat damage to vitamins and other nutrients and the adverse effect on the integrity of the pellets (De Busser et al., 2013b; Jones et al., 1995) and even when achieved may not be sufficient for ingredients with high contamination levels (Fedorka-Cray et al., 1997). The presence of *Salmonella* and *Enterobactericeae* in pelleted feed in the present study may also be due to post-process contamination. Wierup and Häggblom (2010) demonstrated that heat-treated feed may become re-contaminated at different points along the production line. Pelleting systems rely on steam addition to eliminate any pathogens present which adds moisture to pelleted feeds. This moisture is removed via pellet coolers; however, malfunctions in these systems may cause condensation to occur on the interior surfaces of the coolers (Jones, 2011). This increase in moisture within the pellet cooler may provide an environment capable of supporting the growth of *Enterobacteriaceae,* in particular *Salmonella*.

Contamination of feed at the feed mill is also associated with other factors, such as cross-contamination by dust, presence of vectors and poor hygiene conditions (EFSA, 2008). Contamination may also occur during storage at the feed mill (Davies and Wales, 2010). These factors are likely to have had an influence in the present study considering that *Enterobacteriaceae* counts and *Salmonella* prevalence were higher in compound feed than in feed ingredients (even in meal feed, which is not heat-treated), indicating post-process contamination and/or proliferation of surviving cells. Furthermore, it was also interesting that the mill had a significant effect on *Enterobacteriaceae* counts, with the home compounder (Mill E) having particularly high *Enterobacteriaceae* counts and one of the commercial mills (Mill F) having particularly low counts. Lower *Enterobacteriaceae* counts are most likely attributed to better management practises, as similar treatment methods were used across all feed mills. Therefore, a further study is required to obtain information on specific practices at each feed mill. Environmental samples taken from various surfaces within the feed mills may also help to assess the origins of contamination.

5. Conclusions

Salmonella contamination of feed ingredients and compound feeds was observed in this comprehensive Irish feed mill study, albeit at a low prevalence. All of the *Salmonella* isolates recovered were monophasic variants of the serovar Typhimurium, confirming the occurrence of this serotype in new geographical settings. All exhibited some degree of antibiotic resistance, with some multi-resistant isolates found. The data from this study also indicate that a large proportion of the raw materials used for pig feed manufacture are contaminated with high levels of *Enterobacteriaceae* ($>10^4$ cfu/g). Thus, raw materials should be viewed as a critical control point for the entry of pathogenic bacteria into the feed and food chains. While pelleting reduced *Salmonella* prevalence and *Enterobacteriaceae* counts in compound feed, it did not completely eliminate contamination. This, together with the fact that compound feed often had higher *Enterobacteriaceae* counts than ingredients, suggests post-process contamination within the feed mills. Despite a low prevalence of *Salmonella* in pig feed and feed ingredients in the present study still has the potential to affect many herds and may subsequently cause human infection via consumption of contaminated pork. Furthermore, the recovery of an emergent serotype and multi-resistant isolates is a potential cause for concern. In addition, the *Enterobacteriaceae* data generated in the present study show their relevance for the assessment of hygienic standards of feed.

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