Targeted low cost strategies to combat *Salmonella* spp. in finisher pigs and in the slaughterhouse

By:

Kavita Walia, MSc.

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Supervisors:

Dr. Gillian E. Gardiner

Dr. Peadar G. Lawlor

Dr. Geraldine Duffy

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DECLARATION

No element of the work described in this thesis has been previously submitted for a degree at this or any other institution. The work in this thesis has been performed entirely by the author.

Signature: ______________________

Date: February 15, 2017

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LIST OF ABBREVIATIONS

A	Ampicillin
AMP	Ampicillin
ADFI	Average Daily Feed Intake
ADG	Average Daily Gain
ASSuT	Ampicillin, Streptomycin, Sulfonamide, Tetracycline
ASSuTFu	Ampicillin, Streptomycin, Sulfonamide, Tetracycline, Furazolidone
ACGKSSuTTm	Ampicillin, Chloramphenicol, Gentamicin, Kanamycin, Streptomycin, Sulfonamide, Tetracycline, Trimethoprim
a_w	Water Activity
AMR	Antimicrobial Resistance
APC	Aerobic Plate Counts
APHIS	Animal and Plant Health Inspection Service
ATP	Adenosine Triphosphate
AUG	Amoxicillin-Alavulanic Acid
AXO	Ceftriaxone
AZI	Azithromycin
BGA	Brilliant Green Agar
BPW	Buffered Peptone Water
C&D	Cleaning and Disinfecting
C	Chloramphenicol
Ca	Calcium
CDC	Centers for Disease Control and Prevention
CFIA	Canadian Food Inspection Agency
CFU	Colony Forming Units
CIP	Clean-In-Place
CIP	Ciprofloxacin
CHL	Chloramphenicol
CL	Confidence Limit
CQA	Canadian Quality Assurance
CVRL	Central Veterinary Research Laboratory
DAFM	Department of Agriculture Food and Marine
DNA	Deoxyribonucleic Acid
DT	Definitive phage Type
EC	European Commission

ECDC	European Centres for Disease Control and Prevention
EEA	European Economic Area
ELISA	Enzyme-Linked Immunosorbent Assay
EFSA	European Food Safety Authority
EOs	Essential Oils
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FCE	Feed Conversion Efficiency
FIRM	Food Institutional Research Measure
FIS	Sulfisoxazole
FBO	Food Business Operator
FOX	Cefoxitin
FSA	Food Safety Authority
FSAI	Food Safety Authority of Ireland
FSIS	Food Safety and Inspection Service
FSMS	Food Safety Management System
FTU	Phytase Unit
Fu	Furazolidone
G	Gentamicin
GEN	Gentamicin
GI	Gastrointestinal
GIT	Gastrointestinal Tract
GMP	Good Manufacturing Practices
H^+	Hydrogen ion
H_2S	Hydrogen sulphide
HACCP	Hazard Analysis Critical Control Plan
HGT	Horizontal Gene Transfer
HPSC	Health Protection Surveillance Centre
ILN	Ileocaecal Lymph Nodes
ISO	International Organization for Standardization
IU	International Unit
K	Kanamycin
K^+	Potassium ion
KCN	Potassium Cyanide
LAB	Lactic acid bacteria

LIA	Lysine Iron Agar
LOD	Limit of Detection
M cells	Microfold cells
MDR	Multidrug Resistant
MBC	Minimum Bactericidal Concentration
MCFA	Medium-Chain Fatty Acid
MIC	Minimum Inhibitory Concentration
MLVA	Multiple Locus Variable number tandem repeat Analysis
MLN	Mesenteric Lymph Nodes
MPN	Most Probable Number
MS	Member States
MSRV	Modified Semi-solid Rappaport-Vassiliadis
MRD	Maximum Recovery Diluent
N/A	Not Applicable
NA	Nutrient Agar
Na	Sodium
NaCl	Sodium Chloride
NAHMS	National Animal Health Monitoring System
NAL	Nalidixic Acid
No.	Number
NPSCP	National Pig *Salmonella* Control Program
NS	Not Specified
OD	Optical Density
OFFS	On Farm Food Safety
OR	Odds Ratio
P	Phosphorus
PBS	Phosphate Buffered Saline
PCA	Plate Count Agar
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
psi	Pounds per Square Inch
qPCR	Quantitative Polymerase Chain Reaction
QAC	Quaternary Ammonium Compound
QMRA	Quantitative Microbiological Risk Assessment
psi	Pounds per Square Inch

RNA	Ribonucleic Acid
S	Streptomycin
SAS	Statistical Analyses System
SCFA	Short-Chain Fatty Acid
SCVPH	Scientific Committee on Veterinary Measures relating to Public Health
SE	Standard Error
sem	Standard Error of Mean
sed	Standard Error of Difference
S.I.	Statutory Instruments
SOPs	Standard Operating Procedures
SPI-1	*Salmonella* Pathogenicity Island-1
SPI-2	*Salmonella* Pathogenicity Island-2
spp.	Species
SRUC	Scotland Rural College
SSCP	Swine *Salmonella* Control Program
STR	Streptomycin
Su	Sulfonamide
SXT	Trimethoprim/Sulfamethoxazole
T	Tetracycline
TET	Tetracycline
T3SS	Type III Secretion System
Tm	Trimethoprim
TSB	Tryptone Soya Broth
TSI	Triple Sugar Iron
UK	United Kingdom
USA	United States of America
USDA	United States Department of Agriculture
Vi	Virulence
VRBGA	Violet Red Bile Glucose Agar
XLD	Xylose Lysine Deoxycholate
XNL	Ceftiofur
ZAP	Zoonoses Action Plan
ZNCP	Zoonoses National Control Programme
ZNCPig	Zoonoses National Control Plan for *Salmonella* in Pigmeat

ABSTRACT

Salmonella carriage in pigs is a significant food safety issue and low-cost control measures may provide a useful element in reducing the prevalence of *Salmonella* in pigs. This thesis aimed to investigate several pre-harvest *Salmonella* control strategies directed towards finishing pigs (35-110 kg). At farm level, two strategies focusing on dietary supplementation with feed additives: (1) sodium butyrate; and (2) an encapsulated blend of formic acid, citric acid, and essential oils (FormaXOL™) were investigated for their effectiveness to control *Salmonella* shedding and seroprevalence in pigs 28- and 63-days prior to slaughter. In the abattoir, two strategies: (3) cleaning and disinfection (C&D) of lairage pens with a quaternary ammonium chloride or chlorocresol-based disinfectant; and (4) misting pigs with a preoxygen disinfectant at 0.5% were evaluated for their ability to eliminate *Salmonella* in the lairage environment and to topically reduce *Salmonella* prior to slaughter, respectively.

Supplementation with sodium butyrate and FormaXOL™ for 28-days prior to slaughter, not 63-days (for sodium butyrate only), was effective in reducing *Salmonella* shedding and seroprevalence but only in the absence of secondary infections. Both feed additives did not influence intestinal carriage, nor did they reduce seroprevalence to below the cut-off used for the high *Salmonella* risk category in Ireland (50%), or significantly improve growth performance. Treatment with sodium butyrate provided a benefit of €0.04/kg of live-weight gain; while supplementation with FormaXOL™ increased the feed cost/kg of live-weight gain by €0.08.

In the abattoir, drying lairage pens after C&D with a chlorocresol-based disinfectant eliminated *Salmonella*. Additionally, misting with a preoxygen disinfectant might have a role in topical antisepsis for pigs contaminated with *Salmonella* prior to slaughter and as such warrants further investigation.

The findings presented herein, are readily applicable to farmers, abattoirs and regulatory agencies; and have added novel findings to the field of *Salmonella* control in pigs.

CHAPTER 1: Literature Review

1.1 Introduction

When reviewing the literary landscape pertaining to *Salmonella* in pigs, two key themes continually appear. The first is its prevalence at farm level; while the second is the number of control measures required to limit its transmission, especially during the pre-harvest stage of the production chain. Focusing on these topics, the review that follows summarizes the literature to date. It also aims to provide an understanding of the characteristics of *Salmonella*, its persistence at farm level and in the food product, and finally strategies to regulate its ability to remain in both the farm and abattoir environments.

1.2 *Salmonella*

The genus *Salmonella* is a ubiquitous bacterium that causes illnesses in both humans and animals worldwide. The discovery of *Salmonella* began more than a century ago when American scientists, Daniel Salmon and Theobald Smith first isolated *Salmonella choleraesuis*, now known as *Salmonella enterica*, from pigs in 1886 (Schultz, 2008; Wray and Wray, 2000). Since then, 2,579 *Salmonella* serovars have been identified (Table 1.1) and range in their antimicrobial resistance, virulence and pathogenesis (Lipps, 2008; Maurer and Lee, 2005). The following sections discuss these features in detail.

1.2.1 Taxonomy and Characteristics

Generally, *Salmonella* are Gram-negative rods, which are facultatively anaerobic, mostly motile with peritrichous flagella, and non-spore forming (Holt et al., 1994). Most *Salmonella* spp., are also aerogenic (i.e., gas producing), non-lactose fermenting, citrate-utilizing and oxidase-, urease-, acetylmethyl carbinol-, and potassium cyanide-negative (Agbaje et al., 2011). Non-motile serovars exist including *Salmonella* Gallinarum and *Salmonella* Pullorum; and a recent but rare case of a non-motile *Salmonella* Typhimuiurm was isolated from eggs during a foodborne outbreak in France in 2009 (Holt et al., 1994; Le Hello et al., 2012).

Phenotypically, *Salmonella* are mesophilic organisms growing optimally at temperatures between 35 and 37 °C and at optimum pH's of 6.5 to 7.5. Outside of these ideal conditions, the bacterium can grow at temperatures ranging from 5.2 to 46 °C and pH's of 3.8 to 9.5 (Food Safety Authority of Ireland (FSAI), 2011; Holt et al., 1994). In

addition, *Salmonella* are resilient bacteria capable of surviving extreme conditions; for example, in low moisture [i.e., low water activity (a_w)] foods such as peanut butter and chocolate; in desiccated environments such as powdered infant formula; and in highly acidic environments such as those produced by the stomach (Maurer and Lee, 2005).

In the taxonomic hierarchy, *Salmonella* is a member of the *Enterobacteriaceae* family. The genus consists of two species, *Salmonella enterica* and *Salmonella bongori*. The former, is further divided into six subspecies (based on phenotype and genotype) that are generally designated by Roman numerals I, II, IIIa, IIIb, IV, and VI; whereas, *S. bongori*, is often designated by the Roman numeral V (Brenner et al., 2000; Grimont and Weill, 2007). Table 1.1 lists these species and subspecies (referred to as spp. and subsp., respectively) and the number of serovars identified within each to date.

Table 1.1. The species and subspecies of *Salmonella* and their associated number of serovars

Designation	Species and Subspecies	Number of Serovars
I	*S. enterica* subspecies *enterica*	1531
II	*S. enterica* subspecies *salamae*	505
IIIa	*S. enterica* subspecies *arizonae*	99
IIIb	*S. enterica* subspecies *diarizonae*	336
IV	*S. enterica* subspecies *houtenae*	73
VI	*S. enterica* subspecies *indica*	13
V	*S. bongori*	22
		TOTAL = 2579

Source: Grimont and Weill (2007)

1.2.1.1 Identification and Nomenclature of Salmonella

Typically, *Salmonella* isolates are first identified to the genus and species level followed by biochemical testing to determine the subspecies. To further differentiate the seven *Salmonella* species/subspecies, a subtyping method known as serotyping classifies the bacterium based on the presence of 'O' (somatic), 'H' (flagellar), and virulence or 'Vi' (capsular) antigens (Brenner et al., 2000; Velge et al., 2012; Wray and Wray, 2000). Since a substantial amount of diversity exists in these antigens, 2,579 different *Salmonella* serovars have been identified to date (Table 1.1) and approximately 60% of these belong to group I, *Salmonella enterica* subsp. *enterica* (Grimont and Weill, 2007).

The O antigen is a polysaccharide located on the outer lipopolysaccharide membrane of the *Salmonella* bacterium. It is composed of 4-6 sugars and can vary in: (i) its sugar component; (ii) the covalent bonds between the sugars; or (iii) the link

between the subunits that form the antigen (Centers for Disease Control and Prevention (CDC), 2011; Grimont and Weill, 2007; Wray and Wray, 2000). The O antigens are heat stable, and resistant to alcohol and dilute acids (Andrews et al., 2001).

The H antigen on the other hand, is located on the bacterial flagellum and is composed of protein subunits called flagellin. In *Salmonella*, two different flagellin antigens (which are unique to the bacterium) are expressed: Phase 1 and Phase 2. Usually only one antigen is expressed in a single bacterial cell. Monophasic variants of *Salmonella* Typhimurium, referred to as monophasic *S.* Typhimurium, follow this convention by expressing a single flagellin type i.e. they lack expression of flagellar Phase 2 antigens (CDC, 2011; Grimont and Weill, 2007; Wray and Wray, 2000). This phenomenon can occur naturally, but single expression can also result from a loss or lack of expression of the flagellin gene (CDC, 2011; Grimont and Weill, 2007; Wray and Wray, 2000). Unlike O antigens, H antigens are heat labile (Andrews et al., 2001).

The capsular Vi antigen is a carbohydrate, produced by *Salmonella* Typhi*,* *Salmonella* Paratyphi C, and *Salmonella* Dublin. This carbohydrate is expressed as a capsulated surface-bound polysaccharide that, like H antigens, is heat-sensitive (EFSA Panel on Biological Hazards, 2010b).

Independent agglutination assays with antisera are used to detect the O and H antigens via a reaction with either a single antigen or groups of antigens. Once the O and H antigens are identified, the White-Kauffmann-Le Minor Scheme formula is applied to give the serotype designation (CDC, 2011; Grimont and Weill, 2007). The classical format is as follows:

> "Subspecies [space] O antigen [colon] Phase 1 H antigen [colon] Phase 2 antigen" (CDC, 2011).

For example, using this format, the classical designation of *S. enterica* serotype Typhimurium (for which the current abbreviated convention is *Salmonella* Typhimurium) is <u>1</u>,4,[5],12:i:1,2. Monophasic *S.* Typhimurium are denoted with an antigenic structure of 1,4,[5],12:i:-, as they lack expression of flagellar Phase 2 antigens (EFSA Panel on Biological Hazards, 2010b). Both the <u>1</u> and [5] factors can be present or absent in *S.* Typhimurium (EFSA Panel on Biological Hazards, 2010b). The underlined O factor <u>1</u> is determined by phage conversion and is only present if the culture is lysogenized by the corresponding converting phage (EFSA Panel on Biological Hazards, 2010b). Square brackets around the factor [5] indicate that this antigen may or may not be present and this is not related to phage conversion.

Additionally, *Salmonella* serotypes can be further subdivided according to their biochemical characteristics (i.e., biovars/biotypes) and/or their resistance to bacteriophages (i.e., phage types or lystotypes), antibiotics, or heavy metals (FSAI, 2011).

Historically, the formal designation of a *Salmonella* serotype follows one of two formats: either (1) genus-species in italics; or (2) genus-species-subspecies in italics, followed by the term "serotype" or "serovar" and the serotype name/formula for both. For example, *Salmonella enterica* serovar Typhimurium, or *Salmonella enterica* subsp. *salamae* serotype 47:b:1,5 (CDC, 2011). When the serotypes have a name, as is the case with serotypes from subspecies I (e.g. Typhimurium, Typhi or Enteritidis), the serotype designation is not italicized and the first letter is capitalized to signify that it is not a separate species (see above example) (Brenner et al., 2000; Grimont and Weill, 2007). Following this, there are two ways to name *Salmonella*. The formal designation, as described above, is the first way, whilst the second is the current convention of listing the *Salmonella* genus followed by the named serotype e.g. *Salmonella* Derby (abbreviated as *S.* Derby).

Naming of *Salmonella* serotypes has evolved over the years. At first, they were named for the syndrome or relationship they were associated with (e.g. *S.* Typhi, *S.* Paratyphi A, B, C) but due to limitations, this changed to naming based on the geographical location where the serotype was first isolated (e.g., Dublin, London, or Panama) (Brenner et al., 2000; European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC), 2014; Grimont and Weill, 2007).

Clinically, serotypes may also be grouped on the basis of host range. The first group consists of host-restricted serotypes, which are associated with disease in a few hosts but can cause disease in a small number of other species. For example, *Salmonella* Dublin, which is cattle-adapted but infects humans as well; *Salmonella* Choleraesuis, which infects both pigs and humans; *Salmonella* Typhi, which is human-specific; or *Salmonella* Gallinarum, which is restricted to birds [Shivaprasad et al., 2000 as cited in (Velge et al., 2012)]. The second group are broad host range. For example, *Salmonella* Typhimurium and *Salmonella* Enteritidis which infect humans and livestock including cattle, pigs, and poultry worldwide [Velge et al., 2005 as cited in Velge et al. (2012)].

Many of the *Salmonella* serotypes identified to date are known pathogens. The pathogenicity of *Salmonella* is complex, as the bacterium has evolved to overcome the

defence mechanisms of the host in order to survive, proliferate, and become infectious. The following section discusses *Salmonella* pathogenicity in detail.

1.2.2 Pathogenesis and Virulence of *Salmonella*

Microorganisms, and in particular bacteria, are classified as either commensal or pathogenic. Commensal bacteria are not harmful and may even have some beneficial effects, while pathogenic bacteria cause disease (Quinn et al., 2011). For a microorganism to be considered pathogenic, it must satisfy Koch's postulates, which were formulated by the German physician Robert Koch. These set out four criteria that microorganisms must satisfy in order to cause disease (Quinn et al., 2011; Salyers and Whitt, 1994):

i. "The pathogenic microorganism must be present in every case of the disease but absent from the healthy individuals or animals";
ii. "The suspected microorganism must be isolated from the infected individual or animal and grown in pure culture";
iii. "The same disease must occur when the isolated microorganism is injected into healthy susceptible humans or animals"; and
iv. "The same microorganism must be isolated again from the injected human or animal, which developed disease".

However, since *Salmonella* infection is usually sub-clinical with pigs remaining asymptomatic (i.e., showing no signs of clinical disease) (EFSA, 2008; Wray and Wray, 2000), the first criterion of Koch's postulates is not applicable to pigs. Following on from Koch's postulates, for *Salmonella* to survive, replicate and cause disease in its host, it must: (1) survive the acidity of the stomach; (2) adjust to the high pH, anoxic environment and detergents present in the small and large intestines; (3) penetrate the mucosal layer of the intestinal villi; (4) attach and invade intestinal cells; and (5) if the disease becomes septicemic, overcome the defence mechanisms of the host (i.e., macrophages) (Maurer and Lee, 2005).

Generally, *Salmonella* infection in a host, such as pigs and humans, starts by ingestion of the bacterium from contaminated food, water, or faeces, and its subsequent survival in the stomach and during passage to the small intestine. Next, *Salmonella* must adhere to and invade the simple columnar epithelium of the small intestine. This is accomplished by adhesion to two principle cells lining the epithelium – enterocytes and microfold (M) cells (Boyen et al., 2008a; Maurer and Lee, 2005; Muller et al., 2012; Takeuchi, 1967; Velge et al., 2012). Following adhesion, *Salmonella* must invade these epithelial cells in order to cause gastroenteritis and systemic infection.

This invasion step, also known as the Trigger and Zipper mechanisms, occurs when *Salmonella* enters the lumen of the small intestine and senses the surrounding environment (i.e. pH, osmolarity, and oxygen tension) (Lahiri et al., 2010; López et al., 2012; Tegtmeyer et al., 2012; Velge et al., 2012). This mechanism enables type-III secretion system (T3SS-1) genes encoded on the *Salmonella* Pathogenicity Island-1 (SPI-1) to be expressed, subsequently causing the needle complex and export apparatus of the T3SS-1 to be assembled (Velge et al., 2012). This apparatus allows secretory proteins or effectors (SipA, SopA, SopB, SopD and SopE2) to pass through the inner and outer membrane of the bacterial cell. These proteins cause cytoskeleton rearrangements and an outward extension (i.e., ruffle) of the epithelial cell, resulting in *Salmonella's* internalization into the cell (Lahiri et al., 2010; Maurer and Lee, 2005; Velge et al., 2012). Once *Salmonella* invades the epithelial cells, it is surrounded by a vacuole and is transported further into the lamina propria (by actin filaments). There it interacts with, and survives in, other phagocytic cells, namely macrophages [via a type III secretion system (T3SS-2) encoded on the SPI-2] and dendritic cells (Lahiri et al., 2010; López et al., 2012; Velge et al., 2012). It is within these cells that *Salmonella* is taken up and transferred from the gastrointestinal (GI) tract to the spleen, liver, and blood leading to systemic infection and gastroenteritis (also known as salmonellosis) (Maurer and Lee, 2005; Tegtmeyer et al., 2012; Velge et al., 2012).

1.2.2.1 Salmonella in Pigs

Pigs are known to carry *Salmonella* in the intestinal tract (mainly the cecum), lymph nodes and tonsils. Generally, in acute salmonellosis, pigs shed a high level of *Salmonella* with clinical signs that vary from mild diarrhea, acute septicaemia to death (EFSA, 2008). Apart from infection with *Salmonella* Choleraesuis, clinical signs of salmonellosis in finisher pigs are not common with the infections referred to as being sub-clinical (EFSA, 2008; Wray and Wray, 2000). In chronic infection, pigs are considered carriers, intermittently shedding low levels of the bacterium and showing no signs of infection (Wray and Wray, 2000). Of major importance are infected pigs that shed *Salmonella* while remaining asymptomatic (i.e., showing no signs of clinical disease). Asymptomatic finisher pigs are of particular concern, as transfer of *Salmonella* to the carcass at slaughter can lead to contamination of final pork cuts, inadvertently causing human infection upon consumption (Botteldoorn et al., 2003). This results in a food safety issue, and is the focus of this review. The most frequent

Salmonella serotypes isolated from finisher pigs at slaughter in the last 5 years (2010-2014) across the European Union (EU) were *S*. Typhimurium (specifically the DT104, DT120, DT193 and U302 phage types) and its monophasic variant *S*. 4,[5],12:i:-, *S*. Derby, *S*. Altona and *S*. Infantis (Alban et al., 2012; National *Salmonella Shigella* and *Listeria* Reference Laboratory (NSSLRL), 2013; Rostagno and Callaway, 2012). The baseline survey conducted by EFSA (2008) from 2006-2007, showed that one in ten pigs sent to slaughter in 24 member states in the EU were infected with *Salmonella* in the lymph nodes; and one in twelve pig carcasses were contaminated with *Salmonella* (from the 13 member states that also analyzed carcass swabs). As a result, 87 different *Salmonella* serotypes were isolated from lymph nodes with the most frequent being *S*. Typhimurium, *S*. Derby, *S*. Rissen, *S*. 4,[5],12:i:-, and *S*. Enteritidis (EFSA, 2008). While, 30 different *Salmonella* serotypes were isolated from the pig carcasses with *S*. Typhimurium, *S*. Derby, *S*. Infantis, *S*. Bredeney, *S*. Brandenburg being the most frequent (EFSA, 2008). Together, the 9 most frequent *Salmonella* serotypes isolated from slaughter pigs in the EU over an 8-year period (2006-2014) were *S*. Typhimurium, *S*. 4,[5],12:i:-, *S*. Derby, *S*. Altona, *S*. Infantis, *S*. Rissen, *S*. Enteritidis, *S*. Bredeney, *S*. Brandenburg.

1.2.2.2 Salmonella in Humans

Clinical signs of salmonellosis in humans include diarrhea, fever, abdominal cramps, nausea, and vomiting approximately 12 to 72 hours after infection. In most cases, the illness is self-limiting and lasts 4 to 7 days; however, in some patients, a systemic infection such as septicaemia develops requiring hospitalization and antimicrobial treatment. In rare cases, reactive arthritis occurs and this has a low (<1%) mortality rate, i.e., the 2014 mortality rate from 43,995 confirmed non-typhoidal salmonellosis cases was 0.15% or 65 deaths (EFSA and ECDC, 2015b).

In the EU, non-typhoidal salmonellosis is a notifiable disease (Decision No 2119/98/EC). In 2014, 88,715 confirmed cases (from a range of transmission routes including food, travel, pets and direct animal contact) were reported in the EU and non-member states, representing an incidence rate of 23.4 cases per 100,000 population (EFSA and ECDC, 2015b). From these cases, the five most commonly reported serotypes were *S*. Enteritiditis (44.4%), *S*. Typhimurium (17.4%), monophasic *S*. Typhimurium (7.8%), *S*. Infantis (2.5%), and *S*. Stanley (1.0%) (EFSA and ECDC, 2015b). On a national scale, 2013 saw 324 laboratory-confirmed non-typhoidal

salmonellosis cases in Ireland, representing a national crude incidence rate of 7.1 cases per 100,000 population (Health Protection Surveillance Centre (HPSC), 2013). Among the reported serotypes, the five most common were *S.* Typhimurium (22.9%), monophasic *S.* Typhimurium (16.0%), *S.* Enteritidis (14.9%), *S.* Infantis (4.2%) and *S.* Dublin (3.6%) (HPSC, 2013).

It is worth mentioning that monophasic *S.* Typhimurium has been appearing more frequently, both in humans and pigs, in recent years within the EU and USA (EFSA Panel on Biological Hazards, 2010b; Hauser et al., 2010). In fact, they are now among the 10 most frequently isolated serovars from these species (as outlined above and in Section 1.2.2.1). They were first reported in the mid 1980's in chickens, and later in the late 1990's in predominately pigs and bovines.

1.2.3 Antimicrobial Resistance amongst *Salmonella*

Worldwide, antimicrobial agents have been used in agriculture and human medicine for decades. In animal production they are administered as: therapeutics[1], prophylactics[2], metaphylactics[3] and for growth promotion (routine use of the latter was banned in the EU in 2006). In human medicine, they are used as a therapeutic drug and/or as a prophylactic.

Since their development, antimicrobials have become invaluable in reducing morbidity and mortality associated with infectious diseases (White and McDermott, 2001). However, through their use, a selective pressure is imposed (Threlfall, 2002), creating the infamous "survival of the fittest" Darwinian principal, where resistant bacteria are able to survive and multiply (promoting opportunities for resistances to develop), while susceptible bacteria are destroyed.

1.2.3.1 Acquisition of Antimicrobial Resistance

This phenomenon of developing resistance to antimicrobials occurs either via intrinsic or extrinsic mechanisms. The former is due to a natural structural or functional characteristic of the bacterium that allows it to tolerate an antimicrobial drug or class of drugs. This is a form of insensitivity and may be due to any one or more of the following:

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[1] To fight infection/disease
[2] To prevent an infection/disease
[3] A combination of therapy for sick animals and prophylaxis for healthy animals

(i) Enzymatic drug inactivation, whereby specific enzymes act to modify the nucleus of the drug, thereby preventing it binding to the bacterial target;
(ii) Modifying or replacing the drug target site so that the drug is no longer effective;
(iii) Reducing drug uptake by preventing entry into the cell through modification (decreased size or expression) or destruction of the porin channel; or
(iv) Active efflux using efflux pumps that reduce the concentration of an antimicrobial compound within the cytoplasm.

Extrinsic resistance or acquired resistance on the other hand, is the main mechanism that bacteria use to develop antimicrobial resistance (AMR). It is based on a genetic change in the bacterial genome that arises as a consequence of: (i) a mutation; (ii) horizontal acquisition of a genetic element by transduction, transformation or conjugation; or (iii) a combination of the two.

In *Salmonella* spp., the acquisition of multidrug resistance genes (i.e., genes that encode resistance to at least one antimicrobial drug in 3 or more antimicrobial drug categories) is via conjugation, i.e., the movement of foreign deoxyribonucleic acid (DNA) between bacteria by plasmids or transposons as a result of direct cell-to-cell contact (Lipps, 2008; Magiorakos et al., 2012; Wray and Wray, 2000). Using this horizontal gene transfer (HGT) method, *Salmonella* spp., have acquired genes that allow it to survive and proliferate in the presence of the very drugs used to destroy it. Moreover, many serotypes of *Salmonella* carry functional prophages that are capable of generalized transduction (i.e., the transfer of double-stranded DNA by bacteriophages - viruses that infect and replicate in bacteria) of chromosomal host markers and plasmids (Lipps, 2008; Schicklmaier et al., 1998).

Bacterial resistance to antimicrobials is not new and while it has been a topic of concern for many years, it has recently received more attention as the popular antibiotics of the past and new derivatives (e.g., methicillin, tetracycline, carbapenem, fluoroquinolones etc.) are becoming more ineffective in treating bacterial infections. Mono-resistant *Salmonella* strains started emerging in the early 1960s and prevalence has risen, globally, since then. Van Leeuwen et al. (1979) [as cited in Wray and Wray (2000)] described an increase in tetracycline resistance amongst *Salmonella* spp. in the Netherlands from 1959 to 1974 and its subsequent decline after the drug was banned as a growth promoter. In the United States of America (USA), tetracycline resistant

Salmonella isolates obtained from animals and humans first appeared in 1956-1957 and *S.* Typhimurium was observed to have a higher resistance profile than other serotypes (Cherubin, 1981; Wray and Wray, 2000). In the United Kingdom (UK), *S.* Typhimurium definitive phage type (DT) 29 with resistance to five antimicrobial drugs [ampicillin (A), streptomycin (S), sulphonamide (Su), tetracycline (T), and furazolidone (Fu), resulting in the AMR profile ASSuTFu] was first identified in 1964 in calves and humans (Threlfall, 2002). From 1975 to the mid-1980s, the occurrence of multidrug resistant (MDR) *S.* Typhimurium strains increased dramatically in the UK but this time the phage type and drug resistance profile were different to those of the DT29 isolates identified in 1964 (Threlfall, 2002). In addition to possessing the ASSuT resistance profile, *S.* Typhimurium DT193, 204 and 204c also showed resistance to chloramphenicol (C), gentamicin (G), kanamycin (K) and trimethoprim (TM), resulting in the MDR profile ACGKSSuTTm (Threlfall, 2002). It was determined that the *Salmonella* isolates sequentially acquired these specific drug resistance genes from plasmids and transposons carrying them (Threlfall, 2002). Moreover, *S.* Typhimurium DT104, which emerged in the early 1980s from a human case of salmonellosis originating from exotic birds in the UK, demonstrated a penta-resistant pattern of ACSSuT (Threlfall et al., 2000). By the late 1980s, this phage type and resistance pattern had spread to cattle and subsequently over the next 5-years became common to poultry (specifically turkeys), pigs and sheep (Threlfall et al., 2000). In pigs, *S.* Typhimurium infection has been a common occurrence, however in recent years, monophasic variants of S. Typhimurium have emerged with a typical AMR profile, of ASSuT, that is similar to that of *S.* Typhimurium strains (EFSA Panel on Biological Hazards, 2010b; Hauser et al., 2010). However, recent evidence suggests that not all monophasic strains from pigs will present with this classic, ASSuT, AMR profile (Burns et al., 2015).

1.2.4 Diagnostic Methods for Isolation, Quantification and Identification of *Salmonella*

Detection and identification of *Salmonella* from samples, including those of porcine origin, can be achieved using two main approaches – traditional culture-based methods, and modern molecular methods. Both approaches vary in terms of their complexity, technology used, laborious nature and length of time required to determine a definitive *Salmonella*-positive result. Despite this, they are often used in conjunction

with each other. Over the years, newer and faster methods have been developed to detect and quantify *Salmonella* in a sample, yet the gold standards still prevail. The following sections discuss the two approaches in detail.

1.2.4.1 Culture-Based Methods

The traditional culture-based method for *Salmonella* isolation consists of plating an enriched sample onto selective agars to detect the presence of the bacterium, followed by biochemical and serological tests. Table 1.2 lists the typical reactions of *Salmonella* in various biochemical and serological tests used to identify the bacterium. While these tests are numerous, the lysine decarboxylase, urease, growth in potassium cyanide (KCN) broth, and indole tests are sufficient for presumptive identification. Since *Salmonella* strains do not always react to the typical biochemical tests, serological tests are the final confirmatory method to accurately identify the bacterium (Andrews et al., 2001).

When examining samples for the presence of *Salmonella*, it is advisable, and sometimes compulsory, depending on the nature of the work; to follow internationally recognized procedures, such as those set by the International Organization for Standardization (ISO). Different ISO methods are available for *Salmonella* isolation, depending on the type of sample collected.

Table 1.2. Biochemical and Serological Reactions of *Salmonella*

Test/Substrate	Positive Reaction	Negative Reaction	Typical Reaction for *Salmonella**
Glucose test using Triple Sugar Iron (TSI) agar	Yellow butt	Red butt	+
Lysine decarboxylase test using Lysine Iron Agar (LIA)	Purple butt	Yellow butt	+
Lysine decarboxylase test using lysine broth	Purple colour	Yellow colour	+
Hydrogen Sulphide using TSI and/or LIA	Blackening	No blackening	+
Urease test	Purple-red colour	No colour change	-
Phenol red dulcitol broth	Yellow colour and/or gas production	No colour change; no gas production	+
Potassium cyanide (KCN) broth	Growth	No growth	-
Malonate broth	Blue colour	No colour change	-
Indole test	Red colour at the surface	Yellow colour at the surface	-
Phenol red lactose broth	Yellow colour and/or gas production	No colour change; no gas production	-
Phenol red sucrose broth	Yellow colour and/or gas production	No colour change; no gas production	-
Voges-Proskauer test	Pink-to-red colour	No colour change	-
Methyl red test	Diffuse red colour	Diffuse yellow colour	+
Simmons citrate broth/agar	Growth and blue colour	No growth and no colour change	Variable
Polyvalent flagellar test	Agglutination	No agglutination	+
Polyvalent somatic test	Agglutination	No agglutination	+

*These are typical responses. Not all serotypes will react the same way.

Source: Modified from Andrews et al. (2001)

ISO 6579:2002 is used for the detection of *Salmonella* spp. in food and animal feeding stuffs and its 2007 amendment (Amd 1:2007) for animal faeces and environmental samples from primary production. This review focuses on animal faeces, since it is the main sample type analyzed in the subsequent experimental chapters and *Salmonella* isolation from these types of samples requires 6 steps (Table 1.3). The first is a pre-enrichment in non-selective medium to allow resuscitation of any injured cells and multiplication of *Salmonella* (among others) in the sample. This is followed by selective enrichment on a semi-solid medium and plating onto two solid selective media; purification of presumptive *Salmonella* colonies on a non-selective

medium; and finally confirmation using biochemical and serological tests (as listed in Table 1.2). Following these steps, the *Salmonella* isolates can be serotyped using the White-Kauffmann-Le Minor Scheme as discussed in section 1.2.1.1.

The second selective solid medium and media for confirmatory tests are not specified in the ISO method. Instead, a general list of typical media is provided and it is at the discretion of the testing laboratory to select the appropriate ones. As brilliant green agar (BGA) was used as the second selective medium and urea agar slants, *Salmonella* chromogenic agar, and the *Salmonella* latex agglutination kit were used in confirmatory tests when isolating *Salmonella* from porcine samples in subsequent experimental chapters, these are detailed in Table 1.3.

Table 1.3. Isolation, detection and confirmation of *Salmonella* spp. from animal faeces based on ISO 6579:2002/Amd.1:2007

Step	Description
Step 1	• Pre-enrichment of fecal material in buffered peptone water (BPW) for 18-hours ± 2-hours at 37°C ± 1°C.
Step 2	• Selective enrichment of the culture obtained in Step 1 on Modified Semi-solid Rappaport-Vassiliadis (MSRV) agar. • Incubate at 41.5°C ± 1°C for 24-hours ± 3-hours (and a further 24-hours if a plate is negative after the initial 24-hours). • Growth for presumptive *Salmonella* spp. is visible as a grey-white, turbid zone
Step 3	• Selective plating of suspect *Salmonella* colon(ies) obtained from Step 2 on two solid agar media. (1) Xylose lysine deoxycholate (XLD) agar; and (2) One other medium e.g. brilliant green agar (BGA) • Both agars are incubated at 37°C ± 1 °C for 24-hours ± 3-hours. • Typical *Salmonella* colonies on XLD are red with a black centre; while on BGA, they are red/pink with the agar turning a bright pink/red colour.
Step 4	• Purification of isolates on non-selective medium such as nutrient agar (NA) or plate count agar (PCA). • Suspect colonies from Step 3 are plated onto PCA and incubated at 37°C ± 1 °C for 24-hours ± 3-hours.
Step 5	• Biochemical confirmation of colonies from Step 4 using, for example*: (1) Urea agar slants; and (2) *Salmonella* chromogenic agar plates • Incubate at 37°C ± 1 °C for 24-hours ± 3-hours. • Typical *Salmonella* colonies on urea agar will be yellow/orange in colour; while on *Salmonella* chromogenic agar, they are magenta in colour with a raised, smooth morphology.
Step 6	• Serological confirmation of colonies from Step 4 using a *Salmonella* Latex Agglutination Kit (Oxoid®). • A result is positive if agglutination of the test latex occurs within 2 minutes, and no agglutination of the control latex occurs within 2 minutes.

* The tests described above were the typical tests used in the laboratory where the isolation of *Salmonella* from faeces was performed in subsequent chapters. However, any of the tests listed in Table 2.1 can also be used.

Source: International Organization for Standardization (2007)

1.2.4.2 Molecular Methods

The identification of *Salmonella* spp. from a sample takes 4-7 days when using traditional culture-based methods. This is quite lengthy, especially when investigating a *Salmonella* outbreak, as finding the source is essential to limiting infection and spread. Therefore, molecular methods have been developed (and are being continually modified) for the rapid detection, identification, tracking and/or quantification of *Salmonella* spp. in a range of sample matrices. Polymerase chain reaction (PCR), real-

time PCR, pulsed field gel electrophoresis (PFGE), and multiple locus variable number tandem repeat analysis (MLVA) are such methods and are described in further detail below.

1.2.4.2.1 Polymerase Chain Reaction (PCR)

Polymerase chain reaction was developed in the 1980s by Kary Mullis and is an *in vitro* DNA amplification method that is based on using DNA polymerase to synthesize a new strand of DNA complementary to the template strand (i.e., the sample of DNA that contains the target sequence) (Wray and Wray, 2000). This section discusses PCR-based detection/enumeration of *Salmonella*.

The whole PCR process, following initial pre-enrichment and selective enrichment, and DNA extraction steps, can yield results in less than 24 hours versus 4-7-days for the traditional culture-based method. However, a limitation of this procedure is that detection of the amplified sequence is performed at the end of the last PCR cycle. To address this issue, a real-time PCR technique was developed for *Salmonella*.

The real-time quantitative PCR (qPCR) method uses the same procedure as the traditional PCR method but it allows the DNA amplified to be quantified after each cycle and viewed in real-time (i.e., at the same time that it is being amplified). Viewing of the amplification process is via fluorescence. The increase in the fluorescent signal is directly proportional to the number of PCR amplicons generated in the exponential phase of the reaction. By using a thermal light cycler, the change in the fluorescent signal over the course of the reaction can then be measured and plotted against the PCR cycle number.

Several qPCR methods are currently available. The TaqMan® method uses a TaqMan® probe consisting of two dyes – a quencher long-wavelength dye and a reporter short-wavelength dye – and *Taq* polymerase to emit fluorescence (Higuchi et al., 1993). The SYBR® Green method binds a SYBR® Green probe to any double-stranded DNA and emits light when excited (Yin et al., 2001). Lastly, a molecular beacon method uses a reporter probe wrapped into a hairpin structure in close contact with a quencher dye (Tyagi and Kramer, 1996). What all of these methods have in common is that they use fluorescent reporters[4] that are incorporated into the DNA

[4] Fluorescent reporters are double-stranded DNA binding dyes, or other dye molecules attached to PCR primers or probes.

template strand during amplification and emit a measurable fluorescent signal. The use of hybridization probes makes qPCR more robust than conventional PCR.

In the detection/quantification of *Salmonella* a wide range of gene targets have been utilized, with pathogenic, virulence, or biochemical functions. A number of genes are used for the detection of *Salmonella* and include:

- 16S ribosomal RNA – encodes the long component of the 30S small prokaryotic ribosomal subunit, and multiple copies are found throughout the genome. Forward primer sequence (5' to 3') is TGTTGTGGTTAATAACCGCA; reverse primer sequence (5' to 3') is CACAAATCCATCTCTGGA (Lin and Tsen, 1996).
- *sipB* – Located on SPI-1, encodes a secreted protein involved in T3SS. It is a translocation machinery component and is involved in translocating secreted proteins to the host cell, and interacts with SipC. Forward primer sequence (5' to 3') is ACAGCAAAATGCGGATGCTT; reverse primer sequence (5' to 3') is GCGCGCTCAGTGTAGGACTC (Carlson et al., 1999).
- *sipC* – Located on SPI-1, encodes a secreted protein involved in T3SS. It is involved in translocating secreted proteins to host cell and insertion into host cell plasma membrane and interacts with SipB. Forward primer sequence (5' to 3') is ACAGCAAAATGCGGATGCTT; reverse primer sequence (5' to 3') is GCGCGCTCAGTGTAGGACTC (Carlson et al., 1999).
- *invA* – Located on SPI-1. It is a needle complex export protein of the T3SS apparatus. It is also an invasion gene. Forward primer sequence (5' to 3') is GCTGCGCGCGAACGGCGAAG; reverse primer sequence (5' to 3') is TCCCGGCAGAGTTCCCATT (Ferretti et al., 2001).
- *hilA* – Located on SPI-1, and is a T3SS regulator. It activates the expression of invasion genes and activates the expression of prgHIJK, which is part of the T3SS. Forward primer sequence (5' to 3') is CTGCCGCAGTGTTAAGGATA; reverse primer sequence (5' to 3') is CTGTCGCCTTAATCGCATGT (Guo et al., 2000).
- *ttrRSBCA* – Tetrathionate reductase complex. The *Salmonella enterica* tetrathionate complex catalyses the reduction of trithionate but not of sulphur or thiosulfate. Forward ttr-6 primer (5' to 3') is

CTCACCAGGAGATTACAACATGG; reverse ttr-4 primer (5' to 3') is AGCTCAGACCAAAAGTGACCATC (Malorny et al., 2004).

Few studies are available for the direct quantification of *Salmonella* from fecal matter from pigs before pre-enrichment. Many studies have quantified *Salmonella* by qPCR after selective enrichment on MSRV (Eriksson and Aspan, 2007; Gentry-Weeks et al., 2002; Pires et al., 2013) but not before or directly after pre-enrichment. One of the main reasons for this is that faeces contains large amounts of PCR inhibiting compounds such as phenolic metabolic compounds, DNases, proteases and polysaccharides (Malorny and Hoorfar, 2005).

1.2.4.2.2 Pulsed Field Gel Electrophoresis (PFGE)

In contrast to the PCR methods outlined above, PFGE is a subtyping method that generates a PFGE pattern, which acts as a DNA fingerprint, for a bacterial isolate (CDC, 2013). The method involves the use of restriction enzymes to cut the bacterial genome at specific restriction sites, generating restriction fragments of the DNA. These fragments are then separated by size when run on an agarose gel using an electric field (CDC, 2013). Because of the large size of the DNA fragments generated, the DNA extraction, purification and restriction steps must be performed in agarose plugs, and a specialised PFGE apparatus must be used to run the gels. The PFGE patterns produced for each isolate are compared to other patterns (either stored in databases such as PulseNet or for a reference strain on the same gel) to discriminate between isolates. These PFGE patterns are vital in outbreak and epidemiological investigations. For example, PFGE has been used to link *Salmonella* isolates recovered from pigs on the farm to lairage or from lairage to carcass and ceca/lymph nodes at slaughter (Argüello et al., 2012; Duggan et al., 2010; Mannion et al., 2012).

Although PFGE is a time consuming process, and it cannot discriminate closely related strains e.g. clonal *S.* Typhimurium and related variants, it is still the gold standard used by many laboratories/agencies, including the CDC and ECDC in outbreak investigations (CDC, 2013).

1.2.4.2.3 Multilocus Variable Number Tandem Repeat Analysis (MLVA)

Although PFGE has good discriminatory power and is proven highly useful and reliable (Heir et al., 2002), its laborious nature and lack of ability to discriminate all

Salmonella isolates effectively (for example certain phage types such as with DT104), leads to problems in tracing the strains from the source (Murphy et al., 2001). Previous research has therefore focused on the development of the molecular typing scheme Multiple Locus Variable number tandem repeat Analysis (MLVA) as an alternative or a complementary typing tool to PFGE (Larsson et al., 2009). Generally, within the bacterial genome a high percentage of DNA consists of repeats. These repeats vary in size, location, and complexity, and can be clustered in one genomic area or dispersed throughout the genome (Larsson et al., 2009; Lindstedt et al., 2004). Due to the high number of repeats, they become targets for DNA slippage and recombination leading to alterations in the copy number of the repeats (Larsson et al., 2009; Lindstedt et al., 2004). The MLVA method amplifies the genomic sites that undergo rapid alterations, also known as contingency loci, through PCR and analyzes the fragment sizes by high-resolution capillary electrophoresis (Larsson et al., 2009; Lindstedt et al., 2004). From the size estimates, the number of repeats at each locus can be determined, thereby differentiating even highly related strains (Larsson et al., 2009; Lindstedt et al., 2004).

1.3 Epidemiology of *Salmonella* in Pork Production

Globally, *Salmonella* is one of the most common and major causes of foodborne illnesses in humans; and pork, after poultry and eggs, is considered an important source of infection (EFSA, 2006). As outlined in section 1.2.2.1, in pigs, *Salmonella* usually coexists with the intestinal microflora, and normally S*almonella* infection causes little or no clinical signs of disease (Callaway et al., 2008). As such, pigs become reservoirs for *Salmonella* contamination along the production chain (Ojha and Kostrzynska, 2007).

For pigs, the first point of contact with this ubiquitous bacterium is at farm level (Figure 1.3 depicts the typical pig production cycle on the island of Ireland), followed by transport from the farm to slaughter, lairage, and finally the actual slaughter process.

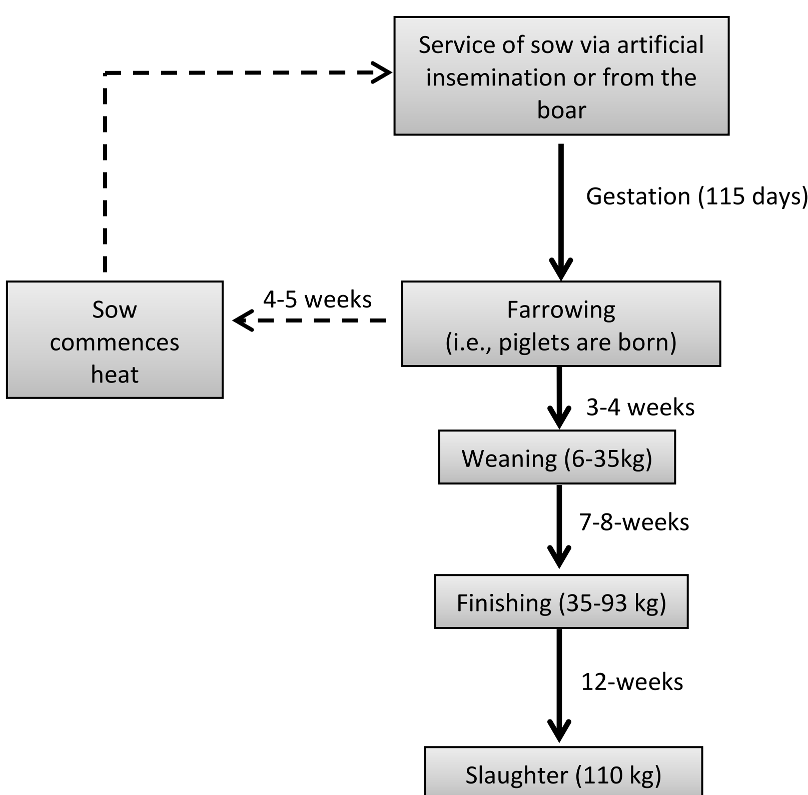

Figure 1.3. The pig production cycle.

Source: Teagasc Pig Development Advisory (2015)

1.3.1 Carriage of *Salmonella* on the Farm

At farm level, the main sources that introduce *Salmonella* to finisher pigs (i.e., pigs between 35 and 93 kg in weight or 12-25 weeks in age) include: (i) purchased piglets; (ii) *Salmonella*-infected breeding pigs; (iii) feed; and (iv) birds and rodents (Alban et al., 2010; EFSA, 2006).

Within the EU, bacteriological surveillance for *Salmonella* spp. is usually based on sampling at the abattoir by means of carcass swabs, sampling of pig meat, lymph nodes, and/or cecal digesta. Serological surveillance is often performed and is normally part of national *Salmonella* control programs (see section 1.3.1.1 for more detail). However, few countries have reported prevalence at farm level based on pig fecal/other sampling (see section 1.3.1.1 for reasons why). According to the 2014 report released by EFSA and ECDC (2015b), the current *Salmonella* prevalence from pigs at farm level across 8 Member States (MS) – Denmark, Estonia, Finland, Germany, Italy, Malta, Netherlands, Sweden and 1 non-MS – Norway – for pig herds, ranged from 0 to 27.3% and on an animal basis, from fecal samples, it ranged from 0 to 21.5%. However, only 9 countries provided information to determine the prevalences and these countries are not known for having high *Salmonella* prevalence as per the 2008 EU baseline survey. Therefore, it is questionable as to how representative these data are of the current trend of subclinical infection among pig herds on the continent. The overall 2014 herd and animal level prevalence of *Salmonella* in the EU was 10.1% and 7.7%, respectively (EFSA and ECDC, 2015b). While not exceedingly high, these levels suggest that even with compliance with EU Regulation (EC) No. 2160/2003 (on the control of *Salmonella* and other specified food-borne zoonotic agents), there are still underlying issues that need to be investigated to obtain better control, especially within those countries with a herd prevalence of >10% .

In Ireland, few studies have been published to determine the prevalence of *Salmonella* in pigs/pig herds at farm level. One such study, conducted in 1999, showed that 30 of 59 (50.8%) farms sampled across the Republic were infected with at least one *Salmonella* serovar, with varying prevalence across production stages (first stage weaner 8.0%; second stage weaner 4.8%; fattener/finisher 5.9%; dry sow 5.1%; and farrowing sow 2.3%) (Rowe et al., 2003). The serovars reported were those frequently associated with Irish pigs: *S.* Typhimurium, *S.* Dublin, *S.* London, *S.* Livingstone, and *S.* Infantis. Similarly, in a more recent study, 9 of 10 farms (with historically high seroprevalence) sampled in the Republic of Ireland from 2012 to 2013, were positive for *Salmonella* in at least one production stage (Burns et al., 2013). High *Salmonella* prevalence was detected among gilts, first and second stage weaners, and finishers (16.7%, 15.3%, 16.7%, and 16.7%, respectively) with lower rates among dry and farrowing sows (6.7%, and 3.8%, respectively) (Burns et al., 2013). The serovars isolated (*S.* Typhimurium; monophasic *S.* Typhimurium; *S.* Derby; *S.* Typhimurium

Copenhagen; and *S.* Infantis) were similar to the strains detected by Rowe and colleagues but a greater occurrence of monophasic *S*. Typhimurium was observed, suggesting that it is currently the predominant serovar among Irish pig herds, at least those with high seroprevalence. As discussed in section 1.2.2.2, monophasic variants of *S.* Typhimurium are frequently emerging in pigs in the EU and USA (EFSA Panel on Biological Hazards, 2010b; Hauser et al., 2010).

In general, *Salmonella* prevalence increases with age – from nursery (i.e., piglets) to slaughter (i.e., market age) – and as such it is these market age pigs that, when sent to slaughter, pose the greatest risk for contamination in the abattoir (Dorr et al., 2009; Rodriguez et al., 2006). The high *Salmonella* prevalence observed in first/second stage weaners by Rowe et al. (2003) and Burns et al. (2013) is indicative of stress-induced infection from weaning, a change in environment, diet, littermates, and no longer getting maternal antibodies from colostrum (Kranker et al., 2003; Roca et al., 2014).

Overall, at farm level, the most commonly reported and/or isolated *Salmonella* serovars in the EU from 2004-2012 were *S.* Typhimurium, *S.* Derby, monophasic *S.* Typhimurium, *S.* Infantis, *S.* Enteritidis, *S.* Rissen, *S.* London, and *S.* Choleraesuis (see section 1.3.3). Less common serovars included: *S.* Typhimurium Copenhagen, *S.* Goldcoast, *S.* Livingstone, *S.* Panama, and *S.* Brandenburg (Burns et al., 2013; Nollet et al., 2005a; Rowe et al., 2003; van Duijkeren et al., 2002). Since no new or surprising variant was detected over this 8-year period, it can be concluded that these serotypes are the main infectious agents in pigs.

1.3.1.1 On-Farm Salmonella Detection: Bacteriology vs. Serology

As mentioned above, few EU member states have evaluated farm level prevalence of *Salmonella* by means of bacteriological/serological sampling. Most use serological tests (from meat-juice or blood samples taken at slaughter) to determine herd prevalence at farm level (see section 1.6 for further detail).

Bacteriological testing of faeces from individual pigs provides a suitable measurement of the current *Salmonella* shedding status of the animals/herds on the farm but since it is too costly in terms of time, labour and money to conduct for on-farm samples, results are extrapolated from testing done at slaughter (Ball et al., 2011). Nevertheless, the current gold standardard method of determining the *Salmonella* status

prior to slaughter is bacteriological testing at farm level conducted as close, as reasonably possible, to the time of delivery to the slaughterhouse.

On the other hand, serological testing via Enzyme-Linked Immunosorbent Assay (ELISA) is a valuable tool for *Salmonella* surveillance at farm level, as it is less laborious and more cost-effective than bacteriological methods. However, since it cannot identify the current *Salmonella* status[5] of the individual pig, its use for the setting of targets is questionable (EFSA, 2008). In a study conducted by Nollet and colleagues (2005b), pig herds that were serologically negative were found to be bacteriologically positive when jejunal and colonic digesta and mesenteric lymph nodes were tested. This outcome suggested that, at the time of sampling, a recent infection occurred with a limited immunological response that was undetectable by the ELISA (Nollet et al., 2005b). This observation was further corroborated by Rostango et al. (2012) who found wide variations in bacteriologic and serologic *Salmonella* prevalence (12.9% and 35.4%, respectively) in the same group of finishing pigs, repeatedly sampled every 1-2 weeks for 12-weeks. Again, it was suggested that recent *Salmonella* infections (i.e. <1-week prior to testing) cannot be detected serologically, and pigs which were infected during the last few days of the finishing period, were not identified when meat samples from the diaphragm were collected at slaughter (Rostagno et al., 2012). Thus, this method, while useful for surveillance at farm level, is limited in terms of identifying high-risk pigs at slaughter (Ball et al., 2011; Rostagno et al., 2012).

1.3.2 Effect of Transport and Lairage Holding on *Salmonella* Shedding

Transport from the farm and lairage holding within the abattoir are, increasingly, reported as critical points for the transmission of *Salmonella* to naïve pigs (Letellier et al., 1999; Rostagno et al., 2003). The increase in stress-induced *Salmonella* shedding from carrier pigs is a common response associated with both transport and lairage. Factors such as feed withdrawal, removal to an unknown environment, the distance travelled, mixing of herds, and temperature are risk factors associated with increased stress in these animals (Williams and Newell 1970; Warriss et al., 1992; Mulder, 1995; Hurd et al., 2002; Martin-Pelaez et al., 2009).

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[5] ELISA detects antibodies to *Salmonella*, which only indicates that the pig was, at one point, exposed to/infected by the bacterium. It does not provide information on the current *Salmonella* status of the animal.

During transport and lairage holding (which is usually 2-6 hours in duration), Berends et al. (1996) showed that the number of finishing pigs excreting *Salmonella* spp. was 1 to 2.4 times greater than when on the farm. The authors suggested that this increase corresponded to: (a) new infections, (b) pigs who were already excreting the organism on the farm and likely to shed in transport, and (c) pigs whose infection was reactivated (likely due to stress) as *Salmonella* was still present in their lymph nodes. Sections 1.5 and 1.6 below list several control measures that have been investigated over the last decade to reduce the transmission of *Salmonella* at these critical parts of the production chain.

1.3.3 *Salmonella* at Slaughter

As discussed in section 1.2.2.1, the most frequent *Salmonella* serotypes isolated from finisher pigs at slaughter in the last 5 years (2010-2014) across the European Union (EU) were *S*. Typhimurium (specifically the DT104, DT120, DT193 and U302 phage types) and its monophasic variant *S*. 4,[5],12:i:-, followed by *S*. Derby, *S*. Altona and *S*. Infantis (Alban et al., 2012; National *Salmonella Shigella* and *Listeria* Reference Laboratory (NSSLRL), 2013; Rostagno and Callaway, 2012). Infection with *Salmonella* at slaughter is a major risk factor for cross-contamination of carcasses on the slaughter line (Duggan et al., 2010). Swanenburg et al. (2001b), determined that carcass contamination was caused by both *Salmonella* infected herds that were slaughtered before *Salmonella*-free herds and from the resident *Salmonella* present in the slaughterhouse. Argüello et al. (2012) and Botteldoorn et al. (2003), detected *Salmonella* on 39.7% and 40% of pre-chilled pig carcasses swabbed from four and five slaughterhouses in Spain and Belgium, respectively. Various points during the slaughtering process have been found to be major sources of *Salmonella* and in turn avenues for carcass contamination. Hald et al. (2003), showed the polishing equipment to be frequently contaminated with *Salmonella* along with the carcass splitter, water outlets and hands of operators. Similarly, Duggan and colleagues (2010) showed that the hands of evisceration operators, conveyor belts, and equipment in the boning hall were major sources of carcass contamination in the abattoir. Likewise, Argüello et al. (2012), found *Salmonella* contamination on the bung dropper, the abdominal open saw, evisceration, the chest saw and carcass splitter. Taken together, these findings show that control measures already in place in abattoirs such as hazard analysis critical control points (HACCP) are not as effective as they should be in limiting cross-

contamination and most likely depend on the incoming level of *Salmonella* contamination from the pigs themselves.

1.3.4 *Salmonella* in Pork and Pig Meat Products

On a whole, it has been estimated that 10 to 20% of all human cases of *Salmonella* infection in the EU may be attributed to pigs (EFSA Panel on Biological Hazards, 2010a). From this, foodborne outbreaks of *Salmonella* have been associated with pork and pig meat products. In 2014, 21/225 (9.3%) foodborne outbreaks with strong-evidence were attributed to *Salmonella* from pig meat and products thereof (EFSA and ECDC, 2015b). In 2013, this figure was lower with 13/314 (4.1%) foodborne outbreaks attributed to *Salmonella* from pig meat and products thereof (EFSA and ECDC, 2015a).

Over a 7-year period (2004 to 2011)[6], *S.* Typhimurium (23,225 isolates) was by far the most frequently reported serovar detected in pig and pig meat products in the EU and non-MS. Other commonly reported serovars included: “other *Salmonella* serovars” – not further specified (5,838); *S.* Derby (5,135 isolates); monophasic *S.* Typhimurium (2,473 isolates); *S.* group B (972 isolates); *S.* Infantis (1,059 isolates); *S.* Rissen (870 isolates); *S.* London (852 isolates); *S.* Enteritidis (854); and *S.* Choleraesuis (696) (EFSA and ECDC, 2013).

In 2012, 12 of the 49 (24.5%) foodborne disease outbreaks caused by *S.* Typhimurium in the EU were attributed to pork and pig meat products, the food vehicle most frequently reported. Of these 12 *S.* Typhimurium-confirmed outbreaks, 7 were attributed to the monophasic form of which 3 were associated with the consumption of pig meat (EFSA and ECDC, 2014).

1.3.4.1 Legislation

The European Commission (EC) has implemented several rules and control measures that require the food business operator (FBO) – the abattoir in the case of this review – to ensure that raw pork leaving the slaughterhouse is safe (i.e., having an acceptable level of microbiological contamination) and fit for human consumption. Of importance for *Salmonella* control are EC Regulation No. 2073/2005 and the

[6] In the 2012 summary report on zoonoses, zoonotic agents and foodborne outbreaks released by EFSA and ECDC (2014), trend data was not provided for the individual *Salmonella* serovars as was the case for the 2011 report. Instead, EFSA and ECDC (2014) stated, “...the trends observed in 2007-2011 continued in 2012.”

amendment EC Regulation No. 218/2014, which implement microbiological criteria and official sampling requirements, respectively, for pig carcases.

1.3.4.1.1 European Commission (EC) Regulation No 2073/2005

Commission regulation No. 2073/2005 lays down microbiological criteria (i.e., acceptability) for certain microorganisms in foodstuffs and the rules that FBO's must comply with when implementing the general and specific hygiene measures of EC regulation 852/2004 (The Commission of the European Communities, 2005). Of particular relevance, is "Chapter 2. Process hygiene criteria; subsection 2.1. Meat and products thereof", which requires the FBO to test 50 samples from pig carcases (from 10 consecutive sampling sessions) after dressing but before chilling for *Salmonella*. The cut-off limit for a satisfactory result is "absence in the area tested per carcase" and only 10% of samples are allowed to fail (i.e., 5 of 50 samples can test positive for *Salmonella*). If results are unsatisfactory, then improvements in slaughter hygiene and review of process controls, animal origin, and farm biosecurity are necessary actions.

1.3.4.1.2 European Commission (EC) Regulation No 218/2014

In addition to EC regulation No. 2073/2005, a new amendment, EC regulation No. 218/2014, was released and requires that the competent authority verify the correct implementation by FBO's of the process hygiene criterion for *Salmonella* on pig carcases specified in EC regulation No. 2073/2005 (see Section 1.4.1).

In order to do this the competent authority is required to perform official sampling of pig carcases by: (a) taking at least 49 random samples (less in smaller abattoirs following a risk evaluation) in the abattoir each year; (b) information should be collected for all samples including the number of *Salmonella* positive samples in accordance with both the microbiological criteria (Article 5(5) of 2073/2005); and (c) the national control program (pork production of 853/2004) regulations (The Commission of the European Communities, 2014). If the process hygiene criterion is not complied with (after several occasions), then the competent authority requires the FBO in question to set forth an action plan and its outcome will be strictly supervised. Finally, the total number and the number of *Salmonella* positive samples, that differentiate between samples from (a), (b) and (c) (as listed above) when applied, are reported in accordance with Article 9(1) of Directive 2003/99/EC.

1.4 On-Farm *Salmonella* Control Measures

The nature of *Salmonella* spp. persistence within the farm environment has led to countless studies striving to find a strategy or set of strategies to regulate its ability to remain on the farm. Various control measures relating to biosecurity, feed, drinking water and transportation to the abattoir have been suggested to date and the sections that follow provide a brief summary of the measures that either proved most successful in reducing the bacterium or have potential (but need further research). In addition, the quantitative microbiological risk assessment (QMRA) of *Salmonella* in slaughter and breeder pigs conducted by the EFSA Panel on Biological Hazards (2010a), sets-forth various control measures that, according to the model, result in a reduction of *Salmonella* prevalence. The results from the QMRA are provided in the sections that follow as further evidence of support for the control strategy.

1.4.1 Farm Biosecurity and Managerial Practices

The nature of biosecurity and in particular, farm biosecurity, is to ensure that our food is of the highest quality, and that animals are healthier and more productive. To achieve this, farm biosecurity generally follows the three stages of risk analysis including risk assessment, risk management, and risk communication. The first aims to identify potential issues and sets to evaluate traffic onto and off the farm, prioritizing the risk of infection as either bioexclusion (i.e., keeping infectious diseases away from the farm) or biocontainment (i.e., reducing infectious diseases within the farm) (Animal Health Ireland, 2014). The second stage refers to implementing a biosecurity plan based on the risk assessment, while the third stage is the follow-through of the actual biosecurity plan (Animal Health Ireland, 2014; Hovingh, 2014). Typically, biosecurity plans are developed on a farm-by-farm basis but usually focus on:

- Access by staff, visitors and machinery;
- Control of rodents/pests, wild animals, birds and pets, and houseflies. For instance, Letellier and colleagues (1999) took 7 fly samples on the same farm, and 6 (or 85.7%) were positive for *Salmonella*. The authors suggested that flies can act as carriers for microorganisms and may be involved in the spread of *Salmonella* within the farm. By preventing *Salmonella* infection from rodents and birds, the QMRA model suggested a 10-20% reduction in

slaughter pig lymph nodes, suggesting pigs, at farm-level are less likely to be carriers (EFSA Panel on Biological Hazards, 2010a).

- Purchases of stock (i.e., gilts etc.), feed and water;
    (a) Sourcing pigs from herds free of *Salmonella,* in addition to age-segregated rearing to reduce *Salmonella* levels on farm (EFSA Panel on Biological Hazards, 2010a).
- Storage, distribution of feed and water; and cleaning/disinfection of feed and water feeders/drinkers, equipment;
- Animal bedding; and
- Location of farm

The 2010 Irish National Pig *Salmonella* Control Program (NPSCP, as discussed in section 1.7.1.1), lists these requirements including training for staff/visitors; having standard operating procedures (SOPs) in place for equipment; using reputable sources with known health status when purchasing stock; isolating stock on the farm to monitor for disease; keeping units and equipment clean/disinfected; and using appropriate pest- and leak-proof storage for afterbirths and dead pigs/fallen stock (Department of Agriculture Food and the Marine (DAFM), 2010). While not every biosecurity plan will be the same, it is imperative and obligatory that each pig farm implement a plan to ensure that potential risks are identified, minimized, and appropriate procedures are put in place to handle such risks should they become evident.

Inherent in every biosecurity plan, is managerial oversight. Within Ireland, the NPSCP lists several key measures that ensure a successful biosecurity plan. To prevent transmission of disease/infection (if any) to piglets, work must flow from clean areas (i.e., from farrowing) to dirty areas (i.e., finishers). In addition, all-in, all-out procedures should be used for movement of pigs. In addition, mixing of pigs from different batches (or herds) should be minimized, in order to reduce stress, thereby potentially reducing *Salmonella* shedding.

1.4.2 Feed and Drinking Water

Strictly speaking, feed and drinking water safety come under the heading of biosecurity. However, they are discussed separately here as both are considered important risk factors for *Salmonella* transmission to finishing pigs, and as such, their

level of contamination, physical properties (i.e. of feed), and formulation are critical issues for pre-harvest *Salmonella* control.

1.4.2.1 Feed and Water Quality

A key feature in feed hygiene is the use of feed and feed ingredients that are free of *Salmonella*. Not only is this important in minimizing contamination within the mill but for the final product as well; as the latter can be a source of *Salmonella* in pigs and indirectly salmonellosis in humans (Harris, 1996; Jones, 2011; Sauli et al., 2005; Wierup and Haggblom, 2010; Wray and Wray, 2000). The QMRA by the EFSA Panel on Biological Hazards (2010a), showed that by feeding pigs with only *Salmonella*-free feedstuffs, a reduction of 10-20% in high prevalence farms and 60-70% in low prevalence farms in the EU member states is possible. Even with adherence to Good Manufacturing Practices (GMPs) and HACCP plans, it is not uncommon to find feed ingredients and feed that are contaminated with *Salmonella*, both at the feed mill and at farm level (see Table 1.4 for summary of feed studies). Berends et al. (1996), estimated that 15-30% of all infections in finishing pigs were due to re-contaminated feed; and as little as 2 CFU/g feed is sufficient for infection. Letellier et al. (1999), found that 40% of feedstuff samples taken from pen feeders on one farm were positive for *Salmonella* but the same feedstuffs were *Salmonella*-negative at the feed mill. This finding points to either re-contamination on the farm, from the feeders/troughs or possibly from the pigs themselves. Moreover, the feed itself can be a source of *Salmonella* to pigs. Molla et al. (2010), showed *Salmonella* strains isolated from feed that originated in the feed bin of the barn (i.e. without access to pigs) possessed similar PFGE profiles from *Salmonella* strains isolated from faeces of pigs in the same barn. Many factors at farm level can contribute to feed contamination/re-contamination including rodents, birds, insects/flies, dust, dirty and wet silos, and dirty feeding troughs (from fecal material) (Jones, 2011).

Table 1.4. Prevalence of *Salmonella* isolated from pig feed sampled at feed mills, during transport, and at farm level

Location	Number of feed mills/pig farms Sampled (% positive)	Total Number of Samples (% positive)	References
Feed mill	N/A	31,359 (0.96%) samples were taken from 1991 to 1996	Haggblom (1997)
Feed mill	N/A	5434 (1.6%)	Baggesen et al. (1997) as cited in Funk and Gebreyes (2006)
Feed mill	523 (27.5%)	3844 (4.8%)	Torres et al. (2011)
Feed truck	25 (22.7%)	549 (0.7%)	Fedorka-Cray et al. (1997)
On-farm	30 (46.7%)	1264 (2.8%)	Harris et al. (1996)
On-farm	135 (25.2%)	1350 (10.02%)	Stege et al. (1997) as cited in Funk and Gebreyes (2006)
On-farm	2 (50%)	800 (0.25%)	Funk et al. (2001)
On-farm	188 (17.6%)	1394 (6.9%)	Lo Fo Wong (2001) as cited in Funk and Gebreyes (2006)
On-farm	1 farm	332 (10.2%)	Korsak et al. (2003)
On-farm	9 (N/A)	275 (3.6%)	Molla et al. (2010)
On-farm	12 (N/A)	143 (29%)	Kich et al. (2011)
On-farm	6 (66.7%)	317 (0.95%)	Burns et al. (2015)

The serovars found in pig feed to date vary and include a number of common strains found in pigs such as *S.* Agona, *S.* Choleraesuis, *S.* Derby, *S.* Enteritidis, *S.* Infantis, *S.* London, *S.* Rissen, *S.* 4,[5],12:i:- and *S.* Typhimurium; and "exotic" strains not commonly found in pigs such as *S.* Anatum; *S.* Cerro; *S.* Cubana; *S.* Heidelberg; *S.* Houtenae; *S.* Livingstone; *S.* Mbandaka; *S.* Newport; *S.* Oranienburg; *S.* Panama; *S.* Schwarzengrund; *S.* Senftenberg; and *S.* Virchow (Burns et al., 2015; EFSA, 2006; Fedorka-Cray et al., 1997; Funk et al., 2001; Keelara et al., 2013; Kich et al., 2011; Torres et al., 2011).

Several interventions have been suggested to control *Salmonella* in feed during both manufacture and storage. These include heat treatment above 80 °C for individual ingredients as well as the final product, keeping storage and manufacturing conditions clean and dry (as *Salmonella* rapidly multiplies in moist feed), microbiological surveillance, changing the feed composition (see Section 1.5.2.2) and the use of additives, such as organic acids and formaldehyde (Haggblom, 1997; Harris, 1996; Sauli et al., 2005; Torres et al., 2011).

Like feed hygiene, a key feature in water hygiene is drinking water that is free of *Salmonella* (and other pathogens). Drinking water is given to all pigs regardless of health status, and is provided during feed withdrawal periods (i.e., before slaughter). It is during this withdrawal period that the animals become stressed and in turn become more susceptible to *Salmonella* infection (Wales et al., 2010). Therefore, because of this fact alone, a hygienic water supply is imperative. Although *Salmonella* contamination is normally via the fecal-oral route, it can enter the water supply directly from human or animal faeces or indirectly through sewage or run-off (Levantesi et al., 2012). Interventions to ensure a *Salmonella*-free water supply include regular testing of the water supply (especially if sourced from a well/borehole) and septic tanks (if present on the farm) as these can be sources of *Salmonella*. Water additives, such as organic acids can also be used (discussed in section 1.5.2.3).

1.4.2.2 Feed Form and Delivery System

Varying feed form and delivery system has been well studied as a means of *Salmonella* control in pigs. Overall, the consensus is to feed coarse non-pelleted (i.e., meal) feed and/or use a liquid feeding system as opposed to a dry feeding system, although conflicting data have been obtained from some studies. Table 1.5 summarises the efficacy of various feed forms and delivery systems in reducing *Salmonella* prevalence at farm level.

Table 1.5. Summary of the various feed sizes, forms and feeding systems evaluated for *Salmonella* control in pigs

Feed Size, Form or Feeding System*	Overall Effect**	References
Fine or Granulated	Not Beneficial	Hotes et al. (2010)
Coarse	Beneficial	Kjeldsen and Dahl (1999)
Coarse	Beneficial	Canibe et al. (2005); Friendship et al. (2006); Jørgensen et al. (2003); Jørgensen et al. (1999); Mikkelsen et al. (2004)
Pelleted	Not Beneficial	Jørgensen et al. (2001a); Kjaersgaard et al. (2001)
Pelleted	Beneficial	Hotes et al. (2010); Jørgensen et al. (2003); Jørgensen et al. (2001b)
Meal	Not Beneficial	Jørgensen et al. (2001a); Kjaersgaard et al. (2001)
Meal	Beneficial	Canibe et al. (2005); Hansen et al. (2001); Jørgensen et al. (1999); Kjeldsen and Dahl (1999); Lo Fo Wong et al. (2004); Mikkelsen et al. (2004)
Liquid	Beneficial	Alban et al. (2012); Farzan et al. (2006); Hotes et al. (2010); van der Wolf et al. (1999)
Dry	Not Beneficial	Farzan et al. (2006); Hotes et al. (2010)
Meal	Beneficial	Letellier et al. (2003); O'Connor et al. (2005)

*Feed size is described as coarse or fine, whereas feed form refers to pelleted or meal feed. Feeding system on the other hand indicates the method of feeding as either liquid, dry, or mash feeding.

** Beneficial = effective in reducing the prevalence of *Salmonella*; Not Beneficial = not effective in reducing the prevalence of *Salmonella*.

Coarsely ground meal has been shown to be more effective than fine meal, and fine and coarse pellets in decreasing the ability of *Salmonella* to survive beyond the stomach (Canibe et al., 2005; Mikkelsen et al., 2004). The reasons for this are two-fold: (1) growth of high levels of anaerobic bacteria, and (2) increased concentrations of lactic acid in the stomach, which reduces the stomach pH (Canibe et al., 2005; Mikkelsen et al., 2004). In a similar manner, liquid feed inhibits *Salmonella* growth better than dry feed due, in part, to its natural fermenting ability. This fermentation step produces lactic and acetic acid, which in turn lowers the feed pH, thus preventing the bacterium's ability to multiply in the stomach (Farzan et al., 2006; van Winsen et al., 2002; van

Winsen et al., 1999). Furthermore, whey (as a potential feed ingredient in liquid feed or as a separate component of the diet) has also been shown to decrease the odds of testing seropositive for *Salmonella* due to an increase in lactic acid bacteria (LAB) produced in the stomach (Farzan et al., 2006; Lo Fo Wong et al., 2004; van der Wolf et al., 2001a; van Winsen et al., 2002).

1.4.2.3 Feed Additives

Many studies have investigated the use of additives in feed or drinking water in order to take advantage of the hurdle effect in decreasing the prevalence of *Salmonella* in pigs. Organic acids and essential oils are natural compounds that are used more often than antibiotics, especially considering the risk of antimicrobial resistance, and the 2006 ban on the routine use (not prescription use per animal) of in-feed antibiotics in the EU. In addition, they are also used as preservatives in feed to prevent contamination (discussed in more detail below). The following sections dicusses the commonly used feed additivies used for controlling *Salmonella* in pigs.

1.4.2.3.1 Organic Acids

Organic acids have been in use in the pig industry since the 1960s, first as a means of carcass decontamination outside of the EU; and later as a useful, relatively non-toxic, natural means of controlling *Salmonella* at farm-level (Van Immerseel et al., 2006). The two main features that organic acids exploit are: (1) ability to decrease the pH of the GIT, thereby preventing *Salmonella* growth; (2) ability to enter the bacterial cell (via the cell membrane), causing decreased invasion into intestinal epithelial cells and eventually cell death (Van Immerseel et al., 2004; Van Immerseel et al., 2006).

The list of organic acids (labelled as “preservatives” or “zootechnical additives” in the 2015 European Commission Register of Feed Additives) that are approved for use in animal feed within the EU is extensive. No list of a similar nature is available for drinking water; however, Regulation (EC) No 1831/2003 on additives for use in animal nutrition, states that feed additives are also considered for use in water and as such the 2015 register of feed additives can also apply to drinking water (The Commission of the European Communities, 2003).

After an extensive search of the literature, Tables 1.6 and 1.7 provide summaries of studies where organic acids were used in feed and drinking water, respectively, to combat *Salmonella* in finishing pigs. Overall, the organic acids (individually or in

combination) showing the most beneficial effects in feed are (in no particular order): (a) potassium diformate; (b) lactic acid + formic acid; (c) sodium butyric acid; and (d) formic acid + propionic acid. Whereas for drinking water, (e) a mixture of lactic acid + formic acid + propionic acid + acetic acid.

Apart from lactic acid, the beneficial acids listed above are classified as short-chain fatty acids (SCFA's) or their salts. Generally, the mechanisms of action for SCFA's are the following:

(i) Decrease ability of *Salmonella* to invade intestinal epithelial cells – butyric and propionic acids suppress invasion by down-regulating the expression of *hilA* and *invF* (genes responsible for activating SPI-1 (section 1.2.2) and *sipC* (gene responsible for *Salmonella's* internalization into the cell) (Boyen et al., 2008b; Durant et al., 2000; Lahiri et al., 2010; Lawhon et al., 2002; Maurer and Lee, 2005; Van Immerseel et al., 2006; Velge et al., 2012).

(ii) Inhibit *Salmonella* growth, which in turns leads to decreased colonization in the cecum and eventually less fecal shedding of the bacterium (Van Immerseel et al., 2004; Van Immerseel et al., 2006).

Table 1.6. Overview of studies that have evaluated organic acids in feed for *Salmonella* control in finishing pigs

Reference	Intervention (Commercial Name)	Type of Study*	Dose, Duration, Inclusion Rate, Cost	Sample Size; Number pigs/pen	Result
Canibe et al. (2005)	Formic acid (Amasil® 85%, BASF)	Non-challenge	1) COARSE diet = coarsely ground meal, non-heated, non-pelleted 2) ACID = finely ground pelleted with 1.8% formic acid 3) STD = finely ground pelleted Duration: 10-days for 60/105 pigs (to reach BW of 63kg); *ad libitum* access to the diets for 45/105 pigs (to reach BW of 99kg) Inclusion rate: 1.8% as-fed basis Cost: €1.0/kg	Growers/Finishers: n=105 21 pens (7 replicates of 5 pigs/pen)	**Beneficial** - feeding a coarsely ground diet, and a finely ground diet with formic acid affected the GI ecology of pigs (mainly by changing the environment in the proximal GIT) and reducing *Enterobacteria* (i.e., *Salmonella* and coliform bacteria)

* In non-challenge studies, pigs were not deliberately infected with *Salmonella,* whereas in challenge studies pigs were deliberately infected prior to evaluation of the organic acid.

Reference	Intervention (Commercial Name)	Type of Study*	Treatments, Dose, Duration, Inclusion Rate, Cost	Sample Size; Number pigs/pen	Result
Creus et al. (2007)	Lactic acid (LacticapP® 50%, ITPSA) Formic acid (Amasil® 85%, BASF) Both lipid microencapsulated Delivered in combination or formic acid used alone	Non-challenge	Experiment 1: - Two pelleted diets, *ad libitum*: (a) Un-acidified standard diet (STD) (b) Diet containing 1.2% as-fed basis of 50:50% lactic–formic acid - Duration: 14-wks - Inclusion rate: 0.6% lactic acid plus 0.6% formic acid -Cost: €1.19 per kg bw gain; ~€2 per pig Experiment 2: - Three pelleted diets, *ad libitum*: (a) STD diet (b) 0.8% as-fed basis of formic acid (c) 0.8% of 50:50% formic–lactic acid - Duration: 8 wks Herd 1 and 9 wks Herd 2 - Inclusion rate: 0.8% formic acid; 0.4% lactic acid plus 0.4% formic acid - Cost: 0.8% of 50:50% formic–lactic acid ~€0.8 per pig	Fatteners/Finishers: Experiment 1: n= 88; 4 pigs/pen (11 pens; 44-pigs/treatment) Experiment 2: Herd 1, n=3000; 10 pigs/pen Herd 2, n=900; 10 pigs/pen	**Beneficial** – lactic acid and formic acid combined significantly decreased *Salmonella* seroprevalence

* In non-challenge studies, pigs were not deliberately infected with *Salmonella,* whereas in challenge studies pigs were deliberately infected prior to evaluation of the organic acid.

Reference	Intervention (Commercial Name)	Type of Study*	Dose, Duration, Inclusion Rate, Cost	Sample Size; Number pigs/pen	Result
Visscher et al. (2009)	Mixture of Formic acid and Propionic acid (Lupro-Mix NC®, BASF); and Potassium diformate (Formi®, ADDCON)	Non-challenge	Farm 1 (f1): • Control group : finely ground feed with 0.4% formic acid and 0.2% propionic acid • Experimental group: coarsely ground feed with 0.4% formic acid and 0.2% propionic acid Farm 2 (f2): • Control group : finely ground feed • Experiment group : coarsely ground feed with 1.2% potassium diformate Duration: Not specified; but from beginning of growing period to just before slaughter (from 30 kg to ~115 kg) Water and feed *ad libitum* Inclusion rate: 0.2% propionic acid;0.4% formic acid;1.2% K-diformate Cost: N/A	Fatteners/Finishers: n=1600 Farm 1 (f1): n=400 Control group : 12 pigs/pen Experimental group : 13 pigs/pen Farm 2 (f2): n=400 Control group : 33 pigs/pen Experimental group : 25 pigs/pen	**Beneficial** – prevalence of *Salmonella* in caecal contents was lower in pigs fed coarse diets + organic acids on f1 ($p<0.05$) & significant reduction in the number of seropositive and distinct seropositive pigs and a significant increase in the number of seronegative pigs. Feeding a coarsely ground diet containing 1.2% K-diformate reduced the prevalence of *Salmonella* on f2 and reduced faecal *Salmonella* shedding immediately pre-slaughter ($p<0.01$).

* In non-challenge studies, pigs were not deliberately infected with *Salmonella,* whereas in challenge studies pigs were deliberately infected prior to evaluation of the organic acid.

Reference	Intervention (Commercial Name)	Type of Study*	Dose, Duration, Inclusion Rate, Cost	Sample Size; Number pigs/pen	Result
Gebru et al. (2010)	1) Chlortetracycline (CT) 2) Anti-*Salmonella* Typhimurium bacteriophage (ASB) 3) *Lactobacillus plantarum* (LP) *4*) Microencapsulated Organic Acids (MOA) 5) Fermented Soybean Meal (FSM)	Challenge with *Salmonella* Typhimurium	6-Treatments: 1) Control (CON) - no antimicrobial agents 2) Positive Control - Chlortetracycline (CT) at 100 mg/kg 3) Anti-*Salmonella* Typhimurium bacteriophage (ASB) $3x10^9$ pfu/kg of feed 4) *Lactobacillus plantarum* (LP) $6.5x10^8$ cfu/kg of feed 5) 0.2% Microencapsulated Organic Acids (MOA) (20% citric acid, 20% fumaric acid, 10% malic acid, 10% phosphoric acid) 6) 5% Fermented Soybean Meal (FSM) (fermented with *Bacillus subtilis* complex) Duration: 28-days (14 days pre-challenge and 14 days post challenge) Inclusion rate: 20% citric acid; 20% fumaric acid; 10% malic acid 10% phosphoric acid 5% fermented soybean meal Cost: N/A	Growers/Finishers (initial wt = 38.7± 6.7kg) n=108 18 pigs/treatment 3 pigs/pen (2 blocks, 3 replicate pens per block)	**Beneficial effects on growth performance only** – "Compared with the control diet, ASB, FSM, and MOA diets had a similar benefit to the antibiotic-supplemented diet in improving the performance of growing pigs, especially after bacterial challenge." The authors did note any differences in *Salmonella* shedding.

* In non-challenge studies, pigs were not deliberately infected with *Salmonella,* whereas in challenge studies pigs were deliberately infected prior to evaluation of the organic acid.

Reference	Intervention (Commercial Name)	Type of Study*	Dose, Duration, Inclusion Rate, Cost	Sample Size; Number pigs/pen	Result
Willamil et al. (2011)	Mixture of Lactic acid (LacticapP® 50%, ITPSA solid blend) and Formic acid (Amasil® 85%, BASF, liquid form) Both lipid microencapsulated	Non-challenge	1) Control diet 2) Non-protected blend (NPB): control diet plus 0.4 % lactic acid and 0.4% formic acid 3) Protected blend (PB): control diet plus lipid microencapsulated blend of 0.14% lactic acid and 0.14% formic acid Duration: Trial 1 = 10-days Trial 2 = 5-weeks Inclusion rate: a) NPB = 1:1 lactic acid (50%) and formic acid (85%); b) PB = 1:1 lactic and formic acid (25%) Cost: N/A	Fatteners/Finishers: Trial 1: n=24 2 pigs/pen (12 pens; 4 replicates per treatment) Trial 2: n=261 8 pigs/pen (3 independent boxes of 8 pigs/pen each)	**Beneficial** - NPB diet showed a significant reduction in *Salmonella* seroprevalence after 5-weeks of treatment; at the abattoir (day 36), the prevalence *of Salmonella* in faeces was significantly lower after feeding NPB and PB diets than after feeding the control diet.

* In non-challenge studies, pigs were not deliberately infected with *Salmonella,* whereas in challenge studies pigs were deliberately infected prior to evaluation of the organic acid.

Reference	Intervention (Commercial Name)	Type of Study*	Dose, Duration, Inclusion Rate, Cost	Sample Size; Number pigs/pen	Result
Calveyra et al. (2012)	Mannanoligosaccharide (12%) (Bio-Mos® Alltech Biotechnology Ltd.) Encapsulated organic acid - contains: 20% fumaric acid, 10% citric acid, 10% malic acid, 10% phosphoric acid (Tetracid® TM-500, Jefo Nutri- tion Inc.) Short chain free organic acid - contains: 26% formic acid, 10% propionic acid, 18% plant fatty acids (Selacid Green Growth® Selko Latin America Ltda.)	Challenge with *Salmonella* Typhimurium	1) Basal Diet (BD) 2) BD + encapsulated organic acids (EOA) 3) BD + 0.2% short chain free organic acids (SOA) 4) BD + mannanoligosaccharide (MOS) *ad libitum* feed and water Duration: NS Cost: N/A	Growers: n=46 (started with 48 but 2 died before inoculation and were not replaced) 1 pig/pen	**Not Beneficial** - No treatment prevented the carrier state, but a tendency for lower fecal excretion was observed in the group treated with MOS. - The low acid concentration (0.2% in feed) may not be sufficient to decrease *Salmonella* shedding rates
Rajtak et al. (2012)	Potassium diformate (Formi®, BASF, ADDCON)	Fecal samples taken from pigs post-treatment and inoculated with *Salmonella* strains [*S.* Typhimurium DT104b (F6); *S.* Typhimurium DT193 (H21); *S.* 4,[5],12:i:- (M5); *S.* Derby (A22); and *S.* Bredeney (L6)] *in vitro* to investigate *Salmonella* survival in faeces of treated pigs.	4 diets: 1) Finely ground meal without K-diformate 2) Finely ground meal with 0.9% K-diformate 3) Finely ground pelleted feed without K-diformate 4) Finely ground pelleted feed with 0.9% K-diformate Duration: 10-weeks Inclusion rate: 0.9% K-diformate Cost: N/A	Finishers: n=24 (6 pigs/treatment) pigs/pen = Not Specified	**Beneficial in terms of reducing *Salmonella* survival in faeces** – supplementation of meal diets with K-diformate reduced the duration of survival ($p<0.1$) and increased rates of decline ($p<0.0001$) of *Salmonella*e in faeces held at 22°C,; the pelleting of feed, compared to feeding meal, reduced ($p<0.1$) the duration of *Salmonella* survival in faeces held at 22°C.

* In non-challenge studies, pigs were not deliberately infected with *Salmonella,* whereas in challenge studies pigs were deliberately infected prior to evaluation of the organic acid.

Reference	Intervention (Commercial Name)	Type of Study*	Dose, Duration, Inclusion Rate, Cost	Sample Size; Number pigs/pen	Result
Argüello et al. (2013)	Potassium diformate (Formi®)	Non-challenge	1) Control group: regular non-pelleted feed 2) Experimental group: regular pelleted feed with 0.5% potassium diformate Duration: Trial B: 52-days Trial C: 49-days Cost: €1.34 per pig	Finishing pigs (last 7 weeks of growth) Trial B: n = 40; 4 pigs/pen; Trial C: n = 40; 4 pigs/pen Samples were taken at beginning of finishing period, first day of treatment, halfway through treatment and last day of treatment	**Beneficial** – In trial B, the prevalence of *Salmonella* shedders was significantly higher in the control group (9/40) vs. the experimental group (1/40) ($p = 0.017$). Fecal shedding was lower in the experimental group at the end of finishing for trials B. No significant differences were observed between the 2 groups in trial C. Higher numbers of seropositive pigs found in the control group than in the experimental group for both trials B and C

* In non-challenge studies, pigs were not deliberately infected with *Salmonella,* whereas in challenge studies pigs were deliberately infected prior to evaluation of the organic acid.

Reference	Intervention (Commercial Name)	Type of Study*	Dose, Duration, Inclusion Rate, Cost	Sample Size; Number pigs/pen	Result
Rasschaert et al. (2016)	1) Butyric acid, uncoated 2) Salts of Formic, Sorbic, Acetic and Proprionic acid and Natural Extracts, all uncoated 3) Meadium-chain-fatty acids: Triglycerides with Caproic and Caprylic acids and Oregano Oil plus Caproic, Caprylic, Lauric and Lactic acids, all uncoated except for oregano oil which was coated	Non-challenge	Control group (dry meal); and 3 Treatment groups (dry meal plus additives 1-3) Duration: Entire Fattening Period (~4.5 months), repeated twice (total 9 months) Inclusion Rate: 1) 1.30 kg/ton 2) 2.92 kg/ton 3) 3.71 kg/ton Cost: N/A	Fattener and finishing pigs from 25 kg until slaughter 4 houses, with 25 pens/house, and 14 pigs/pen (350 pigs/house): n = 1400 pigs Number of pigs per control and treatment groups = 350 pigs Samples collected from 3 pigs per pen, on 2 occasions: (a) 5 weeks after supplementing diets or control group and (b) before being transferred to the abattoir. Ceca and lymph nodes collected from 75 pigs per group at the abattoir.	**Beneficial** – Dietary supplementation with the medium-chain fatty acids (group 3) significantly reduced *Salmonella* prevalence in fecal and ceca/lymph node samples**.** No effect on *Salmonella* prevalence for the other 2 feed additives.

* In non-challenge studies, pigs were not deliberately infected with *Salmonella,* whereas in challenge studies pigs were deliberately infected prior to evaluation of the organic acid.

Table 1.7. Overview of studies that evaluated organic acids and sodium chlorate in drinking water for *Salmonella* control in finishing pigs

Reference	Intervention (Commercial Name)	Type of Study*	Dose, Duration, Inclusion Rate, Cost	Sample Size; Number pigs/pen	Result
van der Wolf et al. (2001a)	Mixture of: Sorbic acid (33%); Ammonium formiate (28%); Formic acid (23%); Acetic acid; Lactic acid (8%); (4%); Propionic acid (3%); Water (1%)	Non-challenge	Control group not treated Duration: not specified Inclusion rate: 2 mL/L Cost: €2.49 per pig	Finishers: n=1040 8-12 pigs/pen (~8-10 pens); some pens had 16 pigs/pen	**Marginal Benefit** - A large and significant treatment effect observed in one herd ($p<0.001$). As a result of the small number of observations and the overall lower seroprevalence in the control groups, the other two herds only showed a statistical trend towards a treatment effect towards the acid mixture ($0.10<p<0.05$).
Howard et al. (2003)	Lactic acid Tylan	Challenge – pigs were placed in pens contaminated with *Salmonella* Typhimurium 4232	1) 0% Lactic acid and 0 g Tylan 2) 0% Lactic acid and 20 g Tylan/907 kg of feed 3) 0.44% Lactic acid and 0 g Tylan 4) 0.44% Lactic acid and 20 g Tylan/907 kg of feed Duration: 7-days post-challenge Inclusion rate: 0.44% lactic acid Cost: N/A	Finishers: 36 pigs pigs/pen = not specified	**Not Beneficial** - no differences ($p>0.05$) were detected in *Salmonella* prevalence among the 4 treatments; no treatment differences were detected in stomach fluid concentrations of total lactic acid, dissociated and un-dissociated lactic acid ion.

* In non-challenge studies, pigs were not deliberately infected with *Salmonella,* whereas in challenge studies pigs were deliberately infected prior to evaluation of the organic acid.

Reference	Intervention (Commercial Name)	Type of Study*	Dose, Duration, Inclusion Rate, Cost	Sample Size; Number pigs/pen	Result
Anderson et al. (2004)	Experimental chlorate preparations (ECP)	Challenge with *Salmonella* Typhimurium	1) Control 2) 1X ad libitum 3) 2X *ad libitum* where X = concentration that delivers a minimal daily effective dose of 30-40 mg/kg bw Duration: 24-h and 36-h post-challenge Inclusion rate: NS Cost: N/A	1 pig/pen (Total finishers n=18)	**Beneficial** - No negative effects of ECP on water intake or animal wellbeing; marginal effects on gut fermentation; rapid bactericidal effect on caecal *Salmonella* within 24-hours (i.e., 1.4 $\log_{10}$ CFU reduction in the 1X group as compared to the control group)
Heylen and Daems (2008)	Mixture of Lactic acid;, Acetic acid, Propionic acid, Formic acid (Agrocid Super, CID-LINES, Belgium)	Non-challenge	pH of water to be 5.8 Duration: 6-months Inclusion rate: NS Cost: N/A	All stages: n=20 (pigs/pen not specified)	**Beneficial –** mean S/P ratio decreased from 1.10 (range 0.21-2.5, SD 0.75) day 0 to 0.27 (range 0.09-0.57, SD 0.14) after 6-months. "The number of *Salmonella* infections in finishing pigs can be reduced by the addition of organic acids to their drinking water." The pH of the drinking water during the trial (pH 5.8) was not as low as general recommended, i.e. 3.5 – 4.1. The higher pH had a better effect on the taste of the drinking water, and was less corrosive. (Results are reported as S/P-ratio's: S/P = (ODsample – ODneg control)/(ODpos control – ODneg control); OD = optical density.)

* In non-challenge studies, pigs were not deliberately infected with *Salmonella,* whereas in challenge studies pigs were deliberately infected prior to evaluation of the organic acid.

Reference	Intervention (Commercial Name)	Type of Study*	Dose, Duration, Inclusion Rate, Cost	Sample Size; Number pigs/pen	Result
De Busser et al. (2009)	Mixture of Formic acid, Propionic acid, Acetic acid, Sorbic acid and a liquid carrier (INVE Nutri-Ad)	Non-challenge	1) Control treatment: - pH 7.8-8.5 2) Acid treatment: - pH 3.6-4.0 - concentration of the acids varied between herds but ranged from 0.25% to 0.40% Duration: 14-days Inclusion rate: 0.25 to 0.40% Cost: N/A	Finishers (last 14 days of growth): 10-14 pigs/pen different herds? Total Finishers n= 600 (300 / treatment)	**Not Beneficial** – *Salmonella* was isolated in 11.9% of samples from slaughterhouse, with the highest frequency in the ileum (18.7%), lymph nodes (17.8%), rectum (7.2%) and carcass swab (3.6%). Overall, no significant difference between the treatment and control groups for the different slaughterhouse samples were observed. Therefore, administration of organic acids 2-weeks before slaughter was not beneficial.
Argüello et al. (2013)	Mixture of Lactic acid (56%), Formic acid (23%), Propionic acid (13%) and Acetic acid (5%) (Acidvall®, MEVET)	Non-challenge	1) Control group: drinking water without acid mixture 2) Experimental group: drinking water with 0.35% acid mixture Dose: 0.035 mL/L in water Duration: 40-days Cost: €1.4 per pig	Finishing pigs (last 6-7 weeks of growth) n = 40, 2-3 pigs/pen	**Beneficial** – percentage of seropositive pigs was higher in the control group than in the experimental group Fecal shedding was lower in the experimental group at the end of finishing compared to the control group ($p<0.01$).

* In non-challenge studies, pigs were not deliberately infected with *Salmonella,* whereas in challenge studies pigs were deliberately infected prior to evaluation of the organic acid.

1.4.2.3.2 Sodium chlorate

Like most members of the *Enterobacteriaceae* family, *Salmonella* possess respiratory nitrate reductase activity that promotes intracellular reduction of chlorate to cytotoxic chlorite (Pichinoty and Piechaud (1968) as cited by Burkey et al., 2004). This property can be exploited through the use of sodium chlorate, which acts as a bactericide against *Salmonella* (Anderson et al., 2004). Few studies have demonstrated the benefit of using sodium chlorate to reduce *Salmonella* in non-challenge trials in weaned to finishing pigs (Anderson et al., 2004; Burkey et al., 2004; Patchanee et al., 2005). Sodium chlorate is, however, not listed in the 2014 EU register of feed additives as being approved for use in water; therefore, its use in Europe is cautioned.

1.4.2.3.3 Essential Oils

Essential oils (EOs) are secondary metabolites extracted from plants that have been shown to have antibacterial, antiparasitic, insecticidal, antiviral, antifungal and antioxidant properties, along with growth-promoting effects in animals (Burt, 2004; Hyldgaard et al., 2012; Langeveld et al., 2014; Oussalah et al., 2007). These natural compounds show much promise in controlling *Salmonella* via feed and drinking water; however, field trials in this area are lacking. Much of the work has focused on the bacteriostatic and bactericidal properties of the three main EOs: carvacrol, eugenol, and thymol against foodborne and spoilage organisms including *S.* Typhimurium. Minimum Inhibitory Concentrations (MICs) against *S.* Typhimurium range from 150-250 μg/mL for carvacrol, 3.18-500 μg/mL for eugenol, and 56.25-150 μg/mL for thymol; while the Minimum Bactericidal Concentration (MBC) for carvacrol is 250 μg/mL (Hyldgaard et al., 2012). The mechanisms of action that these three EOs have in common are: membrane disruption, non-specific permeabilization of cell membranes, leakage of adenosine triphosphate (ATP) and potassium/hydrogen (K^+/H^+) ions, inhibition of ATPase activity, and increase in the fluidity of the phospholipid bilayers (Bakkali et al., 2008; Barbosa et al., 2009; Berge and Wierup, 2012; Burt, 2004; Hemaiswarya et al., 2008; Hyldgaard et al., 2012; Kim et al., 1995; Klein et al., 2013; Langeveld et al., 2014; Oussalah et al., 2007; Smith-Palmer et al., 1998). From this, it can be seen that EOs have mechanistic actions that could inhibit *Salmonella* growth and invasion *in vivo*, and as such their potential as a pre-harvest control measure warrants additional research.

1.4.2.3.4 Probiotics

Probiotics are living microorganisms that, when administered orally, help to maintain the natural balance of microflora in the GIT (Casey et al., 2007). For probiotics to be used as a potential control measure against *Salmonella*, several criteria must be met (Friendship et al., 2006). For example, along with being cost effective for the farmer, the strains should be non-pathogenic and non-toxic (i.e., safe for animals and humans); stable at pH 1 to 4; resist degradation by digestive enzymes such as lysozymes; adhere to epithelial tissue in the GIT; be able to persist (at least for short periods of time) in the GIT; be isolated from the same species as the intended host of the treatment; be able to grow easily, rapidly and survive freeze-drying; and be viable and stable when commercially produced (Friendship et al., 2006). Several *in vitro* and *in vivo* studies have evaluated the efficacy of several probiotic strains in reducing *Salmonella* shedding in pigs with promising results (Baum and Harris, 2000 as cited in Friendship et al., 2006; Casey et al., 2007; Tanner et al., 2014; Yin et al., 2014). However, like essential oils, more research in this area is needed.

1.4.3 Transportation to the Abattoir

Not only are feed and drinking water risk factors for *Salmonella* transmission to finishing pigs, but also the act of transporting pigs to the abattoir is a risk factor that can result in infection of naïve pigs (Alban et al., 2012). As discussed earlier, *Salmonella*-infected pigs are usually asymptomatic carriers showing no clinical signs of disease and only shedding the bacterium in their faeces. During transport, factors such as feed withdrawal; a change in environment; mixing of pen mates; high stocking density, transport time; health status; and adverse weather conditions cause stress and, consequently, induces these asymptomatic pigs to shed *Salmonella* at a higher rate (Williams and Newell 1970; Warriss et al., 1992; Mulder, 1995; Hurd et al., 2002; Martin-Pelaez et al., 2009). Uninfected pigs present in the transport vehicle are then subsequently at risk of infection from *Salmonella*-contaminated faeces (Alban et al., 2012; Ball et al., 2011; Berends et al., 1996; Davies et al., 1999; Gebreyes et al., 2004; Hurd et al., 2002; Magistrali et al., 2008; Mannion et al., 2008; Mannion et al., 2012). Therefore, control of *Salmonella* transmission during transport is necessary and can be achieved by: (1) minimizing the stress imposed on the animals by decreasing the distance travelled and stocking density, segregating *Salmonella*-positive and

Salmonella-negative pigs, and not mixing pigs; and (2) ensuring the transport vehicle is thoroughly cleaned and disinfected before transport and after unloading at the abattoir (Bahnson et al., 2006; De Busser et al., 2013; FCC Consortium, 2013; Mannion et al., 2008).

1.5 Control Measures in the Lairage

The next step in the pre-harvest control of *Salmonella* is within the lairage of the abattoir. The lairage is the area in which pigs entering the abattoir are held prior to slaughter. Generally, the concept of lairage is to allow animals to rest and recover from transport, which in turn affects animal welfare, and subsequently, meat quality (Warriss, 2003). Depending on the size of the abattoir, the lairage can contain multiple pens large enough to hold a large number of pigs (>50 pigs) and a separate pen/area to detain injured/sick pigs. As the pigs entering the lairage are fasted, nipple drinkers for water are normally present along with a toy to minimize boredom. In addition, showers and other misting devices that deliver a fine spray of water to the pigs are used in some abattoirs: (a) calm and clean the animals; (b) regulate body temperature; and (c) improve meat quality (Warriss, 2003).

Several studies have identified the lairage as another point at which uninfected pigs are at high risk of becoming infected with *Salmonella*, which subsequently increases the risk of cross-contamination down the slaughter line (Boughton et al., 2007a, b; De Busser et al., 2013; Duggan et al., 2010; Hurd et al., 2001a; Hurd et al., 2001b; Mannion et al., 2012; Morgan et al., 1987; Rostagno et al., 2003; Warriss, 2003). Stress and a previously contaminated "dirty" environment are considered major factors in the acquisition of *Salmonella* at the lairage; thus, interventions to control *Salmonella* during this stage of the production chain can have a major impact on subsequent pork safety (Hurd et al., 2001b; Rostagno et al., 2003; Swanenburg et al., 2001a).

Aspects such as holding time before slaughter, floor design, length of feed withdrawal, level of contamination of the lairage pens, and seroprevalence of the herd are all factors that influence *Salmonella* prevalence in the lairage. For instance, both Hurd et al. (2001b) and Boughton et al. (2001b) showed that finishing pigs need as little as 2-hours following exposure to a contaminated environment to acquire *Salmonella*. Solid concrete floors resulted in a higher rate of *Salmonella* recovery from the lairage pens than slatted floors (Hurd et al., 2005; Mannion et al., 2012). Duggan et al. (2010) sampled lairage pens before and after the introduction of pigs in three abattoirs in the Republic of Ireland. The researchers found that the lairage pens in all three abattoirs were highly contaminated with several strains of *Salmonella* (of the serotypes Derby, Typhimurium, Manhattan). They also noted that the strains isolated from the carcasses

and intestinal contents of the pigs were the same or similar to those isolated from the lairage pens, providing definitive proof of the role of the lairage in *Salmonella* transmission (Duggan et al., 2010). As a result, in Ireland, the *Salmonella* seroprevalence of pigs entering the abattoir determines the time of day at which the pigs are slaughtered. This is to minimize the risk of cross-contamination during slaughter and in the finished pork product(s) (Department of Agriculture Food and the Marine (DAFM), 2010). Herds with a low seroprevalence are slaughtered at the beginning of the day, whereas those with a >50% seroprevalence are slaughtered at the end of the day (discussed in further detail in section 1.7.1.1).

Since short lairage holding times (i.e., <2 hours) are not always feasible, cleaning and disinfection of lairage pens is needed to limit *Salmonella* contamination. Several cleaning and disinfection methods have been investigated to date; however, even with good intentions, difficulties in eliminating the bacterium still remain. One study recovered *Salmonella* from more than 60% of lairage pen floors immediately after cleaning and disinfection protocols had been employed (Argüello et al., 2011). While a second study was able to show a decrease in the number of *Salmonella*-positive samples recovered after an improved cleaning and disinfection protocol (70-90% before cleaning to 10% after cleaning); however, eliminating the bacterium was still met with difficulty (Swanenburg et al., 2001a). Boughton et al. (2007a), compared the effect of daily washing routines to an intensive cleaning and disinfection protocol on the recovery of *Salmonella* from lairage pens. At the beginning of the slaughter week after intensive cleaning and disinfection, only 3% of the lairage samples were positive, whereas during the week, when only high-pressure cold-water was used between herds, 52% of the samples tested positive for *Salmonella* (Boughton et al., 2007a). Again, although the number of *Salmonella*-positive samples found after cleaning and disinfection was considerably lower, total eradication was not achieved. One possible reason for this is that *Salmonella* produces biofilms in the presence of organic matter, allowing it to survive, thereby lessening the efficacy of the cleaning agents and disinfectants used to destroy it (Boughton et al., 2007a; Corcoran et al., 2014; De Beer et al., 1994; De Busser et al., 2013; Stewart et al., 2001).

1.5.1.1 Cleaning Regimes – Detergents and Disinfectants

The types of cleaning regimes employed in the lairage of pig abattoirs involve just washing (with or without power) to remove gross organic matter or can include the

following: (i) power-wash + detergent + water rinse to remove the detergent + disinfectant, where the disinfectant is either left to dry or, after a sufficient contact time, removed with water; or (ii) power-wash + disinfectant, with or without a water rinse; or (iii) power-wash + detergent + water rinse (Boughton et al., 2007a; Schmidt et al., 2004; Swanenburg et al., 2001a; van der Wolf et al., 2001b). Many studies have demonstrated the usefulness of cleaning and disinfection along with other control measures; however, very few have compared actual cleaning regimes. Moreover, only a handful of studies have looked at the efficacy of actual cleaning and/or disinfecting agents (i.e. detergents and/or disinfectants) used in the lairage area of pig abattoirs. Below is a summary of three studies that looked at the effects of both cleaning regimes and actual cleaning agents.

In the Swanenburg et al. (2001) study mentioned above, the following cleaning regime was used: high-pressure cold-water wash followed by application of an alkaline chloride cleaning solution (Kleencare CF6202) at 30 °C for 1-hour contact time, after which a high-pressure cold-water rinse was employed. This was then followed by the application of a quaternary ammonium compound (QAC), a didecyldimethylammonium chloride disinfectant (Kleencare DS6601) at 30 ºC, and a final high-pressure cold-water rinse. The authors noted that this cleaning regime was successful in decreasing the levels of *Salmonella* but only when visual inspection of drains, holes in the floor, and rough surfaces of floor, walls and corners were completed followed by re-cleaning and disinfection if issues were found.

Schmidt et al. (2004) employed a cleaning regime consisting of a high-pressure cold-water rinse followed by use of an alkaline chloride detergent that was applied and left to sit for 10-minutes, then rinsed with high-pressure cold-water. Next, a hydrogen peroxide, peracetic and octanoic acid sanitizer was applied and after the 10-minute contact time it was rinsed with high-pressure cold-water. Using this cleaning regime, the prevalence of *Salmonella enterica* significantly declined in the pens; however, the authors could not show that this cleaning protocol was capable of reducing subsequent *Salmonella* prevalence in the pigs.

Boughton et al. (2007a) tested a cleaning regime that involves using a cold-water power wash followed by application of a sodium hypochlorite foaming detergent sanitizer (Chlorofoam CTK) with a water rinse and then a final drying step of ~1-day. The researchers found that this intensive cleaning and disinfection protocol decreased the numbers of *Salmonella* from approximately 8 organisms/100 cm^2 before cleaning

(only a high-pressure cold water washing was conducted between batches of pigs) to 1.8 organisms/100cm^2 after cleaning and disinfection.

One key point deduced from these studies is that a high-pressure cold-water wash alone is not effective in eliminating *Salmonella* from the lairage environment.

1.6 *Salmonella* Control Programs

After China, the EU is the second largest exporter of pork in the world; however, with fierce competition from Canada, the USA, and Brazil, the global market for pork is ever diversifying. Given this diversity, it is paramount that control programs are in place to ensure a *Salmonella*- free/reduced product. To achieve this, many EU MS have implemented mandatory or voluntary control programs that emphasize the whole pork production chain, while the countries of North America have programs focusing on control in the abattoir. The following sections discuss some of these control programs in detail.

1.6.1 European Union

The European Commission (EC) Regulation No. 2160/2003 requires that member states set-up national control programs for *Salmonella* serovars in pigs (and poultry) that are considered an important risk to public health (EFSA and ECDC, 2014). Over the years, several MS have had moderate to high *Salmonella* prevalence on pork carcasses (i.e., Denmark, Germany, Netherlands, UK, France, Belgium, and Ireland), while others have historically low levels (i.e., Sweden, Finland and Norway) (EFSA, 2006). The most recent EU-wide baseline surveillance survey, which was conducted in 13 MS, revealed zero prevalence in slaughter pigs in Sweden and Slovenia but Austria, Poland, and Lithuania had between 1.2-1.6% prevalence on carcasses; Cyprus, Denmark, Latvia, and Czech Republic had 3.3-3.7% prevalence; while the UK, France, and Belgium had 13.5%, 17.6%, and 18.8%, respectively. Ireland on the other hand, had the highest prevalence, with 20% of carcasses *Salmonella*-positive (EFSA, 2008).

Despite the mandated regulation, carcass contamination rates in some countries have still not declined. Of unique importance are Ireland and the UK, as both countries are island nations with temperate climates, high precipitation, and as previously mentioned, have considerably high *Salmonella* prevalence on pork carcasses (20% and 13.5%, respectively). On the other hand, Denmark – the largest exporter of pork in the EU – has a well developed and organized pork production system (EFSA, 2008) and low levels of carcass contamination, and as such, its *Salmonella* control program is considered the gold standard in terms of reducing the bacterium in the production chain. For these reasons, this review will focus on the control programs implemented by these

three MS – Ireland, UK and Denmark, as summarised in Table 1.8 and described in detail below.

Table 1.8. *Salmonella* control programs implemented by Ireland, UK and Denmark

Member State	Control Program	Year of Implementation	References
Ireland	National Pig *Salmonella* Control Programme	2002, updated in 2010	Department of Agriculture Food and the Marine (DAFM) (2010)
UK	Zoonoses National Control Program (ZNCP) or Zoonoses National Control Plan for *Salmonella* in Pigmeat (ZNCPig) formally known as Zoonoses Action Plan (ZAP)	2002-2008 for ZAP; 2009 for ZNCP/ZNCPig	Howell and Hilton (2008)
Denmark	Danish *Salmonella* Control Program for pigs	1993 and 1995 for finisher/slaughter pigs	(Alban et al. (2012); Alban et al. (2010); Nielsen (2003))

1.6.1.1 Ireland

Briefly, the NPSCP considers the *Salmonella* prevalence in pigs in two categories ≤ 50% and ≥ 50%. The program focuses on a whole food-chain approach to *Salmonella* control and describes separate requirements that each farm, abattoir, and FBO must adhere to. Currently ~18% of Irish pig herds have a *Salmonella* prevalence of greater than 50% (data extracted from the 2016 NPSCP). Ultimately, the program aims to reduce the risk to the consumer through establishing *Salmonella* herd prevalence and surveillance, while ensuring that levels of the bacterium in pigs sent to slaughter and in the final product, are as low as possible. To achieve the above, each farm is required to have the following pre-harvest controls in place (or face prosecution) (Department of Agriculture Food and the Marine (DAFM), 2010):

(a) A biosecurity plan where: all staff are trained, visitors are logged in, a pest control system is in place, and purchased goods and feed are from reputable sources and isolated/stored where appropriate;

(b) A *Salmonella* Control Plan, through cooperation between the farmer, the private veterinary practitioners (PVPs)/advisor, and the Department of Agriculture;

(c) Gilts and boars are sourced from breeding herds that have a *Salmonella* control plan and a sero-prevalence of ≤ 10%;

(d) All breeding herds must conduct farm bacteriology sampling on an annual basis to establish the *Salmonella* serovars on the farm;

(e) Pigs with ≥ 50% *Salmonella* prevalence must be transported separately to the abattoir; and

(f) Herds with ≥ 50% *Salmonella* prevalence or that do not have an on-farm control plan in place will be excluded from the Bord Bia[7] quality assurance scheme. Farms can be exempted from this, if they are able to demonstrate, by on-farm bacteriological sampling, that antibodies were not due to *Salmonella* serovars (of public health significance).

On the other hand, the abattoir must ensure that pigs with a *Salmonella* prevalence of ≥ 50% are: (i) kept separate in the lairage; (ii) slaughtered at the end of the production day; and (iii) the lairage pens are washed and disinfected after the pigs have been removed. The abattoir and FBO is also required to have a HACCP plan in place that is followed and audited by the facility itself; and a Food Safety Management System (FSMS) that also contains a *Salmonella* control programme. In addition, the NPSCP details that management of the FBO must (Department of Agriculture Food and the Marine (DAFM), 2010):

(a) Conduct carcass swabs twice a week and maintain test results;

(b) Increase the level of *Salmonella* testing on product entering the boning hall;

(c) Have an effective sanitation protocol including thoroughly cleaning and sanitizing loading ramps, holding pens, cutting equipment and all personnel processing equipment; and

(d) Carry out a review of herds that have ≥ 50% prevalence in terms of the farm's biosecurity measures.

In order to establish herd prevalence and to identify herds that are of high-risk (i.e., herds that have ≥ 50% *Salmonella* prevalence), the program requires that, from each abattoir, 6 meat samples/month (up to a maximum of 72 samples/year for herds with ≥ 200 pigs) be taken from the first consignment of pigs sent for slaughter from each

[7] Bord Bia is the Irish Food Board, which sets out to develop a market for Irish food world-wide. Its quality assurance scheme is a programme that indicates a food product is produced in accordance with a set of standards and that the producer or processor of the food in question has been audited against the standard.

farm/month. Herds supplying ≥ 200 fatteners to slaughter must have a herd prevalence based on serological testing (either on meat samples collected in the slaughterhouse; or from 24 blood samples collected at the farm every 4 months). Overall, the abattoir is responsible for maintaining all documentation, sampling, and testing records. Once samples are taken, they must be tested using a recognized serological test as agreed with the Department of Agriculture Food and Marine (DAFM) at an accredited laboratory, within 3-days of sampling. All results are to be maintained in a database and labelled with holding, slaughter plant, date of sampling, date of sample receipt and test result. Finally, upon completion, the laboratory will then forward all serological tests to the national coordinator by the 28th of each month (Department of Agriculture Food and the Marine (DAFM), 2010).

1.6.1.2 United Kingdom

The British Zoonoses National Control Plan for *Salmonella* in Pigmeat (ZNCPig) replaces the Zoonoses Action Plan (ZAP), which operated from 2002 to 2008. Like the Irish control program, the ZNCPig involves a whole chain approach to reducing *Salmonella* in pork and pork products by placing controls in: (1) abattoirs, (2) all herds, (3) breeding herds, and (4) finisher herds. However, unlike the Irish programme, the focus of the UK plan is on processors and final products (Howell and Hilton, 2008).

Generally, the plan requires that all farms follow the "Code of Practice for the Control of *Salmonella* on Pig Farms" and all abattoirs are encouraged to assess their processes using the Food Safety Authority (FSA) tool for hygiene assessment. All farms must have a *Salmonella* control plan in place, which is regularly reviewed (at least once per year); and they must have a detailed review of their meat-juice ELISA results also at least once a year. Those farms that consistently have a *Salmonella* prevalence above the regional average must develop a *Salmonella* Action Plan that details steps' to decrease the bacterium. If they continually have *Salmonella* positives (identified from the ELISA results), the farm may be required to have their veterinarian provide a report on the farm's existing management protocol. If the proposed action is considered inadequate, an improved *Salmonella* control plan will then be required (Howell and Hilton, 2008).

Unlike in Ireland, testing is based on bacteriological detection rather than serological testing of *Salmonella* from meat juice samples. Four samples are taken

monthly from farms supplying finisher pigs in Great Britain and from all herds in Northern Ireland collected at the abattoir. The test results are then provided to the farm three times per year. This bacteriological testing recently came into force following the suspension of serological testing in July 2012. In addition to monthly sampling, veterinarians must confirm on a quarterly basis that a *Salmonella* Control Plan is implemented; while a Farm Assurance Assessor must audit the *Salmonella* Control Plan annually (Howell and Hilton, 2008).

1.6.1.3 Denmark

The Danish Swine *Salmonella* Control Program (SSCP) has been in operation for over a decade, since 1993, and for finisher/slaughter pigs since 1995. As with the Irish and UK programs, the Danish SSCP operates at all stages of the pig production chain including feedstuffs, breeder and multiplier herds, weaner producers, finisher herds with a production of >200 pigs/year, and at the slaughterhouse (Alban et al., 2012; Alban et al., 2010; Nielsen, 2003). However, where the Danish program differs from its Irish and UK counterparts, is that it assigns herds to 1 of 3 levels – 1 "acceptable, low"; 2 "moderate, still acceptable"; or 3 "unacceptable, high" – based on seropositive meat-juice samples from the previous 3-months (Alban et al., 2012; Nielsen, 2003). In addition, all Danish pig herds are assigned a *Salmonella* status (Status A, B, or C), which must be reported to the buyer of pigs. Status A is negative for *Salmonella*; Status B is positive for types of *Salmonella* other than *S.* Typhimurium, *S.* Derby or *S.* Infantis; and Status C is positive for Typhimurium, Derby, or Infantis (Alban et al., 2012). Another significant difference from the Irish and UK programs is the enforcement of a financial penalty for level 2 herds (2% of the carcass value) and level 3 herds (starts at 4%, and increases to 6% and 8% of the carcass value, depending on the number of months the herd has been in level 3) (Alban et al., 2012; Nielsen, 2003). Furthermore, level 2 or 3 herds are considered *Salmonella*-positive for 5-years unless documentation can prove otherwise (Alban et al., 2012). However, like the Irish program, special transport and slaughtering arrangements are required for level 3 pigs.

In order to determine the herd seroprevalence, the Danish program requires that monthly meat-juice samples be taken at slaughter, and that the samples[8] be examined for *Salmonella* antibodies using the Danish Mix-ELISA test (detects O antigens 1, 4-7,

[8] Approximately 25-165 samples are taken per year with the number of samples being dependent upon the herd size (Alban et al., 2012).

12) (Alban et al., 2012). The types of samples collected include: (i) daily swabbing of a 300 cm^2 area of five carcasses (these are then pooled and analyzed as one sample); (ii) three 100 cm^2 areas of carcasses are swabbed with the same gauze; and (iii) blood sampling of breeder and multiplier herds on a monthly basis (Alban et al., 2010).

Apart from pig producers, the Danish program also specifies that all purchased compound feed be in compliance with the rules for *Salmonella*-free production of feed (i.e., heat-treatment to 81 ºC or method(s) equivalent to heat treatment) (Alban et al., 2012).

1.6.2 North America

In contrast to the EU, the USA and Canada have programs in place to control *Salmonella* in pork; however, a greater emphasis is placed on slaughter rather than a whole-chain approach. Farm level control is not compulsory, as is the case with EU MS. Historically, *Salmonella* contamination in North America was an issue more associated with poultry and produce (i.e., vegetables) than with pork, as fears of *Trichinella* infection ensured that pork was more thoroughly cooked (CDC, 2012). The sections that follow briefly discuss pig *Salmonella* control programs in North America.

1.6.2.1 United States of America

The United States Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS) has the responsibility of protecting and promoting agriculture and natural resources in America. Under the USDA-APHIS, it is the Food Safety and Inspection Service (FSIS) that is responsible for ensuring that the country's supply of meat (including pork), poultry, and eggs are: safe, wholesome, labelled, and packaged correctly (FSIS, 2015).

Presently, the FSIS has no pre-harvest, farm-level control program available for *Salmonella* in swine production; and at carcass level, no sampling of pork carcasses is performed (due to past results being consistently low for *Salmonella* positives) (FSIS, 2013b). However, due to an outbreak of *Salmonella* in pulled pork in 2010 and a recent 10-year surveillance study that showed pork as the third most implicated source of *Salmonella* contamination after eggs and chicken, an Action Plan was created to develop (beginning in the second quarter of 2014) a *Salmonella* sampling program for pork products (Folger et al., 2014; FSIS, 2013b; Jackson et al., 2013).

In January 2014, the FSIS released a compliance guideline for official establishments to control and reduce the spread of *Salmonella* in pig slaughter facilities. This guideline, though not mandatory, is published in the Federal Register (Docket No. FSIS-2012-0026), and aims to prevent, eliminate, or reduce the levels of *Salmonella* on pigs at all stages of the slaughter process including: farm rearing, transport, and lairage. For pre-slaughter stages, the recommendations are similar to that of Ireland and Denmark; yet differ at post-slaughter, as the use of organic acids (i.e., lactic, acetic and others) are suggested for the steam/vacuuming, pre-chill rinse, chilling and fabrication stages (FSIS, 2013a). These, and the other recommendations put forth, are essentially GMPs based on HACCP, but are waiting final comments from the public and scientific community, thus revisions are likely.

1.6.2.2 Canada

The Canadian Food Inspection Agency (CFIA) works with Health Canada to ensure that foodborne illnesses are detected early and the public quickly warned. Where Health Canada is responsible for creating food safety standards/policies to minimize the risk of foodborne illnesses, the CFIA administers and enforces the above on, and proper packaging/labelling of Canadian agricultural products (i.e., dairy, egg, processed egg, fruit and vegetables, honey, livestock and poultry carcasses, fish, feed, meat, and maple products).

While compulsory measures to control *Salmonella* are used at slaughter through a HACCP based approach that is regularly audited by CFIA officers; at farm level, the control is voluntary and varies among provinces and within regions. Unlike the EU, much of the on-farm programs in Canada are led by producer-funded organizations in cooperation with, and with recognition from, government. Currently, farms in the country voluntarily follow the Canadian Quality Assurance (CQA) program, which is part of the national pork On Farm Food Safety (OFFS) program, and is based in part on HACCP principles. The CQA is managed by the Canadian Pork Council and certifies pig farms and pork products as meeting the highest food safety standards. Generally, the CQA-OFFS program recommends specific good production practices for purchasing breeding stock; animal, medical, and water management; building design and sanitation; biosecurity including pest control, visitors, and farm personal; traceability and notification; transport; marketing; and training (Canadian Pork Council, 2007; Rajić et al., 2007). In addition, the CQA-OFFS program also provides risk reduction plans for

chemical, biological, and physical hazards along with potential feed and water hazards. However, given these recommendations, no current sampling and/or testing schemes or hazard-minimizing strategies exist at farm level for *Salmonella* (Rajić et al., 2007). Instead, the sampling/testing schemes are performed at slaughter, at least once per year, and end-product testing is only conducted when the safety of a product is believed to have been compromised (Rajić et al., 2007).

1.7 Conclusions

Controlling the prevalence of *Salmonella* in pigs at farm-level and in the lairage of the abattoir is not a simple task. As the present review details, many aspects need to be considered in order to determine 'where', 'what', and 'how' the control strategies should be implemented. Factors such as its epidemiology, managerial practices (on farm and in the abattoir), type of feed and/or drinking water additive, transport to the abattoir, the lairage environment, *Salmonella* control programmes of the country implementing the strategy, and the cost of the strategy itself are just some of the key features that need to be evaluated before any measure is employed.

The chapters that follow evaluate the efficacy of some of the control measures listed above (i.e., organic acid/essential oil feed additives, cleaning and disinfection of the lairage environment, or on disinfecting pigs via misting within the lairage holding pens) to reduce the prevalence of *Salmonella* carriage at farm level and in the abattoir.

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1.9 Overall Research Objectives

In order to determine practical and low-cost control strategies to reduce the prevalence of *Salmonella* in finishing pigs at farm level and in the lairage holding pens in the abattoir, the following research objectives were proposed:

I. To investigate the effectiveness of dietary supplementation with sodium butyrate, an organic acid-based feed additive, to control *Salmonella* shedding and seroprevlaence in finishing pigs.

II. To investigate the effectiveness of dietary supplementation with an encapsulated blend of formic acid, citric acid, and essential oils, to control *Salmonella* shedding and seroprevalence in finishing pigs.

III. To determine the efficacy of dietary supplementation with sodium butyrate or the encapsulated blend of formic acid, citric acid, and essential oils on growth performance of finisher pigs.

IV. To determine the economic value of dietary supplementation with sodium butyrate or the encapsulated blend of formic acid, citric acid, and essential oils by conducting a cost-benefit analysis for each feed additive.

V. To investigate the effectiveness of several cleaning and disinfection protocols to reduce *Salmonella* and *Enterobacteriaceae* in the lairage environment of a pig abattoir.

VI. To investigate the effectiveness of disinfectant misting in lairage to topically reduce *Salmonella* and *Enterobacteriaceae* on pigs before slaughter.

VII. To investigate the efficacy of the combined use of control strategies including use of organic acid-based feed additives with cleaning and disinfection on *Salmonella* shedding and seroprevalence on a commercial farm with a history of high *Salmonella* seroprevalence and secondary infections.

CHAPTER 2: Effect of feeding sodium butyrate in the late finishing period on *Salmonella* carriage, seroprevalence, and growth of finishing pigs

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2.1 Abstract

Pork is an important source of human salmonellosis and low-cost on-farm control measures may provide a useful element in reducing the prevalence of this pathogen in food. This study investigated the effectiveness of dietary supplementation with sodium butyrate administered to finisher pigs for ~4-weeks prior to slaughter to control *Salmonella* shedding on highly contaminated farms.

Two trials (A and B) were conducted on two commercial pig farms, which had a history of high *Salmonella* seroprevalence. In both trials, pens (14 pens of 12 pigs/pen in Trial A and 12 pens of 12-17 pigs/pen in Trial B) were randomly assigned to a control (finisher feed without additive) or a treatment group (the same feed with 3 kg sodium butyrate/t) for 24-28 days, depending on the trial. Faeces were collected from each pig on days 0, 12 and 24/28, blood, caecal digesta and ileocaecal/mesenteric lymph nodes were collected from the slaughterhouse. Pigs were weighed at the start and end of the trials, feed intake was recorded, and carcass quality parameters were recorded at slaughter.

In Trial A, *Salmonella* shedding was reduced in the treatment compared to the control group at the end of the trial (30% versus 57% probability of detecting *Salmonella* in faeces, respectively; $p<0.001$). This reflected the serology results, with detection of a lower seroprevalence in the treatment compared to the control group using the 20% optical density cut-off (69.5% versus 89%; $p=0.001$). However, no effect on faecal shedding or seroprevalance was observed in Trial B, which may be explained by the detection of a concomitant infection with Lawsonia intracellularis. No significant differences in *Salmonella* recovery rates were observed in the caecal digesta or lymph nodes in either trial. Furthermore, feed intake, weight gain, and feed conversion efficiency (FCE) did not differ between groups ($p>0.05$) in either trial. Numerical improvements in weight gain and FCE were found with sodium butyrate treatment, which gave a cost benefit of €0.04/kg of live-weight gain.

Overall, results suggest that strategic feeding of sodium butyrate, at 3 kg/tonne of feed, to finishing pigs for 24-28 days prior to slaughter was effective in reducing *Salmonella* shedding and seroprevalance but perhaps only in the absence of co-infection with other pathogens. However, sodium butyrate supplementation at this rate did not influence intestinal carriage, nor did it reduce seroprevalence to below the cut-off used

for the high *Salmonella* risk category in Ireland (50%), or significantly improve growth performance.

Keywords: Swine, Organic acid, Dietary supplementation, Pig farm, Cost

2.2 Introduction

Asymptomatic intestinal carriage of *Salmonella* in pigs presented for slaughter can result in pork carcass contamination. An EU baseline survey conducted in 2006-2007, showed that Ireland had a high prevalence of *Salmonella* contamination on pork carcasses (20%) (EFSA, 2008). This can be linked to the relatively high prevalence of *Salmonella* in some Irish pig herds (McCarthy et al., 2013; Burns, 2015). In an attempt to reduce this prevalence, the National Pig *Salmonella* Control Program (NPSCP) was updated in 2010 (Department of Agriculture Food and the Marine (DAFM), 2010). Despite this, *Salmonella* herd prevalence has not declined (DAFM personal communication). This highlights a need to find low-cost control measures to reduce *Salmonella* shedding in pigs at primary production, especially finishing pigs (35-100 kg), as carriage rates are high during this stage of production (Burns, 2015) and finishers are a significant source of *Salmonella* in the abattoir (Duggan et al., 2010; Argüello et al., 2013a).

Dietary supplementation with organic acids or their salts is a potential strategy for the control of *Salmonella* in finishing pigs (Creus et al., 2007; Wales et al., 2010). Organic acids can decrease gastrointestinal pH, thus creating an environment, which is hostile to *Salmonella* while favouring the growth of beneficial bacteria such as lactobacilli. The un-dissociated form of various acids can also freely cross the bacterial cell membrane and enter the bacterial cell, causing cell death (Van Immerseel et al., 2006). In addition, some organic acids (e.g., butyric acid and propionic acid) also down regulate the expression of invasion genes (e.g., hilA) in *Salmonella*, thereby suppressing its ability to invade intestinal epithelial cells (Boyen et al., 2008).

Dietary supplementation with sodium butyrate has previously been shown to reduce *Salmonella* shedding and intestinal colonization in weaner pigs, which were deliberately infected with *Salmonella* (Boyen et al., 2008). However, to our knowledge, no field trial has evaluated the effectiveness of sodium butyrate as a *Salmonella* control measure in finishing pigs on farms with historically high levels of the pathogen. In addition, despite the number of field trials that have evaluated organic acids for the control of *Salmonella* in pigs, few have investigated their use for a short-targeted period prior to slaughter and the cost-benefit associated with their use (Gálfi and Bokori, 1990; Creus et al., 2007). Therefore, the objectives of the present study were to conduct a field study on two selected farms with a high *Salmonella* seroprevalence, to investigate

the ability of dietary supplementation with sodium butyrate during the last month of growth pre-slaughter to: (1) reduce faecal shedding and intestinal carriage of *Salmonella* (2) impact growth performance in finisher pigs. Based on the findings, a cost-benefit analysis was also conducted.

2.3 Materials and Methods

2.3.1 Animal Ethics and Experimental Licensing

Two separate feeding trials (Trial A and Trial B) were performed on two commercial pig farms in the last quarter of 2014 and the first quarter of 2015. Ethical approval was obtained from the Waterford Institute of Technology ethics committee and an experimental license was obtained from the Irish Department of Health and Children (number B100/2982). All animals were handled in a humane manner and were slaughtered in a regulated abattoir.

2.3.2 Experimental Procedure

2.3.2.1 Trial A Farm

Trial A was conducted on a 90 sow farrow-to-finish farm. The finisher house in which the trial was conducted consisted of a barn with 14 pens. A total of 169 finisher pigs were used (72 males and 97 females; 12 pigs per pen). Each pig was ear tagged with a unique number for identification purposes. Pigs were housed in pens (each pen was 4.5 m x 2.8 m) with concrete slatted floors and provided with ad-libitum access to water from 2 nipple drinkers per pen. The temperature of the barn was maintained at ~ 20 ºC. Ad-libitum access was provided to dry pelleted feed via single-spaced wet-dry feeders.

This herd had a historically high *Salmonella* seroprevalence (data extracted from the NPSCP); however, the prevalence of the batch of finishing pigs immediately prior to this trial declined to 0%. As a result, pens in the finishing house were artificially contaminated with a monophasic *Salmonella* Typhimurium (*S.* 4,[5],12:i:-) strain with an antimicrobial resistance (AMR) pattern of ASSuT, which had previously been isolated from sows in the same herd. Briefly, a single colony of *S.* 4,[5],12:i:- was inoculated into 90 mL of Tryptone Soya Broth (TSB, Oxoid, Basingstoke, UK), incubated overnight at 37 °C and then diluted in Phosphate Buffered Saline (PBS,

Oxoid, Basingstoke, UK) to a final concentration of ~5 x 10^3 CFU/mL. Five 25 mL vials per pen (each containing ~10^3 CFU/mL of *Salmonella*) were spread at five points: 3 in the defecation area, and 2 near the feeder. The final concentration of *Salmonella* at each inoculation point was 2.5 x 10^4 CFU. Contamination of the pens was performed 7 days before commencing the trial.

2.3.2.2 Trial B Farm

Trial B was conducted on a 180 sow farrow-to-finish farm. The finisher house in which the trial was conducted consisted of a 2-room barn, each with 6 pens per room. A total of 177 finisher pigs were used (86 males and 91 females; 12-17 pigs per pen). Each pig was ear tagged with a unique number for identification purposes. Pigs were housed in pens (each pen was 3.2 m x 3.4 m) with concrete slatted floors and provided with ad-libitum access to water from 2 nipple drinkers per pen. The temperature of each room was maintained at ~ 20 ºC. Ad-libitum access was provided to dry pelleted feed via single-spaced wet-dry feeders.

This farm had a historically high *Salmonella* seroprevalence (i.e. > 50% for 2014), and faecal shedding of *Salmonella* Typhimurium had been confirmed bacteriologically prior to commencement of the trial.

2.3.2.3 Treatment Groups

Approximately 4 weeks before the target slaughter date, pigs in both trials A and B were blocked by sex and weight and randomly assigned to one of two diet groups: a standard finisher feed with no feed additive (control group) or the same finisher feed supplemented with 3 kg per tonne sodium butyrate (Adimix®, Nutriad, Kasterlee, Belgium; treatment group). The composition of the trial diets is shown in Table 2.3.1. In Trial A, the pigs were fed the experimental diets for 28 days and in Trial B, for 24 days. Pigs in both trials were fasted for ~18 h prior to slaughter.

2.3.2.4 Blood and Faecal Sampling and Measurement of Production Parameters

For serological analysis, blood was collected during two occasions: (1) by jugular venipuncture, prior to feeding the experimental diets, and (2) during exsanguination at slaughter. All samples were collected using plastic tubes for whole blood (BD Vacutainer, Becton Dickinson, Oxford, UK). Serum was obtained after coagulation and centrifugation of the tubes (1500 rpm for 10 min) and stored at -20 ºC until analysis.

On day 0 (the day prior to commencing experimental treatments), day 12 and

either day 28 (Trial A) or day 24 (Trial B) (i.e., the final treatment day), faeces (~25 g) was collected into 100 mL sterile bottles (Sarstedt, Nümbrecht, Germany) from each pig by digital rectal stimulation. All samples were collected and handled aseptically to avoid cross-contamination.

Feed intake was recorded throughout each trial and individual body weights were recorded on day 0 and day 28 (Trial A) or day 24 (Trial B). These weights were used to calculate average daily feed intake (ADFI), average daily gain (ADG), and feed conversion efficiency (FCE). Pigs were observed closely at least twice daily. Any pig showing signs of ill health was treated as appropriate. All veterinary treatments were recorded including identity of pig, clinical signs, medication used, and dosage. If a death occurred or antibiotics were administered, the pig(s) were weighed and subsequently removed from the pen(s) and excluded from the trial.

2.3.2.5 Sampling of Trucks and Lairage

Swabs were taken from the trucks immediately prior to loading the pigs, in each trial. Two swabs samples were taken from the floors of the trucks using sterile sponges pre-soaked with Maximum Recovery Diluent (MRD, Oxoid, Basingstoke, UK). Pigs from the treatment group in each trial were compartmentalized on the upper floor of the truck while the control group was on the lower level. Upon arrival at the abattoir, two lairage pens (one for each diet group) were swabbed with sponges, as above, prior to unloading the pigs from Trial B (3 swabs per pen i.e. front, middle and back of each pen). Lairage swabs were not collected in Trial A.

2.3.2.6 Sampling and Carcass Measurements at Slaughter

For each trial, caecal digesta, ileocaecal lymph nodes (ILN), and mesenteric lymph nodes (MLN) were collected from the gastrointestinal tract (GIT) of 88 pigs (45 from the control group and 43 from the treatment group). Caecal digesta (~10 g) was collected via puncture of the blind end of the caecum; while ILN and MLN (≥10 g) were removed from the viscera and pooled for each animal. All samples were collected aseptically to avoid cross-contamination.

The internal organs and digestive tract were removed before measuring hot carcass weight and the head was left on the carcass. The hot carcass weight at harvest was multiplied by 0.98 to obtain the cold carcass weight and is the value reported in this study as carcass weight. Kill out yield was calculated by expressing cold carcass

weight as a percentage of live weight at slaughter. In Trial B, lean meat yield was estimated from back fat and muscle depth measurements taken using a Hennessy Grading probe according to S.I. No. 413 of 2001 (Government Publications, 2001).

Half of the trial pigs (41 control and 47 treatment) in Trial B were sent for slaughter six days after the first half and although no samples were taken from these pigs all other factory measurements were recorded and were used in the analysis of data.

2.3.2.7 Salmonella Isolation from Faecal, Digesta and Intestinal Samples, and Truck and Lairage Swabs

All samples were kept at 4 °C and processed the same day or within 24 h for the presence or absence of *Salmonella* according to the International Organization for Standardization (ISO) 6579:2007 (Amendment 1: Annex D) method (International Organization for Standardization, 2007). The ILN and MLN were first processed according to EC Regulation 668/2006 (Regulation (EC) No 668/2006 (06.10.2006), 2006) by removing the fat and capsula followed by immersion in 90% ethanol (v/v). They were then flamed to sterilize the outer surface, and cut into small pieces using sterile scissors to an approximate weight of 10 g.

All *Salmonella* isolates recovered were banked onto beads and stored at -80°C for further characterization.

2.3.3 Serotyping and Antimicrobial Resistance Determination of *Salmonella* Isolates

All presumptive *Salmonella* isolates was first tested using the real-time polymerase chain reaction (PCR) assay for the identification and differentiation of *Salmonella enterica* serotype Typhimurium and *S.* 4,[5],12:i:- as described by Prendergast et al. (2013). If isolates were not identified as *S.* Typhimurium or its monophasic variant, then serotyping was performed according to the White-Kauffmann-Le Minor scheme (Grimont and Weill, 2007) using commercial antisera (Pro-Lab Diagnostics, Cheshire, UK; SIFIN Institute, Berlin, Germany; and Statens Serum Institute, Copenhagen, Denmark).

The antimicrobial resistance (AMR) pattern of each isolate was determined using the Sensititre^{TM} Gram Negative NARMS Plate (Thermo Scientific, Waltham, MA, USA). The following antimicrobials were tested: amoxicillin-clavulanic acid (AUG), ampicillin (AMP), azithromycin (AZI), cefoxitin (FOX), ceftiofur (XNL),

ceftriaxone (AXO), chloramphenicol (CHL), ciprofloxacin (CIP), gentamicin (GEN), nalidixic acid (NAL), streptomycin (STR), sulfisoxazole (FIS), tetracycline (TET), and trimethoprim/sulfamethoxazole (SXT). Minimal Inhibitory Concentrations (MICs) were interpreted using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) epidemiological cut-off values.

2.3.4 *Salmonella* Serological Analysis

Serum samples were analyzed in duplicate using an in-house indirect Enzyme-Linked Immunosorbent Assay (ELISA). Testing was performed by the Department of Agriculture Food and the Marine (Ireland) in accordance with the methods used for serological monitoring in the current NPSCP. The crude optical density (OD) values of the unknown samples were adjusted with OD values of the positive and negative controls [((sample – negative control)/(positive control – negative control)) X 100]. The mean of the adjusted OD values of tested samples were used to compare the control and treatment groups. Cut-offs were fixed at ODs of 20% and 40%, according to previous studies (Nielsen et al., 1995; Argüello et al., 2013c).

2.3.5 Statistical Analysis

Pens of pigs were blocked on sex and weight. Entire males or gilts and feed with or without sodium butyrate supplementation were used in a 2 x 2 factorial arrangement with seven pen replicates per diet group in Trial A and six pen replicates per diet group in Trial B.

For *Salmonella* prevalence and serology, data were analyzed using the GLIMMIX procedure in Statistical Analyses System (SAS, V9.3, 2011). The pig was the experimental unit. To avoid potential clustering and effects of over-dispersion, pen was included as a random effect in the model. Means for *Salmonella* prevalence and serology were separated using the Tukey-Kramer least square means adjustment for multiple comparisons and evaluated as the probability of detecting *Salmonella* in faeces, caeca, ILN/MLN, or *Salmonella* antibodies in serum. The *Salmonella* prevalence on day 0 (before control and treatment diets were administered) was used as a covariate in the Tukey-Kramer least square means adjustment.

For growth performance, data were analyzed using the mixed models procedure in SAS. The experimental unit was the pen of pigs. Fixed effects were diet group, sex

and day and block was included as a random effect. Where significant in the model initial weight or carcass weight was included as a covariate in the analysis.

Residual checks were made to ensure that the assumptions of the analyses were met. For all analyses, significance in difference was established at $\alpha = 0.05$.

2.4 Results

2.4.1 Faecal Shedding of *Salmonella*

For Trial A, one female pig was removed from the treatment group following the administration of penicillin. The probability of detecting *Salmonella* was similar in control and treatment groups on day 12. By the end of the trial (day 28), the administration of sodium butyrate to finishing pigs had resulted in a decline in the probability of detecting *Salmonella* compared to the control group (30% versus 57%, respectively; $p < 0.05$; Table 2.4.1). Table 2.4.2 details the number of *Salmonella*-positive faecal samples recovered and the resultant *Salmonella* prevalence was calculated for each of the 3 sampling time points (days 0, 12, and 28). Between day 12 and day 28, supplementation with sodium butyrate reduced the probability of detecting *Salmonella* shedding from 66% to 30%, ($p < 0.001$; Table 2.4.1). However, the probability of detecting *Salmonella* was 50% and 57%, on day 12 and day 28, respectively, for the control group ($p > 0.05$; Table 2.4.1).

For Trial B, no effect of sodium butyrate treatment was observed either on day 12 or 24 ($p > 0.05$); neither was there an effect of treatment over time ($p > 0.05$; Table 2.4.1).

The *Salmonella* serotype recovered from pigs in Trial A was *S.* 4,[5],12:i:-; while in Trial B, all of the isolates were typed as *S.* Typhimurium.

2.4.2 *Salmonella* Serology

The pig sera samples were analyzed with 20% and 40% OD cut-off values, which are commonly used in *Salmonella* control programmes (Table 2.4.3).

In Trial A, all of the pigs (82 per group) were seronegative at the beginning of the trial. In agreement with the reduction in *Salmonella* prevalence observed in faecal samples, significantly lower seroprevalence was detected in the sodium butyrate-treated group at slaughter using the 20% OD cut-off as compared to the control group (69.5%

versus 89.0%, respectively; $p < 0.001$). When using the 40% OD cut-off, no significant difference in seroprevalence was detected between groups ($p > 0.05$). When adjusted OD values from the control (mean OD value 65.1; sem = 4.31) and treatment group (47.7; sem = 4.67) pigs were compared, significant differences were detected between groups ($p < 0.05$).

In Trial B, blood was collected from 40 control pigs and 36 sodium butyrate-treated pigs at the start of the finishing period. Half of the pigs from the control group had seroconverted at this stage and the serological prevalence (or OD values) was higher in the control group compared to the sodium butyrate-treated group ($p < 0.01$). At the end of the trial, most pigs had seroconverted, indicating the presence of infection in both groups. During this second sampling, no differences in prevalence were detected between groups with either cut-off value (20% OD $p > 0.05$; and 40% OD $p > 0.05$), or when the mean of the adjusted OD values of the control group (mean OD 66.7; sem = 7.13) was compared with that of the sodium butyrate-treated group (mean OD 87.0; sem = 9.27) ($p > 0.05$).

2.4.3 *Salmonella* from Truck and Lairage Swabs

For both Trials A and B, the truck swabs taken prior to loading of pigs were *Salmonella* negative. Swabbing of the lairage pens to which pigs from Trial B were allocated prior to slaughter showed *Salmonella* in both pens (one sample of the three taken per pen was positive). The serotype recovered from these two pens was *S.* 4,[5],12:i:.

2.4.4 *Salmonella* in Intestinal Samples

Table 2.4.2 details the number of *Salmonella*-positive caecal and ILN/MLN samples recovered and the resultant *Salmonella* prevalence calculated for each trial. Overall, no differences in the probability of detecting *Salmonella* in the caecal digesta were observed between control and treated pigs for Trial A (85% versus 84%, respectively; sem = 0.060; $p > 0.05$) and Trial B (91% versus 83%, respectively, sem = 0.052; $p > 0.05$). Similarly for the pooled ILN/MLN samples, no differences in the probability of detecting *Salmonella* were observed between control and treated pigs in Trial A (35% versus 36%, respectively; sem = 0.14; $p > 0.05$) and Trial B (42% versus 28%, respectively; sem = 0.073; $p > 0.05$).

In Trial A, the *Salmonella* serotype recovered from all *Salmonella*-positive caecal digesta and ILN/MLN samples was *S.* 4,[5],12:i:-.

In Trial B, isolates recovered were identified as both *S.* Typhimurium and *S.* 4,[5],12:i:-. However, only a small number of pigs in the control group (7 pigs) and treatment group (4 pigs) were positive for *S.* 4,[5],12:i:-. Five samples of caecal digesta, from 3 control pigs and 2 treatment pigs, and 6 samples of lymph nodes, from 4 control pigs and 2 treatment pigs, were positive for *S.* 4,[5],12:i:-. The *S.* 4,[5],12:i:- isolates when recovered from the caecal digesta were not recovered from the lymph nodes of the same pig and vice-versa.

2.4.5 Production Parameters

No significant differences between groups were observed for ADFI, ADG or FCE in either trial (Table 2.4.4). Despite this, pigs in the sodium butyrate-treated group in both trials had numerically higher ADG than those in the control group (Table 2.4.4). A numerical increase of 7% and 2.6% in ADG was found in Trials A and B, respectively as a result of feeding sodium butyrate. Moreover, the FCE for pigs in both trials was numerically better in the sodium butyrate-treated group as compared to the control group. A numerical improvement of 8.5% and 4.3% in FCE was found in Trials A and B, respectively as a result of feeding sodium butyrate. Although the growth performance of pigs in either trial was not significantly affected by treatment, the numerical differences observed, particularly in Trial A, were of biological importance. For this reason the cost-benefit of supplementing the diet with sodium butyrate was determined by considering the increased feed cost associated with incorporating sodium butyrate into the diet and the feed efficiency of pigs during the trial period (Table 2.4.4). From Table 2.4.5, the final feed cost per kg live-weight gain during Trial A was €0.89 and €0.85 for the control and treatment groups, respectively; and for Trial B it was €0.91 and €0.92 for the control and treatment groups, respectively.

2.5 Discussion

Decreasing *Salmonella* at farm-level can be considered an initial step in any overall control strategy to limit its spread throughout the pig production cycle if non-negligible levels are present (Goldbach and Alban, 2006; Ojha and Kostrzynska, 2007; Alban et al., 2012). However, ensuring that *Salmonella* is not introduced in the first instance should be the initial step, especially for herds that are negligible for the

pathogen (Alban et al., 2012). Control strategies at farm-level not only decrease the infection pressure during production but the resultant reduction in *Salmonella* carriage can also lessen cross contamination in transport vehicles and lairage – two points at which pigs are prone to acquiring *Salmonella* (Berends et al., 1996). This should also result in a reduction in pork carcass contamination at slaughter.

Organic acids and/or their salts, as a potential *Salmonella* control measure, have been tested in challenge and non-challenge trials in pigs at various stages of growth (Canibe et al., 2005; Creus et al., 2007; Boyen et al., 2008; Visscher et al., 2009; Gebru et al., 2010; Willamil et al., 2011; Calveyra et al., 2012; Argüello et al., 2013b). However, results from these studies are inconclusive and the success of the interventions depends on the product used, its concentration and the duration of administration (Creus et al., 2007; Argüello et al., 2013b). The dietary supplement evaluated in the present study was a commercially available sodium butyrate feed additive used at the manufacturer's recommended inclusion rate with a relatively short treatment period, i.e., approximately the last four weeks prior to slaughter. As feed intake is high during the finisher period and diet acidification is expensive, the latter was done to evaluate the efficacy of sodium butyrate while minimizing its impact on feed costs. This particular additive was chosen as it is in a coated form, which ensures delivery to the lower GIT. In addition, there is good evidence for its mechanism of action in terms of reducing *Salmonella* (Van Immerseel et al., 2006). Butyric acid is a short-chain-fatty-acid (SCFA), which down regulates the expression of several *Salmonella* invasion genes including hilA, and invF, leading to reduced invasion of intestinal epithelial cells (Gantois et al., 2006). As a result, *Salmonella* uptake into the cytosol of epithelial cells is diminished along with caecal colonization (Gantois et al., 2006; Van Immerseel et al., 2006). However, scientific literature on the usefulness of butyric acid and/or its salts for *Salmonella* control in livestock animals, specifically pigs, is scarce, with only one trial reported, in which weaner pigs were supplemented with coated butyrate (Boyen et al., 2008).

The present study is the first on-farm trial to evaluate the efficacy of sodium butyrate as a control measure to reduce *Salmonella* shedding and intestinal carriage in finishing pigs on farms with a history of high seroprevalence. Results showed that the additive was successful in decreasing *Salmonella* shedding over a 28-day period on a highly contaminated farm (Trial A) in the absence of a secondary infection. This is in agreement with previous research, which showed that 12 days of dietary

supplementation with coated butyrate tended to reduce *Salmonella* shedding for 3 days post-infection in weaner pigs deliberately infected with *Salmonella* (Boyen et al., 2008). However, the fact that *Salmonella* prevalence increased in the first half of the treatment period in the first trial, provides clear evidence that supplementation with sodium butyrate is not a 'quick fix' in terms of controlling *Salmonella* at farm-level. Similar to earlier studies, a reduction in prevalence was observed only after several weeks of treatment (Creus et al., 2007; Argüello et al., 2013b), which supports the idea that the duration of administration is one of the key factors affecting the success of *Salmonella* control using in-feed organic acids or their salts. Cost is also an important issue for the primary producer and increasing the treatment period increases feed costs. Therefore, it is necessary to identify the minimum period for efficacy of any feed additive for it to be commercially viable.

However, in the second trial, sodium butyrate did not reduce faecal shedding of *Salmonella*. As diarrhoea was common in pigs during Trial B, laboratory analysis was performed on faecal samples to investigate the presence of other intestinal pathogens, i.e. *Brachyspira hyodisenteriae, Brachyspira pilosicoli* and *Lawsonia intracellularis*. Interestingly, *L. intracellularis* was detected in these samples (data not shown). Infection with multiple pathogens is commonly observed as part of the porcine intestinal disease complex (Brockmeier et al., 2002) and may help to explain why sodium butyrate had no effect on *Salmonella* shedding during the second trial. This suggests that factors such as concomitant diseases may impact the efficacy of sodium butyrate used to control *Salmonella*. Laboratory diagnostic investigations should therefore be performed during feeding trials, when clinical signs are present. Moreover, in this second trial, animals were already infected with *Salmonella* at the start of the finisher period, whereas pigs were artificially exposed to the organism one week before the study commenced in the first trial. This difference in infection onset may have also contributed to the lack of effect from sodium butyrate administration in the second trial.

All faecal, caecal and ILN/MLN isolates obtained from the first trial were identified as *S.* 4,[5],12:i:-, a monophasic variant of *S.* Typhimurium. This was expected, as all finisher pens in this trial had been artificially contaminated with this serotype to mimic natural environmental contamination. This serotype has become widespread in pigs in recent years (Mueller-Doblies et al., 2013; Argüello et al., 2014; Burns, 2015) and is increasingly associated with human salmonellosis (EFSA, 2010). In the second trial, the *Salmonella* isolates recovered from the caecal digesta and the

ILN/MLN were identified as both *S.* Typhimurium and *S.* 4,[5],12:i:-, although the former predominated. Interestingly, *S.* 4,[5],12:i:- was not recovered from faecal samples on the farm but was recovered from the two lairage pens at the abattoir, prior to entry of the pigs. This suggests that the pigs may have acquired a new infection in the lairage.

The serological results confirmed the successful establishment of *Salmonella* infection in both trials. All pigs in the first trial were seronegative before the trial commenced, which was why artificial environmental contamination was performed. Significant differences in seroprevalence were detected at the end of the trial using a 20% OD cut-off. This is one of two cut-off values most commonly used in the *Salmonella* control programme in Ireland and Denmark (Alban et al., 2002; Department of Agriculture Food and the Marine (DAFM), 2010). While a lower seroprevalence was observed in the sodium butyrate-treated group, in agreement with faecal shedding data, pigs in this group would still be considered high seroprevalence, i.e. > 50%. In the second trial the serology data revealed that half, or more than half, of the control pigs and only 1-3 pigs in the sodium butyrate group were already infected by *Salmonella* at the beginning of the finishing period when using the 20% and 40% OD cut-off values. The fact that most of the pigs had seroconverted by the end of the trial indicates that the infection pressure was similar in both groups and is in agreement with the bacteriological results obtained. As in the first trial, the pigs fed sodium butyrate were high seroprevalence, i.e. > 50% at the end of the trial. Such high values mean a number of restrictions during slaughter would apply to pigs from this herd according to the Irish NPSCP regulations. These findings are in contrast to those of other studies, which showed that dietary supplementation with organic acids and/or their salts reduces seroprevalence to below the cut-off for high risk herds used in serology-based control programmes in Europe (Creus et al., 2007; Visscher et al., 2009; Argüello et al., 2013b). Creus et al. (2007) and Argüello et al. (2013b) showed that finishing pigs supplemented with a combination of lactic and formic acid in feed for 14 weeks and potassium diformate in feed for 7 weeks, respectively, resulted in a reduction of *Salmonella* prevalence. It is possible that a longer duration of treatment in the present study would have reduced *Salmonella* seroprevalence to below the high seroprevalence threshold; however this would certainly have increased the financial cost of the intervention.

Ideally, control measures used on-farm should reduce *Salmonella* carriage in pigs at slaughter. Numerous studies have shown a reduction of *Salmonella* in caecal

digesta and/or lymph nodes when acidified feed is used (Creus et al., 2007; Boyen et al., 2008; Visscher et al., 2009; Willamil et al., 2011; Argüello et al., 2013b); however, there are others that failed to show a significant effect (De Busser et al., 2009; Michiels et al., 2012; Argüello et al., 2013b). The fact that no significant differences in *Salmonella* detection in the caecal digesta or ILN/MLN were observed for either trial in the present study adds to the inconclusive nature of the evidence. Factors such as, stress, the period of feed withdrawal and mixing of pigs, which can lead to contamination during transport and lairage can suppress effects seen at farm-level (Argüello et al., 2012; Mannion et al., 2012). The fact that a new serotype was isolated from some of the pigs post-slaughter and that this serotype was also isolated from the lairage holding pens prior to entry of the trial pigs supports this suggestion.

In addition to evaluating the efficacy of in-feed sodium butyrate as a *Salmonella* control measure, effects on growth performance and an associated cost-benefit analysis were also investigated. Many studies have shown that dietary supplementation with organic acids and/or their salts is beneficial to the growth performance of pigs (Gálfi and Bokori, 1990; Partanen and Mroz, 1999; Øverland et al., 2000; Mroz et al., 2002; Partanen et al., 2002; Lawlor et al., 2005; Lawlor et al., 2006; Creus et al., 2007; Walsh et al., 2007; Øverland et al., 2009; Gebru et al., 2010; Htoo and Molares, 2012; Upadhaya et al., 2014). Few studies, however, have investigated the cost-benefit of diet acidification (Gálfi and Bokori, 1990; Creus et al., 2007). Goldbach and Alban (2006) provided an economic analysis of four different *Salmonella* control strategies in Denmark, including the use of acidified feed for slaughter pigs. While the authors noted that acidified feed reduced *Salmonella* prevalence, they determined that it did so at a net financial cost to the primary producer (Goldbach and Alban, 2006). These authors, however, failed to perform a cost-benefit analysis, which considered improvements in growth and feed efficiency as a result of diet acidification. Gálfi and Bokori (1990) had earlier showed that sodium butyrate was an effective growth promoter in pigs between weaning and slaughter when it reduced feed costs by 9% and increased sales receipts by 13% (Gálfi and Bokori, 1990). While the present study had a much shorter treatment period, a 7% increase in growth rate and an 8% improvement in FCE were found during the 28 day trial in response to feeding sodium butyrate in Trial A. Although sodium butyrate supplementation added €0.71 to the feed cost of a pig during the trial, when growth and FCE are considered, strategic dietary supplementation with sodium butyrate for approximately 28 days prior to slaughter

reduced feed cost per kg live weight gain by €0.04. As the numerical improvements in ADG and FCE were less in Trial B, supplementation with sodium butyrate increased feed cost per kg live weight gain during the trial by a marginal €0.01. The reason for the latter was most likely a consequence of the *Lawsonia* infection detected in pigs during this trial.

2.6 Conclusions

Overall, strategic feeding of sodium butyrate to finishing pigs for a relatively short period of time (< 30 days) immediately prior to slaughter was effective in reducing *Salmonella* shedding and seroprevalance in one of two trials. Lack of efficacy in the second trial may be explained by a concomitant infection with *L. intracellularis*. Sodium butyrate supplementation did not reduce intestinal carriage, nor did it reduce seroprevalence to below the cut-off used for the high *Salmonella* risk category in Ireland (50%). Although it did not significantly improve growth performance, the numerical improvements found, for both growth rate and FCE, were sufficient to reduce feed cost by €0.04 per kg of live-weight gain in the absence of concomitant enteric infections.

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Table 2.3.1. Declared composition of diets used in Trials A and B (on an air-dry basis, %)

Item	Amount (on an as-fed basis, %)[a]	
	Trial A	Trial B
Maize	25.0	20.0
Wheat	22.0	20.0
Barley	20.0	31.7
Soya (Bean) Meal Dehulled	16.4	14.3
Pollard	6.0	-
Rapeseed Meal	3.5	8.0
Soya Hulls	2.0	-
Soya Oil	1.65	2.21
Sugar Cane Molasses	1.0	1.0
Mineral and Vitamin Premix	2.45[1]	2.79[2]
Chemical Composition		
Dry Matter	87.5	87.9
Crude Protein	17.0	16.2
Crude Oils and Fats	3.80	3.42
Crude Fiber	4.30	4.21
Crude Ash	4.90	4.60
Lysine	1.10	1.15
Digestible Energy, MJ/kg	-	14.0

[1]Premix provided per kg of complete diet: vitamin A, 1000 IU; vitamin D3, 2000 IU; vitamin E, 90 IU; calcium iodate anhydrous, 6.14 mg; zinc oxide, 124 mg; sodium selenite, 0.55 mg; manganese oxide, 65 mg; ferrous sulphate monohydrate, 380 mg; cupric sulphate pentahaydrate, 60 mg; endo-1.4 beta-xylanase, 100 IU; Ca, 8.5 g; Na, 2.0 g; P, 5 g; methionine, 3.0 g; Phytase, 1500 FTU.

[2]Premix provided per kg of complete diet: vitamin A, 1000 IU; vitamin D3, 2000 IU; vitamin E, 90 IU; calcium iodate anhydrous, 2.86 mg; zinc oxide, 111 mg; sodium selenite, 6.6 mg; manganese oxide, 81 mg; ferrous sulphate monohydrate, 400 mg; cupric sulphate pentahydrate, 50 mg; endo-1.4 beta-xylanase, 10 IU; butylated hydroxyanisole, 0.45 mg; ethoxyquin, 0.45 mg; Ca, 6.5 g; NaCl, 5.5 g; P, 5.0 g; methionine, 3.4 g; threonine, 6.9 g; tryptophan, 2.0g; Phytase, 5000 FTU.

Table 2.4.1. The effect of dietary supplementation with sodium butyrate on the probability of detecting *Salmonella* in faeces from finisher pigs on day 12 and day 24/28 for Trials A and B on two commercial pig farms (LS means +/- sem)

	Trial A				**Trial B**			
	Day 12	**Day 28**	**sem**	**p-value**	**Day 12**	**Day 24[a]**	**sem**	**p-value**
Control (%)	50	57	0.063	0.80	30	29	0.053	1.00
Treatment (%)	66	30	0.059	< 0.001	23	23	0.049	1.00
sem	0.062	0.060			0.052	0.050		
p-value	0.26	0.018			0.81	0.86		

[a] A sex by treatment by day effect ($p<0.001$) was observed for the probability of finding *Salmonella* in faeces. On day 24, the probability of finding *Salmonella* in faeces was reduced in males when sodium butyrate was added to feed; whereas in females the probability increased.

Table 2.4.2. *Salmonella* prevalence in faeces, caecum and pooled ileocaecal and mesenteric lymph nodes (ILN/MLN), collected from finisher pigs fed either a control diet or a diet supplemented with sodium butyrate on days 0, 12, 24/28 (on farm) and days 26/29 (slaughter) for Trials A and B on two commercial pig farms

			No. Pigs Positive for *Salmonella*/No. Pigs Sampled (% *Salmonella* Prevalence)		
			Faeces	**Caecum**	**ILN/MLN**
Trial A	**Day 0**	**Control**	15/80 (18.8)	- [a]	-
		Treatment	35/79 (44.3)	-	-
	Day 12	**Control**	43/83 (51.8)	-	-
		Treatment	53/81 (65.4)	-	-
	Day 28	**Control**	47/81 (58)	-	-
		Treatment	28/78 (35.9)	-	-
	Day 29	**Control**	-	37/45 (82.2)	20/45 (44.4)
		Treatment	-	35/43 (81.4)	17/43 (39.5)
Trial B	**Day 0**	**Control**	17/83 (20.5)	-	-
		Treatment	5/88 (5.7)	-	-
	Day 12	**Control**	27/82 (32.9)	-	-
		Treatment	20/87 (23)	-	-
	Day 24	**Control**	28/86 (32.6)	-	-
		Treatment	20/88 (22.7)	-	-
	Day 26	**Control**	-	41/45 (91.1)	19/45 (42.2)
		Treatment	-	36/43 (83.7)	12/43 (27.9)

[a] - indicates no samples were taken.

Table 2.4.3. *Salmonella* seroprevalence at the start of the finishing period and at the end (at slaughter) in finisher pigs fed either a control diet or a diet supplemented with sodium butyrate[1]

	Entry to finisher house				Slaughter			
	OD 20		OD 40		OD 20		OD 40	
	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment
Trial A								
No. Positive Pigs	0	0	0	0	73	57	54	43
No. Negative Pigs	82	82	82	82	9	25	28	39
***Salmonella* Prevalence (%)**	0	0	0	0	89.0[a]	69.5[b]	65.9	52.4
Trial B								
No. Positive Pigs	20	3	16	1	39	30	33	25
No. Negative Pigs	20	33	24	35	1	6	7	11
***Salmonella* Prevalence (%)**	50.0[a]	9.1[b]	66.7[x]	2.9[y]	97.5	83.3	82.5	69.4

[1] Trial A=82 pigs per group; Trial B=40 pigs in the control group, 36 pigs in the treatment group

[a, b, x, y] Within a row for the same OD value and same stage of sampling, values with different superscripts are significanly different ($p<0.01$).

Table 2.4.4. The effect of dietary supplementation with sodium butyrate on growth, feed efficiency, and carcass quality in finisher pigs on Trials A and B conducted on two commercial pig farms

	Trial A[a]				Trial B[a]			
	Control	Treatment	sem	p-value	Control	Treatment	sem	p-value
Weight - Day 0 (kg)	86.2	88.8	5.58	0.43	78.5	75.4	1.92	0.14
Weight - Day 24 (kg)	N/A	N/A	N/A	N/A	101	101	0.7	0.96
Weight - Day 28 (kg)	113	115	1.5	0.3	N/A	N/A	N/A	N/A
Average Daily Feed Intake (g)	2781	2832	80.61	0.66	2718	2666	79.1	0.65
Average Daily Gain (g)	919	984	62.7	0.29	840	862	24.1	0.5
Feed Conversion Efficiency (g/g)	3.15	2.88	0.11	0.12	3.24	3.1	0.08	0.25
Carcass Weight (kg)	86.2	87.4	1.24	0.52	80.2	79.6	0.58	0.54
Kill Out Yield (%)	76.2	74.9	0.66	0.14	78.7	78.3	0.32	0.35
Lean Meat Yield (%)	N/A	N/A	N/A	N/A	57.1	56.7	0.26	0.39

[a] N/A – indicates not applicable.

Table 2.4.5. Cost-benefit analysis of dietary supplementation of finisher pigs with sodium butyrate on Trials A and B conducted on two commercial pig farms

	Trial A		Trial B	
	Control	**Treatment**	**Control**	**Treatment**
Weight Gain (kg)	25.7	27.6	20.2	20.7
Feed Conversion Efficiency (kg/kg)	3.15	2.88	3.24	3.10
Cost of Sodium Butyrate (€/kg)	-	5	-	5
Inclusion Rate of Sodium Butyrate (kg/t)	-	3	-	3
Total Cost of Sodium Butyrate (€/t)	-	15	-	15
Cost of Sodium Butyrate (€/pig)	-	1.19	-	0.96
Finisher Feed Price in Ireland for July 2015 (€/t)	281	281	281	281
Finisher Feed Price with/without added Sodium Butyrate (€/t)	281	296	281	296
Total Feed Intake (kg/pig)	81.1	79.3	65.3	64.1
Finisher Feed Cost (€/kg)	0.281	0.296	0.281	0.296
Finisher Feed Cost per pig (€/pig)	22.78	23.49	18.35	18.98
Total Finisher Feed Cost per kg Live Weight Gain (€/kg live weight gain)	0.89	0.85	0.91	0.92

CHAPTER 3: Effect of strategic administration of an encapsulated blend of formic acid, citric acid and essential oils on *Salmonella* carriage, seroprevalence, and growth of finishing pigs

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3.1 Abstract

Controlling *Salmonella* at farm level can act as the first line of defence in reducing salmonellosis from pork. This study investigated the efficacy of an encapsulated blend of formic acid, citric acid, and essential oils (FormaXOL™) administered to finisher pigs for 28 days prior to slaughter in controlling *Salmonella* shedding on a commercial farm with a history of high *Salmonella* seroprevalence.

Fourteen pens of 8-10 pigs/pen were randomly assigned to a control (finisher diet without additive) or a treatment group (the same diet with 4 kg/t of FormaXOL™) for 28 days. Faeces were collected from each pig on days 0, 14, and 28, while on day 29 blood, caecal digesta and ileocaecal-mesenteric lymph nodes were collected at slaughter. Pigs were weighed at the start and end of the trial, feed intake was recorded, and carcass quality parameters were recorded at slaughter.

On day 14, *Salmonella* shedding was reduced in the treatment compared to the control group (27.9% versus 51.7% probability of detecting *Salmonella* in faeces, respectively; $p = 0.001$). However, on day 28, no reduction was observed (20.6% versus 35.9% probability of detecting *Salmonella* in faeces, respectively; $p = 0.07$). Interestingly, *Salmonella* shedding rates in the treated pigs remained stable throughout the trial compared to the control group. This suggests that the feed additive prevented additional pigs from acquiring the *Salmonella* infection. A lower *Salmonella* seroprevalence was detected at slaughter in the treatment compared to the control group using the 40% optical density cut-off (64.5% versus 88.5%, respectively; $p = 0.01$). However, no significant differences in *Salmonella* recovery rates were observed in the caecal digesta or lymph nodes between treated and control groups. Treated pigs had a lower feed intake than pigs fed the control diet ($p = 0.001$); however, average daily gain and feed conversion efficiency were not affected by treatment ($p = 0.45$ and 0.55, respectively). Consequently, supplementing the diet with FormaXOL™ for 28 days increased the feed cost per kg of live-weight gain by €0.08.

Overall, results suggest that strategic administration of an encapsulated blend of formic acid, citric acid, and essential oils, to finishing pigs for 28 days prior to slaughter has potential to prevent increased *Salmonella* shedding at certain time points as well as seroprevalence. However, this additive did not lower intestinal carriage, nor did it reduce seroprevalence to below the cut-off used for the high *Salmonella* risk category in Ireland (50%) or improve growth performance.

Keywords: Organic acid; Dietary supplementation; Swine; Pig farm; Cost

3.2 Introduction

Globally, *Salmonella* is one of the most common causes of foodborne disease in humans and pork is considered an important source of human salmonellosis (EFSA, 2008). In the latest summary report on trends and sources of foodborne outbreaks within the European Union (EU), 225 foodborne outbreaks were linked to *Salmonella* (EFSA, 2015). Of these, 9.3% were linked to the consumption of pork, the third most commonly reported food vehicle after eggs and egg products and bakery products. The non-typhoidal *Salmonella* serotypes that cause human infection are usually carried asymptomatically in pigs, causing little or no clinical signs of disease (Callaway et al., 2008). As such, pigs become reservoirs for *Salmonella* contamination along the production chain (Rodriguez et al., 2006; Ojha and Kostrzynska, 2007; Dorr et al., 2009; Duggan et al., 2010). The most recent EU survey in slaughter pigs showed that *Salmonella* prevalence in intestinal lymph node samples was 10.3% and that 8.3% of carcasses were contaminated, indicating the extent of the problem (EFSA, 2008).

Controlling the introduction, persistence, and transmission of *Salmonella* at farm level is therefore often the first line of defence in reducing human salmonellosis. Various control measures have been investigated in pigs to date, including dietary supplementation with organic acid feed additives (Berge and Wierup, 2012; De Busser et al., 2013; Walia et al., 2016). Generally, these organic acids are short- and medium-chain fatty acids (SCFA, MCFA), which, when used in an un-dissociated form ultimately disrupt vital metabolic processes within the bacterial cell, leading to cell death (Van Immerseel et al., 2006). Essential oils have also been shown to exhibit anti-*Salmonella* activity, mainly acting via membrane disruption, non-specific permeabilization of cell membranes, leakage of adenosine triphosphate (ATP) and potassium/hydrogen ions, inhibition of ATPase activity, and an increase in the fluidity of phospholipid bilayers (Burt, 2004; Oussalah et al., 2007; Bakkali et al., 2008; Barbosa et al., 2009; Berge and Wierup, 2012; Hyldgaard et al., 2012; Langeveld et al., 2014).

However, to our knowledge, only three *in vivo* studies to date have investigated essential oils as a dietary strategy for *Salmonella* reduction in pigs (Ahmed et al., 2013; Michiels et al., 2012; Rasschaert et al., 2016). Furthermore, despite the number of field studies that have evaluated in-feed organic acids for the control of *Salmonella* in pigs, only two of the studies above evaluated an essential oil in combination with organic

acids and only one was conducted in finishers. Moreover, none of these studies performed a cost-benefit analysis. Additionally, no field trial to our knowledge, has evaluated the efficacy of an encapsulated blend of formic acid, citric acid, and essential oils as a dietary additive for *Salmonella* control in finishing pigs. Previous studies showed success in reducing *Salmonella* in finishing pigs when supplemented with various organic acid feed additives, i.e., potassium diformate, lactic-formic acid, formic-propionic acid for a minimum of 7 weeks (Creus et al., 2007; Visscher 2009; Argüello et al., 2013a). Yet, few have evaluated a shorter duration of feeding (i.e., < 30 days) as a low-cost approach to controlling *Salmonella* at farm level (Walia et al., 2016). Additionally, the economic value of administering a formic-citric acid and essential oil blend to finishing pigs for such a short period prior to slaughter, is absent from published literature. Therefore, given these knowledge gaps, the present study aimed to investigate the ability of targeted dietary supplementation with an encapsulated blend of formic acid, citric acid, and essential oils, during the last 28 days of the finishing period, to reduce faecal shedding, intestinal carriage, and *Salmonella* seroprevalence, together with an evaluation of its impact on growth performance.

3.3 Materials and Methods

3.3.1 Animal Ethics and Experimental Licensing

The feeding trial was performed on a commercial pig farm in the last quarter of 2015. Ethical approval was obtained from the Waterford Institute of Technology ethics committee and an experimental license was obtained from the Irish Department of Health and Children (number B100/2982). All animals were handled in a humane manner and were slaughtered in a regulated abattoir.

3.3.2 Experimental Procedure

The feeding trial was conducted on a 90 sow farrow-to-finish farm. The finisher house in which the trial was conducted consisted of a barn with 14 pens. One hundred and twenty four finisher pigs (70 males and 54 females; in 14 pens of 8-10 same gender pigs per pen), managed as a single all-in-all-out group, were used in the experiment. Each pig was ear tagged with a unique number for identification purposes. Each pen was 4.5 m x 2.8 m with concrete slatted floors and ad-libitum access to water was

provided from 2 nipple drinkers per pen. The temperature of the barn was maintained at ~ 20 °C. Ad-libitum access was provided to dry pelleted feed via single-spaced wet-dry feeders.

This herd had a historically high *Salmonella* seroprevalence [data extracted from the National Pig *Salmonella* Control Programme (NPSCP)]; however, the prevalence of the batch of finishing pigs immediately prior to this trial had declined to 0%. In order to guarantee *Salmonella* carriage in the pigs, pens in the finishing house were artificially contaminated with a *S.* 4,[5],12:i:-, which had previously been isolated from sows in the same herd and had an antimicrobial resistance (AMR) profile of ASSuT. Briefly, a single colony of *S.* 4,[5],12:i:- was inoculated into 90 mL of Tryptone Soya Broth (TSB, Oxoid, Basingstoke, UK), incubated overnight at 37 °C and then diluted in phosphate buffered saline (PBS) to a final concentration of ~5×10^3 CFU/mL. Five 25 mL vials (each containing ~5×10^3 CFU/mL of *Salmonella*) were spread at five points in each pen: 3 in the defecation area, and 2 near the feeder. The final concentration of *Salmonella* at each inoculation point was therefore expected to be 2.5×10^4 CFU/mL. Contamination of the pens was performed 7 days before commencing the trial.

3.3.2.1 Diets

Approximately 4 weeks before the target slaughter date, pens of pigs were blocked (7 blocks) by sex and weight and randomly assigned within block, using a random number generator in Excel, to one of two dietary treatments: a standard finisher diet with no feed additive (control group) or the same finisher diet supplemented with 4 kg per tonne of an encapsulated blend of formic acid, citric acid, and essential oils from citrus fruit extract, cinnamon, oregano, thyme, and capsicum (FormaXOL^{TM}, Kemin Industries, Inc. Southport, Merseyside, UK). The composition of the trial diets is shown in Table 3.3.1. The pigs were fed the experimental diets for 28 days and were fasted for ~18 h prior to slaughter.

3.3.2.2 Blood and Faecal Sampling and Measurement of Production Parameters

For serological analysis, blood was collected by jugular venipuncture, prior to feeding the experimental diets, and during exsanguination at slaughter. All samples were collected using plastic vacutainers for whole blood (BD Vacutainer, Becton Dickinson, Oxford, UK). Serum was obtained after coagulation and centrifugation of the tubes (1500 rpm for 10 min) and was stored at -20 °C until analysis.

On day 0 (the day prior to commencing experimental treatments), day 14 and day 28 (i.e., the final treatment day), faeces (~25 g) was collected from each pig by digital rectal stimulation into 100 mL sterile bottles (Sarstedt, Nümbrecht, Germany). All samples were collected and handled aseptically to avoid cross-contamination.

Feed intake was recorded throughout the trial and individual live weights were recorded on day 0 and day 28. These weights were used to calculate the average daily feed intake (ADFI), average daily gain (ADG), and feed conversion efficiency (FCE). In addition, weight gain, FCE and feed intake over the 28-day feeding period, together with industry prices for the feed and feed additive were used in the cost-benefit analysis for the two experimental diets. Pigs were observed closely at least twice daily. Any pig showing signs of ill health was treated as appropriate. All veterinary treatments were recorded including identity of pig, clinical signs, medication used, and dosage. If a death occurred or antibiotics were administered, the pig(s) were weighed and subsequently removed from the pen(s) and excluded from the trial.

3.3.2.3 Sampling of Truck Floors and Lairage Pens

Swabs were taken from the truck used to transport pigs to the abattoir immediately prior to loading the pigs. Four swabs were taken from the floors of the truck, two from the back of the truck and two from the front of the truck, using sterile sponges pre-soaked with maximum recovery diluent (Technical Services Consultants Ltd, Lancashire, UK). Each swab covered a 40 cm x 40 cm area. Pigs from the treatment group were compartmentalised in the back of the truck, while the control group were confined in the front. Upon arrival at the abattoir, two lairage pens (one for each diet group) were swabbed with sponges, as above, prior to unloading the pigs (3 swabs per pen i.e. front, middle and back of each pen, with each swab covering a 40 cm x 40 cm area).

3.3.2.4 Sampling and Measurements Collected at Slaughter

Caecal digesta, ileocaecal lymph nodes (ILN), and mesenteric lymph nodes (MLN) were collected from the gastrointestinal tract (GIT) of 74 pigs (42 control pigs and 32 treated pigs). Caecal digesta (~25 g) was collected via puncture of the blind end of the caecum, while ILN and MLN (≥ 10 g) were removed from the mesentery and pooled for each animal. All samples were collected aseptically into sterile containers to avoid cross-contamination.

The internal organs and digestive tract were removed before measuring hot carcass weight (the head was left on the carcass). The hot carcass weight at harvest was multiplied by 0.98 to obtain the cold carcass weight, which is the value reported in this study as carcass weight. Kill out yield was calculated by expressing cold carcass weight as a percentage of live weight prior to slaughter. Lean meat yield was estimated from back fat and muscle depth measurements taken using a Hennessy Grading probe according to S.I. No. 413 of 2001 (Government Publications, 2001).

3.3.2.5 Salmonella Isolation and Serotyping

All samples were kept at 4 °C and tested the same day or within 24 h for the presence of *Salmonella* according to the International Organization for Standardization (ISO) 6579:2007 (Amendment 1: Annex D) method (International Organization for Standardization, 2007). All media were obtained from Oxoid.

The ILN and MLN were first processed according to EC Regulation 668/2006 (Regulation (EC) No 668/2006 (06.10.2006), 2006) by removing the fat and capsula followed by immersion in 90% ethanol (v/v). They were then flamed to sterilize the outer surface, and cut into small pieces using sterile scissors to an approximate weight of 10 g.

Briefly, 25 g of each faecal or digesta sample was homogenized in 225 mL of buffered peptone water (BPW) and 10 g of ILN/MLN was homogenized in 90 mL of BPW. All BPW suspensions were incubated at 37 ºC for 19 hours, after which 100 µL of each enrichment was inoculated onto modified semi-solid rappaport-vassiliadis (MSRV) agar plates and incubated at 42 ºC for 24 hours. If the MSRV plate was negative, it was incubated for a further 24 hours. Presumptive *Salmonella* growth was then streaked onto xylose lysine deoxycholate (XLD) and brilliant green (BG) agar plates and incubated at 37 ºC for 24 hours. Suspect colonies from XLD or BG agar plates were then streaked onto plate count agar (PCA), and incubated at 37 ºC for 24 hours. Urea agar slants and *Salmonella* chromogenic agar plates were then inoculated with colonies from the PCA plates and incubated at 37 ºC for 24 hours. Serological confirmation of colonies from PCA was performed using a *Salmonella* latex agglutination kit (Oxoid). All presumptive *Salmonella* isolates recovered were banked onto beads and stored at -80 °C for further characterization.

All presumptive *Salmonella* isolates were first tested using the real-time polymerase chain reaction (PCR) assay for the identification and differentiation of

Salmonella enterica serotype Typhimurium and *S.* 4,[5],12:i:- as described by Prendergast et al. (2013). If isolates were not identified as *S.* Typhimurium or its monophasic variant, then serotyping was performed according to the White-Kauffmann-Le Minor scheme (Grimont and Weill, 2007) using commercial antisera (Pro-Lab Diagnostics, Cheshire, UK; SIFIN Institute, Berlin, Germany; and Statens Serum Institute, Copenhagen, Denmark).

3.3.3 *Salmonella* Serological Analysis

Serum samples were analysed in duplicate using an in-house indirect Enzyme-Linked Immunosorbent Assay (ELISA) (Nielsen et al., 1995). Testing was performed by the Department of Agriculture Food and Marine (Ireland) in accordance with the methods used for serological monitoring in the current NPSCP. The crude optical density (OD) values of the unknown samples were adjusted with OD values of the positive and negative controls [((sample – negative control)/(positive control – negative control)) X 100]. The mean of the adjusted OD values of tested samples were used to compare the control and treatment groups. Cut-offs were fixed at ODs of 20% and 40%, according to previous studies (Nielsen et al., 1995; Argüello et al., 2013b).

3.3.4 Statistical Analysis

The experiment was a randomised complete block design with treatment applied at the pen level. Within block, pens of pigs were fed with or without FormaXOL^{TM} in the diet.

For *Salmonella* prevalence and serology, data were analysed using the GLIMMIX procedure in Statistical Analyses System (SAS, V9.3, 2011), while for the adjusted OD values from sera, data were analysed using the mixed models procedure in SAS. The model assumed a binary response distribution using the logit link function, with pen being used as a random effect for correlation within pen. The pig was the experimental unit. Means for *Salmonella* prevalence and serology were separated using the Tukey-Kramer least square means adjustment for multiple comparisons and evaluated as the probability of detecting *Salmonella* in faeces, caecal digesta, ILN-MLN, or the presence of *Salmonella* antibodies in serum. The *Salmonella* prevalence on day 0 (before the diets were administered) was used as a covariate in the Tukey-Kramer least square means adjustment.

For growth performance parameters data were analysed using the GLIMMIX model procedure in SAS. The experimental unit was the pen. Fixed effects were dietary group, sex, and day. Block was included as a random effect in the model and adjustment was also made for pen effect. Initial weight at day 0 was used as a covariate in the analysis of pig weight at day 28, average daily feed intake, average daily gain, and feed conversion efficiency. Carcass weight was included as a covariate in the analysis for lean meat yield, muscle depth, and fat depth.

Residual checks were made to ensure that the assumptions of the analyses were met. For all analyses, statistical significance was established at $\alpha = 0.05$ and 95% confidence intervals (CI) were reported.

3.4 Results

3.4.1 *Salmonella* Shedding in Faeces

On day 0, before the experimental diets were administered, faecal shedding of *Salmonella* was comparable between the control and treatment groups (26.7% versus 23.0%, respectively; Table 3.4.1). Two weeks later, *Salmonella* shedding increased in the control compared to the treatment group, with the probability of detecting *Salmonella* in faeces being 51.7% versus 27.9%, respectively ($p = 0.001$; Table 3.4.2). Faecal *Salmonella* prevalence decreased in both groups by day 28 compared to day 14 and there was a tendency for a decrease in the probability of detecting *Salmonella* in the faeces of the treatment group compared to the control group (20.6% versus 35.9%, respectively; $p = 0.07$; Table 3.4.2). When comparing the probability of detecting *Salmonella* in the faeces over time for each group, no differences were detected between day 14 and day 28 for the treatment group (27.9% versus 20.6%; $p = 0.24$) unlike the control group, in which the probability of detecting *Salmonella* was lower on day 14 compared to day 28 (51.7% versus 35.9%; $p = 0.03$; Table 3.4.2). Table 3.4.3 details the pen-level prevalence of *Salmonella* shedding over the three sampling days. The serotype of all of the faecal isolates recovered from the pigs was *S.* 4,[5],12:i:-.

3.4.2 *Salmonella* Serology

All pigs, 61 in the control group and 62 in the treatment group, were seronegative at the beginning of the trial. A lower seroprevalence was found in the

treatment group compared to the control group (64.3% versus 89.2%, respectively; 95% CI = 47.1-78.6 versus 76.4-95.5, respectively; p = 0.01) at the end of the experiment using the 40% OD cut-off. However, no significant reduction was detected in the treatment group as compared to the control group (88.2% versus 98.0%, respectively, 95% CI = 66.5-96.6 versus 85.9-99.8, respectively; p = 0.13) when the 20% OD cut-off was used. When adjusted mean OD values from both groups were compared, the treatment group showed a significantly lower adjusted mean OD than the control group (62.4 versus 94.6, respectively; 95% CI = 42.0-82.9 versus 74.1-100, respectively; p = 0.03).

3.4.3 *Salmonella* from Truck and Lairage Swabs

Twenty five percent (1/4) of the truck swabs taken from the floor at the back of the transport truck prior to loading the treatment pigs were positive for *Salmonella*. The remaining swabs, including 2 taken from the floor at the front of the truck, where the control pigs were carried, were *Salmonella*-negative. Swabbing of the lairage pens prior to unloading the pigs at the abattoir showed the presence of *Salmonella* in one pen (one swab of the three taken from this pen was positive). Pigs from the treatment group were randomly allocated to this pen. The serotype recovered from both the truck and lairage pen swabs was *S.* 4,[5],12:i:-.

3.4.4 *Salmonella* in Caecal Digesta and Lymph Nodes

The number of *Salmonella*-positive caecal and pooled ILN-MLN samples found and the calculated *Salmonella* prevalence is shown in Table 3.4.1. Overall, no difference in the probability of detecting *Salmonella* in the caecal digesta was observed between the control and treatment groups (72.5% versus 83.9%, respectively; 95% CI = 56.5-84.2 versus 66.3-93.2, respectively; p = 0.26). Likewise, for the pooled ILN-MLN, no significant differences were observed when the control and treatment groups were compared (27.5% probability of detecting *Salmonella* versus 19.4%, respectively; 95% CI = 15.8-43.5 versus 8.8-37.3, respectively; p = 0.43).

The serotype recovered from all *Salmonella*-positive pooled ILN-MLN samples and 55/57 *Salmonella*-positive caecal digesta samples was *S.* 4,[5],12:i:-. *Salmonella* Derby and *S.* Typhimurium were recovered from the two other *Salmonella*-positive caecal digesta samples; one obtained from a control pig and the other from a pig in the treatment group.

3.4.5 Production Parameters

No differences in ADG (p = 0.45) or FCE (p = 0.55) were detected between control and treatment groups (Table 3.4.4). However, pigs in the treatment group had lower ADFI as compared to the control group (p = 0.001), along with lower carcass weight (p = 0.25), and kill-out yield (p = 0.04). Although, pigs fed the treatment diet were leaner than those fed the control diet (p = 0.02), supplementing the diet with the organic acid-essential oil blend was not cost beneficial in this trial. The total feed cost per kg of live-weight gain for the duration of the experiment was €0.76 for the control group and €0.84 for the treatment group (Table 3.4.5).

3.5 Discussion

Research on the efficacy of dietary supplementation with organic acid-essential oil combinations as a pre-harvest *Salmonella* control strategy in pigs is scarce, with only two trials reported to date (Michiels et al., 2012; Rasschaert et al., 2016). Instead, much of the current literature focuses on the use of organic acids and/or their salts alone or in combination (Canibe et al., 2005; Creus et al., 2007; Boyen et al., 2008; Visscher et al., 2009; Gebru et al., 2010; Willamil et al., 2011; Calveyra et al., 2012; Rajtak et al., 2012; Walsh et al., 2012 ; Argüello et al., 2013a; Walia et al., 2016). The present study is the first on-farm trial to evaluate the efficacy of an encapsulated blend of formic acid, citric acid, and essential oils as a dietary treatment to reduce *Salmonella* shedding and intestinal carriage in finishing pigs. The feed additive used is a commercial product and was used at the manufacturer's recommended inclusion rate for a short treatment period (28 days) prior to slaughter. Feed accounts for ~70% of the total cost of producing a pig (Teagasc Agriculture and Food Development Authority, 2015), and as such, identifying cost-effective dietary solutions that limit the persistence and transmission of *Salmonella* during the finisher stage will increase profitability. The targeted 28-day administration period employed in the present study was chosen to evaluate the efficacy of the feed additive for *Salmonella* control while reducing its impact on feed costs.

Results demonstrated the efficacy, albeit somewhat limited, of the organic acid-essential oil treatment in controlling *Salmonella* on-farm. While inclusion of the feed additive did not prevent *Salmonella* infection per se, shedding in the treatment group was lower than in the control group, in which as many as half of the pigs were *Salmonella*-positive at one point during the trial. Interestingly, *Salmonella* shedding

rates in the treated group remained stable throughout the trial, as opposed to the control group, in which *Salmonella* prevalence spiked two weeks into the trial. This suggests that the additive provided protection against *Salmonella* by preventing acquisition of infection in at least some of the pigs in the treated group. Our findings are contrary to those of a previous study which showed that 26-27 days of dietary supplementation with a formic-citric acid-essential oil combination did not reduce *Salmonella* shedding in weaner pigs when compared to an untreated control group (Michiels et al., 2012). However, a direct comparison is not possible due to the different stage of production and the fact that the pigs were deliberately infected with *Salmonella.* On the other hand, our results are supported by those of a recent study in fattening pigs from a high *Salmonella* seroprevalence farm that showed a reduction in *Salmonella* shedding on supplementation with an organic acid-essential oil blend. However, the feed additive used was different to that fed in the present study in that it contained MCFAs, lactic acid, and oregano oil (Rasschaert et al., 2016). Furthermore, *Salmonella* reductions were only seen after supplementation for the entire fattening period, with earlier faecal samples taken 5 weeks into treatment showing no differences in shedding between treatment and control groups. The latter highlights the fact that better efficacy may have been seen in our study had the duration of treatment been extended. As such, additional field trials with this feed additive are warranted, especially given the fact that a reduction in *Salmonella* shedding was only detected at certain time points in the current study.

Prior to commencing the present study, all pigs were seronegative, which is why the initial artificial contamination of pens was required. Significant differences in seroprevalence were detected at slaughter, using a 40% OD cut-off, which is the cut-off value used in the Irish NPSCP (Department of Agriculture Food and the Marine (DAFM), 2010). This finding correlates with the bacteriological results discussed above and demonstrates that the feed additive did reduce infection pressure. However, the lower seroprevalence observed in the treated group, would still be considered high, i.e., > 50%, and therefore in the high prevalence category according to the Irish NPSCP, and as such restrictions during slaughter would apply to pigs from this herd. It is possible that the treatment duration was too short to elicit a seroprevalence below 50% and it is also possible that using the additive during successive batches of pigs might reduce environmental *Salmonella* contamination and ultimately seroprevalence in pigs over time. This finding is similar to that obtained in a recent study from our group,

which showed that dietary supplementation with sodium butyrate for 24-28 days prior to slaughter reduced seroprevalence but not to below the cut-off used for high-risk herds in Ireland (Walia et al., 2016). A treatment duration of at least 7 weeks maybe necessary to reduce seroprevalence beyond that found in the present study (Creus et al., 2007; Visscher et al., 2009; Argüello et al., 2013a).

It is well documented that *Salmonella* infection can occur at any point during the growth of pigs and as such this presents a challenge as to where interventions should be focused for effective control. The main purpose in using pre-harvest *Salmonella* control strategies in pigs is to reduce the incidence of *Salmonella* carriers presented at slaughter (Arguello et al., 2013c). In this regard, much research has focused on the effect of dietary supplementation with organic acids at farm level to reduce the prevalence of *Salmonella* in the GIT of pigs at slaughter with conflicting results. Certain acid additives (sometimes used in combination with essential oils) have been successful in reducing *Salmonella* in the caecal digesta and/or lymph nodes (Creus et al., 2007; Boyen et al., 2008; Visscher et al., 2009; Willamil et al., 2011; Argüello et al., 2013a; Rasschaert et al., 2016), while others have not (De Busser et al., 2009; Willamil et al., 2011; Michiels et al., 2012; Argüello et al., 2013a; Walia et al., 2016; Rasschaert et al., 2016). The inability of the additive used in the present study to reduce *Salmonella* prevalence in the caecal digesta and ILN-MLN further illustrates the importance of additive selection and duration of feeding regarding control of *Salmonella* carriage in pigs. On the one hand, no significant differences were detected in *Salmonella* shedding at the end of the trial, which could explain the lack of differences observed in the caecal digesta and ILN-MLN. On the other hand, the fact that *Salmonella* was detected on the truck and in one lairage pen prior to introducing the animals, together with the fact that *S.* Derby and *S.* Typhimurium, two serotypes not present on the farm, were recovered from the caecum of two pigs, demonstrates that pigs could potentially have acquired a new infection during transport to the abattoir and/or in the lairage (Duggan et al., 2010; Arguello et al., 2014). It is also possible that multiple *Salmonella* serotypes were present, but undetectable, on the farm, although this is probably unlikely, as the finisher pens on this farm were artificially contaminated with *S.* 4,[5],12:i:- due to the fact that the pen and faecal samples collected prior to commencing the study were *Salmonella*-negative on multiple occasions. Nonetheless, factors such as the presence of multiple serotypes and/or acquisition of new infections immediately pre-slaughter may mask the success of control measures used at farm level. However, on-farm interventions are still

considered a necessary first step in the overall hurdle approach to controlling *Salmonella* in pigs (Ojha and Kostrzynska, 2007). Moreover, it is possible that a longer treatment period with the formic-citric acid-essential oil additive used, i.e., > 40 days, is needed in order to reduce intestinal *Salmonella* carriage. This demonstrates the importance of finding the correct balance between efficacy and cost-effectiveness. Additional feeding trials with a longer duration of treatment are therefore warranted.

Previous studies have demonstrated growth benefits as a result of dietary supplementation with organic acids, sometimes in combination with essential oils (Gálfi and Bokori, 1990; Partanen and Mroz, 1999; Øverland et al., 2000; Mroz et al., 2002; Partanen et al., 2002; Lawlor et al., 2005; Lawlor et al., 2006; Creus et al., 2007; Walsh et al., 2007; Øverland et al., 2009; Gebru et al., 2010; Htoo and Molares, 2012; Upadhaya et al., 2014; Zeng et al., 2015; Walia et al., 2016). Therefore, in addition to evaluating the efficacy of the feed additive as a *Salmonella* control measure, effects on growth performance and an associated cost-benefit analysis were investigated in the present study. Few studies have investigated the cost-benefit of dietary acidification in relation to *Salmonella* control (Creus et al., 2007; Walia et al., 2016) and none have evaluated it when essential oils are also present. Although pigs in the control group were heavier at the start of the study compared to those in the treatment group, the growth performance variables were adjusted for these weight differences and as such they do not impact the results and comparisons reported. Moreover, while treated pigs were leaner than pigs fed the control diet, they had numerically lower ADG and lighter carcasses, due to a significant reduction in feed intake. It therefore appears that the additive may have reduced feed acceptability. Consequently, supplementing the diet for 28 days with the formic-citric acid-essential oil blend increased the feed cost per kg of live-weight gain by €0.08. Therefore, for the present study, the organic acid-essential oil feed additive used was not cost beneficial, despite its efficacy in reducing *Salmonella* prevalence, albeit only at certain time points.

3.6 Conclusions

Overall, the results suggest that dietary supplementation with an encapsulated blend of formic acid, citric acid and essential oils, at 4 kg/tonne of feed, to finishing pigs for a strategic 28-day period prior to slaughter has potential to prevent increased *Salmonella* shedding at certain time points and seroprevalence. However, supplementation at this rate and for this duration did not influence intestinal carriage,

nor did it reduce seroprevalence to below the cut-off used for the high *Salmonella* risk category in Ireland (50%). Furthermore, it did not improve growth performance and, in fact, increased the feed cost per kg live-weight gain during the trial. A longer duration of dietary supplementation is perhaps warranted, although the cost-benefit of this would have to be determined.

3.7 Acknowledgments

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Table 3.3.1. Declared composition of finisher diet used (on an air-dry basis, %)

Ingredient	
Maize	25.0
Wheat	22.0
Barley	20.0
Soya (Bean) Meal Dehulled	16.4
Pollard	6.0
Rapeseed Meal	3.5
Soya Hulls	2.0
Soya Oil	1.65
Sugar Cane Molasses	1.0
Mineral and Vitamin Premix	2.45[1]
Chemical Composition	
Dry Matter	87.5
Crude Protein	17.0
Crude Oils and Fats	3.80
Crude Fiber	4.30
Crude Ash	4.90
Lysine	1.10
Digestible Energy, MJ/kg	9.8

[1]Premix provided per kg of complete diet: vitamin A, 1000 IU; vitamin D3, 2000 IU; vitamin E, 90 IU; calcium iodate anhydrous, 6.14 mg; zinc oxide, 124 mg; sodium selenite, 0.55 mg; manganese oxide, 65 mg; ferrous sulphate monohydrate, 380 mg; cupric sulphate pentahydrate, 60 mg; endo-1.4 beta-xylanase, 100 IU; Ca, 8.5 g; Na, 2.0 g; P, 5 g; methionine, 3.0 g; Phytase, 1500 FTU.

Table 3.4.1. ***Salmonella*** **prevalence in faeces, caecum and pooled ileocaecal and mesenteric lymph nodes (ILN-MLN), collected from finisher pigs fed either a control diet or a diet supplemented with an encapsulated blend of formic acid, citric acid, and essential oils**

		No. Pigs Positive for *Salmonella*/No. Pigs Sampled (% *Salmonella* Prevalence)		
		Faeces	**Caecum**	**ILN-MLN**
Day 0	**Control**	16/60 (26.7)	- [a]	-
	Treatment	14/61 (23.0)	-	-
Day 14	**Control**	31/62 (50.0)	-	-
	Treatment	17/62 (27.4)	-	-
Day 28	**Control**	22/61 (36.1)	-	-
	Treatment	12/60 (20.0)	-	-
Day 29	**Control**	-	30/42 (71.4)	11/42 (26.2)
	Treatment	-	27/32 (84.4)	6/32 (18.8)

[a] '-' indicates no samples were taken.

Table 3.4.2. The effect of dietary supplementation with an encapsulated blend of formic acid, citric acid and essential oils on the probability of detecting *Salmonella* in faeces from finisher pigs on days 14 and 28 on a commercial pig farm)[a]

	Day 14[b]	**Day 28[b]**	**p-value**
Control (95% CI)	51.7% (39.0-64.2)	35.9% (24.6-49.0)	0.03
Treatment (95% CI)	27.9% (17.9-40.6)	20.6% (12.0-32.3)	0.24
p-value	0.001	0.07	

[a] The values reported are based on statistical analysis from a single model containing effects of diet, day and the interaction of diet by day. The interaction between diet and day was only statistically significant for the control group. Confidence intervals (95%) are given for the estimated probabilities.

[b] The *Salmonella* prevalence at day 0 was used as a covariate in the analysis of the probabilities for the control and treatment groups.

Table 3.4.3. Pen-level prevalence of *Salmonella* shedding in faeces collected from finisher pigs fed either a control diet or a diet supplemented with an encapsulated blend of formic acid, citric acid, and essential oils over three sampling days (days 0, 14 and 28) at farm-level.

				Sampling Period					
				Day 0		**Day 14**		**Day 28**	
Pen	**Diet**	**Sex**	**No. Pigs/Pen**	**No. Pigs Positive for *Salmonella***	**% *Salmonella* Prevalence**	**No. Pigs Positive for *Salmonella***	**% *Salmonella* Prevalence**	**No. Pigs Positive for *Salmonella***	**% *Salmonella* Prevalence**
1	Control	Male	9	0	0.0	3	33.33	0	0.0
2	Treatment	Male	9	1	11.1	1	11.11	0	0.0 [a]
3	Control	Female	10	0	0.0	3	30.00	3	30.0
4	Treatment	Female	8	1	14.3[a]	0	0.00	0	0.0
5	Control	Male	8	7	100.0[a]	5	62.50	3	37.5
6	Treatment	Male	9	2	22.2	0	0.00	0	0.0
7	Control	Female	9	3	33.3	1	11.11	6	66.7
8	Treatment	Female	9	0	0.0	9	100.00	8	100.0 [a]
9	Control	Male	9	0	0.0	9	100.00	7	87.5 [a]
10	Treatment	Male	9	6	66.7	4	44.44	2	22.2
11	Control	Female	9	5	62.5[a]	3	33.33	0	0.0
12	Treatment	Female	9	3	33.3	1	11.11	1	11.1
13	Control	Male	8	1	12.5	7	87.50	3	37.5
14	Treatment	Male	9	1	11.1	2	22.22	1	11.1

[a] *Salmonella* prevalence is based on 1 less pig per pen for these pens on these sampling days, as faeces could not be collected from all pigs in the pen.

Table 3.4.4. The effect of dietary supplementation with an encapsulated blend of formic acid, citric acid, and essential oils on growth, feed efficiency, and carcass quality of finisher pigs on a commercial pig farm[a]

	Control (95% CI)	Treatment (95% CI)	p-value
Weight - Day 0 (kg)	80.5 (75.4-85.6)	76.4 (71.3-81.5)	0.04
Weight - Day 28 (kg)[b]	111 (108-114)	109 (106-112)	0.45
Average Daily Feed Intake (g)[b]	3037 (2992-3090)	2943 (2888-2985)	0.001
Average Daily Gain (g)[b]	1160 (1053-1263)	1107 (1001-121)	0.45
Feed Conversion Efficiency (g/g)[b]	2.71 (2.53-2.89)	2.78 (2.60-2.96)	0.55
Carcass Weight (kg)	81.1 (78.9-83.2)	79.4 (77.2-81.5)	0.25
Kill Out Yield (%)	73.4 (72.8-73.9)	72.672.1-73.1)	0.04
Lean Meat Yield (%)[c]	56.4 (55.8-57.0)	57.5 (56.9-58.1)	0.02
Muscle Depth (mm)[c]	50.6 (49.5-51.8)	52.6 (51.4-53.7)	0.004
Fat Depth (mm)[c]	13.37 (12.7-14.1)	12.44 (11.7-13.2)	0.07

[a] The statistical model used for comparison of the growth performance variables listed in the table included dietary group, sex, and day. Block was included as a random effect and adjustment was also made for pen effect. Confidence intervals (95% CI) are provided for each growth performance/carcass quality variable.

[b] Initial body weight at day 0 was used as a covariate in the analysis.

[c] Carcass weight was used as a covariate in the analysis.

Table 3.4.5. Cost-benefit analysis of dietary supplementation with an encapsulated blend of formic acid, citric acid, and essential oils to finisher pigs on a commercial pig farm

	Control	Treatment
Weight Gain (kg)	32.5	31.0
Feed Conversion Efficiency (kg/kg)	2.71	2.78
Cost of Formic acid, Citric acid, and Essential oils (€/kg)	-	5
Inclusion Rate of Formic acid, Citric acid, and Essential oils (kg/t)	-	4
Total Cost of Formic acid, Citric acid, and Essential oils (€/t)	-	20
Cost of Formic acid, Citric acid, and Essential oils (€/pig)	-	1.72
Finisher Feed Price in Ireland for July 2015 (€/t)	281	281
Finisher Feed Price with/without added Formic acid, Citric acid, and Essential oils (€/t)	281	301
Total Feed Intake (kg/pig)	88.0	86.2
Finisher Feed Cost (€/kg)	0.281	0.301
Finisher Feed Cost per pig (€/pig)	24.73	25.93
Total Finisher Feed Cost per kg Live Weight Gain (€/kg live weight gain)	0.76	0.84

CHAPTER 4: The efficacy of different cleaning and disinfection procedures to reduce *Salmonella* and *Enterobacteriaceae* in the lairage environment of a pig abattoir

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4.1 Abstract

This study investigated several cleaning and disinfection protocols for their ability to eliminate *Salmonella* and to reduce levels of *Enterobacteriaceae,* within the lairage pens of a commercial pig abattoir.

Eight protocols were evaluated in each of 12 lairage pens at the end of the slaughtering day on 3 occasions (36 pens/protocol): (P1) high-pressure cold water wash (herein referred to as high-pressure wash); (P2) high-pressure wash followed by a quaternary ammonium compound (QAC)-based disinfectant without rinsing; (P3) high-pressure wash followed by a chlorocresol-based disinfectant without rinsing; (P4) high-pressure wash followed by a sodium hydroxide/sodium hypochlorite detergent with rinsing; (P5) P4 followed by P2; (P6) P4 followed by P3; (P7) P5 with drying for 24-48 hours; and (P8) P6 with drying for 24-48 hours. Two floor swabs and one wall swab were taken from each lairage pen before and after each protocol was applied, and examined for the presence of *Salmonella* and enumeration of *Enterobacteriaceae.*

High-pressure washing alone (P1) did not reduce the prevalence of *Salmonella* in the lairage pens. When high-pressure washing, the probability of detecting *Salmonella* following application of the chlorocresol-based disinfectant (P3) was lower than with the QAC-based disinfectant, P2 (14.2% versus 34.0%, respectively; $p < 0.05$). The probability of detecting *Salmonella* after the combined use of detergent and the chlorocresol-based disinfectant (P6) was also lower than application of detergent followed by the QAC-based disinfectant, P5 (2.2% versus 17.1%, respectively; $p < 0.05$). Drying of pens (P7 and P8) greatly reduced the probability of detecting *Salmonella.* Only 3.8% of swabs were *Salmonella*-positive 48-hours after cleaning with detergent and the QAC-based disinfectant (P7); while an eradication of *Salmonella* was achieved 24-hours after cleaning with detergent and the chlorocresol-based disinfectant, P8. A reduction in *Enterobacteriaceae* counts to below the limit of detection (LOD; 10 CFU/cm^2) was achieved following cleaning with detergent and disinfection with the chlorocresol-based disinfectant, regardless of drying ($p < 0.05$), whereas, applying detergent and the QAC-based disinfectant (P7) did not reduce *Enterobacteriaceae* counts to below the LOD.

Therefore ensuring that lairage pens are allowed to dry after intensive cleaning with detergent and a chlorocresol-based disinfectant is recommended as the most

effective hygiene routine to eliminate *Salmonella* and reduce *Enterobacteriaceae* counts.

Keywords: Lairage; Quaternary ammonium compound; Drying; Chlorocresol; Eliminate

4.2 Introduction

Following transport from the farm, the next stage in the pre-harvest control of *Salmonella* in pigs is within the lairage of the abattoir. Lairaging pigs in the abattoir, provides a buffer for the slaughter line, allowing pigs to recover from the stress of transport, and improves meat quality (Warriss, 2003). It is well documented that finishing pigs need as little as 2 hours following exposure to a contaminated environment to acquire *Salmonella* (Boughton et al., 2007b; Hurd et al., 2001). As such, there is a clear risk of pigs acquiring *Salmonella* from the lairage, if the environment is contaminated. This fact has been highlighted in previous molecular typing studies that investigated *Salmonella* in abattoirs. Duggan et al. (2010) found that the lairage pens in all three Irish abattoirs investigated were highly contaminated with several serovars of *Salmonella enterica* (Derby, Typhimurium, and Manhattan). More importantly, strains isolated from pig carcasses and intestinal contents have been shown to be indistinguishable from those isolated from lairage pens (Argüello et al., 2013; Bolton et al., 2013; Duggan et al., 2010; Mannion et al., 2012; Rostagno et al., 2003).

One way to limit the occurrence and spread of *Salmonella* within the lairage environment is through appropriate cleaning and disinfection regimes. Several approaches have been investigated (Argüello et al., 2011; Boughton et al., 2007a; Swanenburg et al., 2001); however, difficulties in eliminating *Salmonella* remain. Reasons for this include production of biofilms, or developed resistance to the cleaning agents and/or disinfectants, or harboring sites (i.e., cracks and holes in the lairage pens, drains) that are not easily cleaned or disinfected, all of which allow *Salmonella* to survive (Boughton et al., 2007a; Corcoran et al., 2014; De Beer et al., 1994; De Busser et al., 2013; McLaren et al., 2011; Stewart et al., 2001).

While many studies highlight the usefulness of cleaning and disinfection in the lairage to reduce the level of *Salmonella* carriage in pigs before slaughter, very few have compared cleaning regimes (Boughton et al., 2007a; Schmidt et al., 2004; Swanenburg et al., 2001; van der Wolf et al., 2001). Moreover, no study to date has investigated the various combinations of power washing with detergent and disinfectants and a subsequent drying step as a means to eliminate *Salmonella* from lairage pens. Additionally, only a limited number of studies have examined the efficacy of specific detergent and/or disinfectant agents against *Salmonella* when used in the lairage area of pig abattoirs (Boughton et al., 2007a; Schmidt et al., 2004; Swanenburg

et al., 2001). Quaternary ammonium compound (QAC) disinfectants are commonly used biocides, as their broad-spectrum of activity means that they are effective against a wide range of bacterial species (Hegstad et al., 2010; Holah et al., 2002; Sidhu et al., 2002). The main mode of action of QAC's against Gram-negative bacteria is disruption of the lipid bilayer of the cytoplasmic membrane and outer membrane leading to leakage of cytoplasmic components and eventually cell lysis (Quinn et al., 2011). Similarly, chlorocresol acts by causing a loss of cell membrane integrity and coagulation of cytoplasmic components, most likely due to protein denaturation (McLaren et al., 2011). Furthermore, chlorocresol was shown as the superior disinfectant as it consistently killed *Salmonella* in wet environments, albeit with poultry faeces (McLaren et al., 2011), which is typical of lairage pens in pig abattoirs. For these reasons, and the fact that chlorocresol is not widely used in pig abattoirs as a disinfectant, its effect was compared to that of a QAC-based disinfectant in the present study.

Therefore, the objective of this study was to evaluate several cleaning and disinfection protocols, specifically with QAC-based or chlorocresol-based disinfectants, for their ability to eliminate *Salmonella* and to improve overall hygiene, as determined by measuring *Enterobacteriaceae* counts, within the lairage pen environment of a commercial pig abattoir.

4.3 Materials and Methods

4.3.1 Abattoir and Lairage Area

One pig abattoir in the Republic of Ireland participated in this study. This abattoir routinely operates a Monday to Friday schedule, slaughtering approximately 2000 pigs per day from herds across the country. The lairage area consists of 12 main pens with solid concrete floors and walls. Each pen was 2.05 m x 14.95 m, with a capacity for holding 65 pigs during the day. During a slaughtering day, each pen was filled and emptied on multiple occasions with pigs from different herds.

4.3.2 Cleaning and Disinfection Protocols

Eight different cleaning protocols were evaluated in this study (Table 4.3.1). Each protocol consisted of the following: (P1) high-pressure cold (15-20 ºC) water

wash (herein referred to as high-pressure wash) to remove gross faecal matter; (P2) high-pressure wash followed by application of a QAC-based disinfectant (Holquat®, Holchem Laboratories Limited, UK) at a dilution rate of 2% without subsequent rinsing; (P3) high-pressure wash followed by application of a chlorocresol-based disinfectant (Interkokask®, Hysolv, UK) at a dilution rate of 2-3% without subsequent rinsing; (P4) high-pressure wash followed by an alkyl dimethyl amine oxide, sodium aryl sulphonate, sodium hydroxide and sodium hypochlorite detergent (Rapier®, Holchem Laboratories Limited, UK) at a dilution rate of 5%, with a contact time of 20 minutes followed by a high-pressure water rinse; (P5) protocol P4 followed by protocol P2; (P6) protocol P4 followed by protocol P3; (P7) combining protocol P5 with a drying step for 24-48 hours; (P8) combining protocol P6 with a drying step for 24-48 hours.

Each protocol was implemented in each of the 12 lairage pens at the end of the slaughtering day on three occasions (36 pens per protocol).

4.3.3 Sample Collection

The 12 lairage pens were sampled, before and after implementation of the protocols listed above. In each pen, after it was emptied of pigs and before the protocol was implemented, two floor swabs (sterile sponges, 100 cm^2, pre-moistened with 10 mL Maximum Recovery Diluent (MRD), (Technical Services Consultants Ltd, Lancashire, UK) and one wall swab were collected ('Before Power Wash'). Each swab covered a 40 cm x 40 cm area. Immediately after applying P1 ('After Power Wash') or 25 minutes after applying P4 ('After Detergent') another 3 swabs per protocol were collected following the same procedure as above. Ten minutes after applying P2 ('After QAC Disinfectant') or after applying P3 ('After Chlorocresol Disinfectant'), 6 swabs per protocol containing a neutralizing buffer were used [four floor (2 per area) and two wall; sterile sponges, 50 cm^2, pre-moistened with 10 mL of neutralizing buffer; Technical Service Consultants Ltd, Lancashire, UK]. The neutralizing buffer consisted of the following compounds: Tween (Polysorbate) 80, Saponin, Sodium chloride, Sodium thiosulphate, Lecithin, L-Histidine and Deionized water. Ten minutes after applying P5 ('After Detergent + QAC Disinfectant'), or 10 minutes after applying P6 ('After Detergent + Chlorocresol Disinfectant) only, P7 ('After QAC Drying'), and P8 ('After Chlorocresol Drying'), 3 swabs, as above, were taken after each protocol, using either the MRD swabs after application of the detergent or 6 swabs containing the

neutralizing buffer after application of the disinfectants. All swabs were collected aseptically, kept at 4 ºC and processed within 24-hours.

4.3.4 Microbiological Analysis

Each MRD sponge swab was suspended in 90 mL of buffered peptone water (BPW; Oxoid Limited, Hampshire, UK) and homogenized in a stomacher for 2 minutes. For the neutralizing buffer swabs, since 4 floor swabs were collected from 2 different areas in the pen, and 2 wall swabs were collected from the same area in the pen, the 2 swabs per floor or wall area were pooled and suspended in 90 mL of BPW and homogenized in a stomacher for 2 minutes, as above.

Isolation of *Salmonella* was carried out in accordance with International Organization for Standardization (ISO) 6579:2007 (Amendment 1: Annex D) method (International Organization for Standardization, 2007). All media and agar were obtained from Oxoid Limited (Hampshire, UK). Briefly, BPW swab suspensions were incubated at 37 ºC for 19-hours, after which 100 µL of each sample was pipetted onto modified semi-solid rappaport-vassiliadis (MSRV) agar plates and incubated at 42 ºC for 24-hours. If the MSRV plate was negative, it was incubated for a further 24-hours. Presumptive *Salmonella* growth was streaked onto xylose lysine deoxycholate (XLD) and brilliant green (BG) agar and incubated at 37 ºC for 24-hours. Suspect colonies from XLD or BG agar plates were then streaked onto plate count agar (PCA) and incubated at 37 ºC for 24-hours. Afterwards, urea agar slants and *Salmonella* chromogenic agar plates were inoculated with colon(ies) from PCA and incubated at 37 ºC for 24-hours. Serological confirmation of colonies from PCA was performed using a *Salmonella* Latex Agglutination Kit (Oxoid).

In addition, *Enterobacteriaceae* counts from the floor swabs only were obtained before and after the implementation of each of the 8 protocols as follows. Ten-fold serial dilutions of the BPW swab suspensions were performed in MRD and appropriate dilutions pour-plated on violet red bile glucose agar (VRBGA; Oxoid). Plates were overlaid with VRBGA and incubated at 37 ºC for 24-hours. The limit of detection (LOD) was 10 CFU/cm^2.

4.3.5 Serotyping and Antimicrobial Resistance Determination of *Salmonella* Isolates

All presumptive *Salmonella* isolates recovered after the implementation of each of the 8 protocols was first tested using the real-time polymerase chain reaction (PCR) assay for the identification and differentiation of *Salmonella enterica* serotype Typhimurium and *S.* 4,[5],12:i:- as described by Prendergast et al. (2013). If isolates were not identified as *S.* Typhimurium or *S.* 4,[5],12:i:-, then serotyping was performed according to the White-Kauffmann-Le Minor scheme (Grimont and Weill, 2007) using commercial antisera (Pro-Lab Diagnostics, Cheshire, UK; SIFIN Institute, Berlin, Germany; and Statens Serum Institute, Copenhagen, Denmark).

The antimicrobial resistance (AMR) pattern of each isolate was determined using the Sensititre™ Gram Negative NARMS Plate (Thermo Scientific, Waltham, MA, USA). The following antimicrobials were tested: amoxicillin-clavulanic acid (AUG), ampicillin (AMP), azithromycin (AZI), cefoxitin (FOX), ceftiofur (XNL), ceftriaxone (AXO), chloramphenicol (CHL), ciprofloxacin (CIP), gentamicin (GEN), nalidixic acid (NAL), streptomycin (STR), sulfisoxazole (FIS), tetracycline (TET), and trimethoprim/sulfamethoxazole (SXT). Minimal Inhibitory Concentrations (MICs) were interpreted using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) epidemiological cut-off values.

4.3.6 Crystal Violet Biofilm Assay of *Salmonella* Isolates

Analysis of biofilm formation was performed according to Chagnot et al. (2014) and an in-house methodology (Teagasc, Food Research Centre, Ashtown, Ireland) on selected isolates recovered after the implementation of all protocols except P8 ('After Chlorocresol Drying'), as no *Salmonella* was recovered following this protocol. Isolates were selected for analysis based on the uniqueness of their AMR profiles and serotypes, focusing on serotypes that are of concern in the Irish National Pig *Salmonella* Control Program (NPSCP) but also focusing on isolates that were obtained after application of the protocols that combined detergent and disinfectant with or without drying.

Isolates were grown on Luria-Bertani (LB; Oxoid) agar plates and incubated at 37 ºC for 19-hours. One colony from each LB agar plate was inoculated into 5 mL of LB without salt and incubated at 37 ºC for 19-hours. This overnight culture was diluted

into 5 mL of fresh LB without salt so as to achieve an optical density ($OD_{600\ nm}$) of 0.02. Two hundred microliters of the OD-adjusted samples were then transferred into 4 wells of a 96-well microplate (for 4 technical replicates per isolate; F bottom microplate, NUNC™, ThermoFisher Scientific, Roskilde, Denmark) and incubated at 15 ºC (to mimic the average temperature in the lairage of the abattoir) and at 37 ºC (optimal temperature for *Salmonella* growth) for 48 hours. After incubation, the liquid was removed and the wells washed with 200 µL of tryptone salt (Oxoid). Afterwards, 300 µL of pure ethanol (99.2%, Merck, Darmstadt, Germany) was added to each well and left for 20 min before being removed and allowing the well to air-dry for 1-hour. Next, 200 µL of 0.1% crystal violet (Sigma-Aldrich Ireland Limited, Arklow, Ireland) was added to each well and left for 10 min at room temperature, after which it was removed and the wells washed twice with distilled water. Two hundred microliters of 33% acetic acid (Sigma-Aldrich Ireland Limited) was added to each well and the microplate agitated for 5 min on an orbital shaker (Stuart Scientific, Staffordshire, UK). Afterwards, 150 µL of this solution was transferred to a new 96-well microplate and the OD_{595nm} values were determined for each well using a plate reader (Multiskan™ FC Microplate Photometer, Thermo Scientific, Paisley, UK). For each isolate, this entire procedure was repeated for 4 biological replicates.

The biofilm formation ability of each isolate was determined according to Chelvam et al. (2014) as follows: no biofilm (OD isolate </= OD cut-off), weak biofilm producer (OD cut-off < OD isolate </= 2 x OD cut-off), moderate biofilm producer (2 x OD cut-off < OD isolate </= 4 x OD cut-off), and strong biofilm producer (4 x OD cut-off < OD isolate). The OD cut-off was defined as three standard deviations above the mean OD_{595nm} of the negative control wells.

Resistance to the QAC-based or chlorocresol-based disinfectants was performed according to Andrews (2001) and Gantzhorn et al. (2014) on select isolates recovered from protocols P5 to P7. No isolates were recovered from P8. The concentrations that were tested to determine the minimum inhibitory concentration (MIC) of the QAC-based disinfectant were 2%, 1%, 0.5%, 0.25%, 0.125% and 0.0625%; while for the chlorocresol-based disinfectant the concentrations tested were 3%, 2%, 1%, 0.5%, 0.25%, 0.125% and 0.0625%.

4.3.7 Statistical Analysis

Salmonella prevalence data and odds ratios were analyzed using the GLIMMIX procedure in Statistical Analyses System (SAS, V9.3, 2011), while *Enterobacteriaceae* data were log transformed and analyzed using the Mixed models procedure in SAS. In all cases the 'Before Power Wash' results were tested as a covariate in analysis and Tukey-Kramer least squares means adjustment for multiple comparisons was used to separate the treatment (protocol) means.

Residual checks were made to ensure that the assumptions of the analyses were met. For all analyses, statistical significance was established at $\alpha = 0.05$.

4.4 Results

4.4.1 Detection of *Salmonella* after Application of the Cleaning and Disinfection Protocols

If any one of the three swabs taken from each pen was found to contain *Salmonella*, then the pen was considered *Salmonella*-positive. Overall, all pens were *Salmonella*-positive before applying the cleaning protocols (Table 4.4.1), with just under 83% (450/543) of the swabs positive for *Salmonella* (Table 4.4.2). Likewise, all pens after power washing, after application of detergent, and after sole use of the QAC-based disinfectant were *Salmonella*-positive (Table 4.4.1). Power washing alone had no effect on *Salmonella* prevalence, with 87.2% (157/180) of swabs positive for *Salmonella*; while, using detergent alone after power washing resulted in a reduction in the number of *Salmonella*-positive swabs to 58/108 (54%, Table 4.4.2).

Ten pens were *Salmonella*-positive after sole use of the chlorocresol-based disinfectant (Table 4.4.1). Eighteen percent (13/72) of swabs taken after application of the chlorocresol-based disinfectant were *Salmonella*-positive as compared to 49/123 (40%) after application of the QAC-based disinfectant (Table 4.4.2). The probability of detecting *Salmonella* following power washing and application of the chlorocresol-based disinfectant was lower when compared with power washing and application of the QAC-based disinfectant ($p < 0.05$, Table 4.4.2).

Nine pens were *Salmonella*-positive after the combined use of the detergent and the QAC-based disinfectant (Table 4.4.1). In contrast, only one pen was *Salmonella*-positive following the combined use of the detergent and the chlorocresol-based

disinfectant. The combined use of detergent and the QAC-based disinfectant resulted in a decline in the probability of detecting *Salmonella* ($p < 0.05$, Table 4.4.2) with 17/72 (24%) of swabs positive for *Salmonella*. A greater reduction in the number of *Salmonella*-positive swabs 1/72 (1%) was detected following the combined use of detergent and the chlorocresol-based disinfectant, resulting in a reduction in the probability of detecting *Salmonella* ($p < 0.05$, Table 4.4.2).

Only two pens were *Salmonella*-positive after drying following cleaning with detergent in combination with use of the QAC-based disinfectant, whereas all pens were *Salmonella*-negative after drying following cleaning with detergent and use of the chlorocresol-based disinfectant (Table 4.4.1). Allowing pens to dry greatly reduced the probability of detecting *Salmonella* ($p < 0.05$, Table 4.4.2). All swabs were *Salmonella*-negative 24 hours after cleaning with detergent and use of the chlorocresol-based disinfectant followed by drying; whereas 2/72 (3%) of the swabs were *Salmonella*-positive 48 hours after cleaning with detergent and use of the QAC-based disinfectant followed by drying.

4.4.2 Odds of *Salmonella* Contamination after Application of the Cleaning and Disinfection Protocols

Odds ratios for comparing the likelihood of *Salmonella* contamination in the lairage pens following application of each cleaning/disinfection protocol were also determined (Table 4.4.3). Overall, power washing did not reduce *Salmonella* contamination in the lairage pens. Power washing alone was more likely to result in *Salmonella* contamination than when sole use of detergent, QAC- or chlorocresol-based disinfectants, combined detergent and QAC- or chlorocresol-based disinfectants, and drying after combined use of detergent and the QAC- or chlorocresol-based disinfectants, respectively, were applied after power washing of the pens ($p < 0.05$, Table 4.4.3). Allowing the lairage pens to dry for 48 hours following application of detergent and the QAC-based disinfectant was less likely to result in *Salmonella* contamination than sole use of the QAC-based disinfectant, the detergent after power washing, or power washing alone, respectively ($p < 0.05$, Table 4.4.3). Allowing the pens to dry for 24 hours after applying the detergent and the chlorocresol-based disinfectant was likely to result in *Salmonella* contamination than sole use of the chlorocresol-based disinfectant, the detergent after power washing, or power washing alone, respectively ($p < 0.05$, Table 4.4.3).

4.4.3 *Enterobacteriaceae* Counts

The effect of the different cleaning and disinfection protocols on *Enterobacteriaceae* counts in the lairage pens is presented in Table 4.4.4. In line with the *Salmonella* results, a reduction in *Enterobacteriaceae* counts to below the LOD (10 CFU/cm^2) was achieved following application of the chlorocresol-based disinfectant after power washing as compared to a 0.86 $\log_{10}$ CFU/cm^2 reduction after application of the QAC-based disinfectant after power washing ($p < 0.05$). Sole use of detergent after power washing resulted in a 1.82 $\log_{10}$ CFU/cm^2 reduction in *Enterobacteriaceae* counts, as compared to a 0.59 $\log_{10}$ CFU/cm^2 reduction after the combined used of detergent and the QAC-based disinfectant ($p < 0.05$). On the other hand, a reduction in *Enterobacteriaceae* counts to below the LOD was also achieved following combined use of detergent and the chlorocresol-based disinfectant regardless of whether the pens were dry or wet ($p < 0.05$). A 2.9 $\log_{10}$ CFU/cm^2 reduction after pens were dried following application of the detergent and the QAC-based disinfectant was not enough to reduce *Enterobacteriaceae* counts to below the LOD.

4.4.4 Serotyping and Antimicrobial Resistance of *Salmonella* Isolates

Serotypes of the *Salmonella* isolates obtained from swabs taken after the use of detergent and/or disinfectants are listed in Table 4.4.5. Six serotypes were detected; *S.* 4,[5],12:i:-; *S.* Brandenburg, *S.* Bredeney; *S.* Derby; *S.* Typhimurium and *S.* Panama. Overall, as the protocol intensified, the number of different *Salmonella* serotypes recovered decreased; however, *S.* 4,[5],12:i:- was recovered after implementation of all of the protocols listed in Table 4.4.5.

The AMR profiles of the same isolates recovered after cleaning and/or disinfection are also listed in Table 4.4.5. A total of 16 unique AMR profiles were detected amongst the various *Salmonella* serotypes, with most (i.e. 40 isolates) being multidrug resistant, showing resistance to ampicillin, streptomycin, trimethoprim/sulfamethoxazole and tetracycline; while fewer showed resistance towards cefoxitin (3 isolates), chloramphenicol (8 isolates), and gentamicin (3 isolates). None of the *S.* 4,[5],12:i:- or *S.* Typhimurium isolates showed the typical ASSUT (ampicillin, streptomycin, sulphonamide, tetracycline) resistance profile associated with Typhimurium. All isolates were susceptible to the following antimicrobials:

amoxicillin-clavulanic acid, azithromycin, ceftiofur, ceftriaxone, ciprofloxacin, nalidixic acid, and sulfisoxazole.

4.4.5 Biofilm Formation of *Salmonella* Isolates

The biofilm forming capability of selected isolates from within the six serotypes isolated after the various cleaning and/or disinfection protocols are detailed in Table 4.4.5.

At 15 ºC (average lairage temperature), after sole use of the QAC-based disinfectant, a *S.* 4,[5],12:i:- was isolated and shown to be weak biofilm former. None of the *S.* 4,[5],12:i:- that were isolated after application of the chlorocresol-based disinfectant, either in combination with detergent or without, or after pens were allowed to dry following application of the detergent and the QAC-based disinfectant, were capable of forming biofilms. Likewise, none of the *S.* Derby or *S.* Panama isolates that were recovered after any of the protocols were shown to form biofilms at 15 ºC. As the protocols intensified, the isolates recovered including *S.* Bredeney, showed moderate biofilm forming capability; while *S.* Typhimurium isolates recovered after application of the QAC-based disinfectant were better biofilm formers than isolates recovered after the detergent was applied in combination with the QAC-based disinfectant. At both 15 ºC and 37 ºC, *S.* Brandenburg isolates recovered after drying following cleaning with detergent and the QAC-based disinfectant showed moderate biofilm forming ability.

At 37 ºC (optimum growth temperature for *Salmonella*), as the protocols intensified, *S.* 4,[5],12:i:-, *S.* Bredeney, *S.* Derby and *S.* Panama isolates showed weak to moderate biofilm forming capability. On the other hand, *S.* Typhimurium recovered after sole use of the QAC-based disinfectant, acted in a similar manner to the results at 15 ºC. It possessed moderate biofilm forming ability, while those recovered after treatment with detergent and the QAC-based disinfectant showed weaker biofilm forming ability.

All 23 isolates recovered after the combined use of detergent and the QAC-based disinfectant or the chlorocresol-based disinfectant with or without a drying step were susceptible to the QAC-based and the chlorocresol-based disinfectants.

4.5 Discussion

This study evaluated eight cleaning and disinfection protocols for their ability to eliminate *Salmonella* and to reduce levels of *Enterobacteriaceae*, an indicator of overall hygiene, within the lairage pen environment of a pig abattoir in the Republic of Ireland.

The abattoir in the present study adhered to the recommendation that herds with a low *Salmonella* seroprevalence are slaughtered at the beginning of the day, whereas those with > 50% seroprevalence are slaughtered at the end of the day in order to minimize the risk of cross contamination (Department of Agriculture Food and the Marine (DAFM), 2010). However, no pen(s) within the lairage of the study abattoir was specifically allocated to herds with *Salmonella* seroprevalence > 50%. All pens at one point in time, during the study, did, however, contain pig herds with at least 60% seroprevalence and more than half were allocated to herds with greater than 80% *Salmonella* seroprevalence. This was most likely the reason that all pens were contaminated with *Salmonella,* as stress from transport may have initiated shedding even in pigs from low prevalence herds (Hurd et al., 2001, 2002; Williams and Newell, 1970).

Our results show that high-pressure washing alone was ineffective in reducing *Salmonella* in the lairage pens, and is in agreement with findings from previous research (Argüello et al., 2011; Boughton et al., 2007a; Schmidt et al., 2004; Swanenburg et al., 2001). Application of the chlorocresol-based disinfectant alone after power washing was better in terms of reducing *Salmonella* prevalence than the QAC-based disinfectant or sole use of detergent but results still showed the presence of *Salmonella* in the lairage pens. These results are also supported by the enumeration of *Enterobacteriaceae* and are also similar to those of previous work. For example, Boughton et al. (2007a) showed that reduction but not elimination of *Salmonella* was only achieved in lairage pens at weekends after intensive cleaning and disinfection was performed and pens were allowed to dry. Moreover, our finding that the chlorocresol-based disinfectant had better efficacy than the QAC-based disinfectant is supported by McLaren et al. (2011) and Gosling et al. (2016) who also found that chlorocresol was more effective than QAC's at reducing *Salmonella* in wet and dry environments. However, these studies were performed in the laboratory with inoculated poultry faeces, and to our knowledge, the current study is the first to evaluate the use of a chlorocresol-based disinfectant in a commercial abattoir as a means of reducing *Salmonella* prevalence.

Our results are also in agreement with earlier research, which suggest that use of detergent and disinfectant is not fully effective in removing *Salmonella* from the lairage environment (Boughton et al., 2007a; Schmidt et al., 2004; Small et al., 2006; Swanenburg et al., 2001). The present study, however, illustrates that a 4-step protocol consisting of combined use of detergent and a chlorocresol-based disinfectant with subsequent drying for 24 hours was the most successful in terms of removing *Salmonella* from the lairage pens. This is a novel finding that has not been shown to date. A previous study investigating the effect of different cleaning regimes on recovery of *Clostridium perfringens* from poultry crates, found that pressure washing with a QAC followed by drying for 48 hours greatly reduced the amount of *C. perfringens* as compared to the cleaning regimes without a drying step (McCrea and Macklin, 2006). Although the study differed from the present study in that it focused on Gram-positive bacteria, poultry containers and a 3-step cleaning procedure, it nonetheless showed the effectiveness of drying in terms of reducing bacterial contamination. Further work in this area is needed, especially since our findings demonstrate that drying is a critical step in terms of elimination versus reduction of *Salmonella* from the lairage environment. Moreover, the relevance of a clean lairage is perhaps questionable for pig herds with high *Salmonella* prevalence.

This finding, however, highlights the issue of the practicality of allowing lairage pens to dry for 24-48 hours and highlights the risks associated with overnight accommodation of pigs in lairage pens when the environment is not dry. Considering the risk that the lairage represents in terms of acquisition of new *Salmonella* infections (Argüello et al., 2014; Duggan et al., 2010; Mannion et al., 2012), continual cleaning with detergent followed by efficient disinfection and drying for as long as possible is required to reduce levels of *Salmonella* in the lairage environment. While the pens in this study were naturally air dried, time is limited for this process and thus it may not be effective, particularly in cool weather with high humidity. As such, we recommend that heaters or other means of artificially drying the pens after cleaning and disinfection be used between batches of animals to shorten the drying time, although this would introduce a cost.

Despite the increasing amount of literature on bacterial resistance to QAC's (Hegstad et al., 2010), none of the isolates recovered in this study were resistant to the QAC-based disinfectant or the chlorocresol-based disinfectant in MIC tests. The fact that *Salmonella* was recovered after various cleaning and/or disinfection protocols were

employed, in spite of showing no resistance to the disinfectants, highlights that more attention should perhaps be given to cleaning and disinfection procedures in the lairage, with appropriate use of the chemical agents (i.e., recommended concentrations and contact time) rather than focusing on disinfectant resistance. This finding is supported by several studies describing inadequate evidence of disinfectant resistant isolates from studies conducted *in vitro*, at farm level, or in abattoirs (Aarestrup and Hasman, 2004; Gantzhorn et al., 2014; Holah et al., 2002; Karatzas et al., 2007; McLaren et al., 2011). Emphasis of the cleaning and disinfection approach should therefore be directed towards problem areas in the pen including cracks and holes in the concrete flooring and walls, and cleaning/disinfecting the walls to the same standard as the floors.

In the transition to biofilm status, some characteristics of bacteria change, including their adherence, invasion, virulence, and resistance (Liu et al., 2014). Therefore, it is extremely difficult to eradicate biofilm-related contamination using routine cleaning methods such as disinfectants. The present study showed that a number of isolates, among the 6 serotypes of *Salmonella* recovered, were able to form biofilms at temperatures representative of those found in Irish abattoirs as well as at the optimal growth temperature for the organism (37 ºC). Although the ability to form biofilms was variable, and depended not only on temperature but by the intensity of the cleaning protocol, combining detergents with disinfectants. Moreover, all isolates recovered after the combined use of detergent and the two disinfectants with or without a drying step were susceptible to the QAC-based and the chlorocresol-based disinfectants. As such, in this study, while it is possible that these two *Salmonella* isolates, *S.* Brandenburg and *S.* 4,[5],12:i:-, were recovered as a result of their ability to form biofilms, it is more probable that they were recovered as a result of inadequate cleaning and disinfection (Brooks and Flint 2008; Krysinski et al., 1992; Marin et al., 2009). These findings, suggest that a rigorous cleaning protocol, with for instance a chlorocresol-based disinfectant in combination with a drying step, can remove *Salmonella* from the lairage pen environment.

4.6 Conclusions

Overall, power washing alone was not successful in reducing the prevalence of *Salmonella* in the lairage pens of a commercial pig abattoir. The key recommendation from the present study is to ensure that lairage pens are allowed to dry after intensive cleaning and disinfection with a chlorocresol-based disinfectant in order to ensure that

Salmonella is eliminated and *Enterobacteriaceae* counts reduced. Moreover, the ability of *Salmonella* isolates recovered from the lairage pens to form biofilms was variable, and was most common among *S.* Typhimurium, some of which were recovered after multiple step cleaning protocols.

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Table 4.3.1. The eight different cleaning and disinfection protocols employed and sample collection conducted in each of 12 lairage pens in a commercial pig abattoir

Cleaning and Disinfection Protocols [a]	Sampling Day
(P1) High-Pressure Wash ('After Power Wash') (P2) High-Pressure Wash + QAC [b] Disinfectant ('After QAC Disinfectant') (P3) High-Pressure Wash + Chlorocresol [c] Disinfectant ('After Chlorocresol Disinfectant')	Mid-Week (Tuesday/Wednesday)
(P4) High-Pressure Wash + Detergent [d] ('After Detergent') (P5) High-Pressure Wash + Detergent + QAC Disinfectant ('After Detergent + QAC Disinfectant') (P6) High-Pressure Wash + Detergent + Chlorocresol Disinfectant ('After Detergent + Chlorocresol Disinfectant')	End of Week (Friday/Saturday)
(P7) Drying following cleaning with High-Pressure Wash + Detergent + QAC Disinfectant ('After QAC + Drying') (P8) Drying following cleaning with High-Pressure Wash + Detergent + Chlorocresol Disinfectant ('After Chlorocresol + Drying')	Sunday

[a] 2 floor and 1 wall swabs were taken after each cleaning and disinfection protocol was applied.

[b] QAC, Quaternary Ammonium Compound disinfectant was Holquat®, Holchem Laboratories Limited, UK.

[c] Chlorocresol disinfectant was Interkokask®, Hysolv, UK.

[d] Detergent was Rapier®, Holchem Laboratories Limited, UK.

Table 4.4.1. *Salmonella*-prevalence of all 12 lairage pens in a commercial pig abattoir sampled before and after several cleaning and disinfection protocols were applied

		***Salmonella* Prevalence [No. *Salmonella*-positive swabs/No. swabs taken (%)]**								
Pen	**Surface**	**Before Power Wash**	**After Power Wash**	**After Detergent**	**After QAC Disinfectant**	**After Chlorocresol Disinfectant**	**After Detergent + QAC Disinfectant**	**After Detergent + Chlorocresol Disinfectant**	**After QAC Drying**	**After Chlorocresol Drying**
1	Floor	26/30 (87%)	10/10 (100%)	3/6 (50%)	6/6 (100%)	0/4 (0%)	3/4 (75%)	0/4 (0%)	0/4 (0%)	0/4 (0%)
	Wall	13/15 (87%)	5/5 (100%)	1/3 (33%)	2/3 (67%)	0/2 (0%)	1/2 (50%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
	Total	39/45 (87%)	15/15 (100%)	4/9 (44%)	8/9 (89%)	0/6 (0%)	4/6 (67%)	0/6 (0%)	0/6 (0%)	0/6 (0%)
2	Floor	29/32 (91%)	8/10 (80%)	5/6 (83%)	4/6 (67%)	0/4 (0%)	2/4 (50%)	0/4 (0%)	0/4 (0%)	0/4 (0%)
	Wall	12/16 (75%)	4/5 (80%)	0/3 (0%)	2/3 (67%)	1/2 (50%)	1/2 (50%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
	Total	41/48 (85%)	12/15 (80%)	5/9 (56%)	6/9 (67%)	1/6 (17%)	3/6 (50%)	0/6 (0%)	0/6 (0%)	0/6 (0%)
3	Floor	31/32 (97%)	9/10 (90%)	3/6 (50%)	5/6 (83%)	0/4 (0%)	0/4 (0%)	0/4 (0%)	0/4 (0%)	0/4 (0%)
	Wall	13/16 (81%)	3/5 (60%)	1/3 (33%)	0/3 (0%)	1/2 (50%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
	Total	44/48 (92%)	12/15 (80%)	4/9 (44%)	5/9 (56%)	1/6 (17%)	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)
4	Floor	32/32 (100%)	10/10 (100%)	5/6 (83%)	5/8 (63%)	0/4 (0%)	2/4 (50%)	0/4 (0%)	0/4 (0%)	0/4 (0%)
	Wall	12/16 (75%)	4/5 (80%)	3/3 (100%)	2/4 (50%)	1/2 (50%)	0/2 (0%)	0/2 (0%)	1/2 (50%)	0/2 (0%)
	Total	44/48 (92%)	14/15 (93%)	8/9 (89%)	7/12 (58%)	1/6 (17%)	2/6 (33%)	0/6 (0%)	1/6 (17%)	0/6 (0%)
5	Floor	30/32 (94%)	10/10 (100%)	5/6 (83%)	4/8 (50%)	0/4 (0%)	2/4 (50%)	0/4 (0%)	0/4 (0%)	0/4 (0%)
	Wall	11/16 (69%)	4/5 (80%)	1/3 (33%)	0/4 (0%)	1/2 (50%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
	Total	41/48 (85%)	14/15 (93%)	6/9 (67%)	4/12 (33%)	1/6 (17%)	2/6 (33%)	0/6 (0%)	0/6 (0%)	0/6 (0%)
6	Floor	31/32 (97%)	10/10 (100%)	4/6 (67%)	6/8 (75%)	0/4 (0%)	2/4 (50%)	0/4 (0%)	0/4 (0%)	0/4 (0%)
	Wall	14/16 (88%)	5/5 (100%)	1/3 (33%)	1/4 (25%)	2/2 (100%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
	Total	45/48 (94%)	15/15 (100%)	5/9 (56%)	7/12 (58%)	2/6 (33%)	2/6 (33%)	0/6 (0%)	0/6 (0%)	0/6 (0%)
7	Floor	25/30 (83%)	10/10 (100%)	4/6 (67%)	3/8 (38%)	0/4 (0%)	0/4 (0%)	0/4 (0%)	0/4 (0%)	0/4 (0%)
	Wall	12/15 (80%)	2/5 (40%)	1/3 (33%)	1/4 (25%)	2/2 (100%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
	Total	37/45 (82%)	12/15 (80%)	5/9 (56%)	4/12 (33%)	2/6 (33%)	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)
8	Floor	27/30 (90%)	10/10 (100%)	5/6 (83%)	2/8 (25%)	0/4 (0%)	1/4 (25%)	0/4 (0%)	0/4 (0%)	0/4 (0%)
	Wall	11/15 (73%)	3/5 (60%)	0/3 (0%)	1/4 (25%)	1/2 (50%)	0/2 (0%)	1/2 (50%)	0/2 (0%)	0/2 (0%)
	Total	38/45 (84%)	13/15 (87%)	5/9 (56%)	3/12 (25%)	1/6 (17%)	1/6 (17%)	1/6 (17%)	0/6 (0%)	0/6 (0%)
9	Floor	24/30 (80%)	8/10 (80%)	2/6 (33%)	1/8 (0%)	0/4 (0%)	1/4 (25%)	0/4 (0%)	0/4 (0%)	0/4 (0%)
	Wall	11/15 (73%)	3/5 (60%)	0/3 (0%)	1/4 (25%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
	Total	35/45 (78%)	11/15 (73%)	2/9 (22%)	1/12 (8%)	0/6 (0%)	1/6 (17%)	0/6 (0%)	0/6 (0%)	0/6 (0%)
10	Floor	23/28 (82%)	9/10 (90%)	5/6 (83%)	1/6 (17%)	0/4 (0%)	1/4 (25%)	0/4 (0%)	0/4 (0%)	0/4 (0%)
	Wall	8/14 (57%)	4/5 (80%)	0/3 (0%)	0/3 (0%)	1/2 (50%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
	Total	31/42 (74%)	13/15 (87%)	5/9 (56%)	1/9 (11%)	1/6 (17%)	1/6 (17%)	0/6 (0%)	0/6 (0%)	0/6 (0%)
11	Floor	19/28 (68%)	10/10 (100%)	5/6 (83%)	1/6 (17%)	1/4 (25%)	1/4 (25%)	0/4 (0%)	1/4 (25%)	0/4 (0%)
	Wall	8/14 (57%)	3/5 (60%)	0/3 (0%)	0/3 (0%)	1/2 (50%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
	Total	27/42 (64%)	13/15 (87%)	5/9 (56%)	1/9 (11%)	2/6 (33%)	1/6 (17%)	0/6 (0%)	1/6 (17%)	0/6 (0%)
12	Floor	21/26 (81%)	10/10 (100%)	3/6 (50%)	1/4 (25%)	0/4 (0%)	0/4 (0%)	0/4 (0%)	0/4 (0%)	0/4 (0%)
	Wall	7/13 (54%)	3/5 (60%)	1/3 (33%)	0/2 (0%)	1/2 (50%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
	Total	28/39 (72%)	13/15 (87%)	4/9 (44%)	1/6 (17%)	1/6 (17%)	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)

Table 4.4.2. Number of *Salmonella*-positive samples and the probability of detecting *Salmonella* from 12 lairage pens in a commercial pig abattoir sampled before and after several cleaning and disinfection protocols were applied

	***Salmonella* Prevalence** [No. *Salmonella*-positive swabs/No. swabs taken (%)]			**Probability of detecting *Salmonella* (%)** [1]	**sem (%)**
Cleaning and Disinfection Protocols	**Floor**	**Wall**	**Total**		
Before Power Wash	318/362 (87.8%)	132/181 (72.9%)	450/543 (82.9%)	N/A [e]	N/A [e]
(P1) After Power Wash	114/120 (95.0%)	43/60 (72%)	157/180 (87.2%)	87.9 [a]	2.7
(P2) After QAC Disinfectant	39/82 (47%)	10/41 (24%)	49/123 (40%)	34.0 [b]	5.0
(P3) After Chlorocresol Disinfectant	1/48 (2%)	12/24 (50%)	13/72 (18%)	14.2 [c]	5.7
(P4) After Detergent	49/72 (68%)	9/36 (25%)	58/108 (54%)	45.8 [b]	5.9
(P5) After Detergent + QAC Disinfectant	15/48 (31%)	2/24 (8%)	17/72 (24%)	17.1 [c]	5.4
(P6) After Detergent + Chlorocresol Disinfectant	0/48 (0%)	1/24 (4%)	1/72 (1%)	2.2 [d]	1.8
(P7) After QAC + Drying	1/48 (2%)	1/24 (4%)	2/72 (3%)	3.8 [d]	2.2
(P8) After Chlorocresol + Drying	0/48 (0%)	0/24 (0%)	0/72 (0%)	1.2 [d]	1.2

[1] Values presented are probability of detecting *Salmonella* from total mean values of floor and wall swabs.

[a, b, c, d] Protocols sharing the same superscript are not significantly different ($p < 0.05$).

[e] N/A = not applicable, as data from 'Before Power Wash' were used as a covariate in the analysis.

Table 4.4.3. Odds ratios for the efficacy of the cleaning and disinfection protocols in removing *Salmonella* from 12 lairage pens in a commercial pig abattoir

Cleaning and Disinfection Protocols	Odds Ratios [a]							
	After Power Wash	After Detergent	After QAC Disinfectant	After Chlorocresol Disinfectant	After Detergent + QAC Disinfectant	After Detergent + Chlorocresol Disinfectant	After QAC Drying	After Chlorocresol Drying
After Power Wash	N/A [b]	8.62 ($p < 0.001$)	14.15 ($p < 0.001$)	44.00 ($p < 0.001$)	35.71 ($p < 0.001$)	333.3 ($p < 0.001$)	186.9 ($p < 0.001$)	500.0 ($p < 0.001$)
After QAC Disinfectant	0.07 ($p < 0.001$)	0.61 ($p = 0.80$)	N/A [b]	3.11 ($p = 0.35$)	2.49 ($p = 0.43$)	23.26 ($p = 0.006$)	13.21 ($p = 0.001$)	41.67 ($p = 0.007$)
After Chlorocresol Disinfectant	0.023 ($p < 0.001$)	0.196 ($p = 0.35$)	0.322 ($p = 0.35$)	N/A [b]	0.80 ($p = 1.0$)	7.46 ($p = 4.0$)	4.26 ($p = 0.54$)	13.33 ($p = 0.28$)
After Detergent	0.116 ($p < 0.001$)	N/A [b]	1.64 ($p = 0.80$)	5.09 ($p = 0.04$)	4.08 ($p = 0.04$)	37.93 ($p = 0.001$)	21.62 ($p < 0.001$)	66.67 ($p = 0.001$)
After Detergent + QAC Disinfectant	0.028 ($p < 0.001$)	0.245 ($p = 0.04$)	0.401 ($p = 0.43$)	1.25 ($p = 1.0$)	N/A [b]	9.26 ($p = 0.21$)	5.30 ($p = 0.26$)	16.67 ($p = 0.15$)
After Detergent + Chlorocresol Disinfectant	0.003 ($p < 0.001$)	0.026 ($p = 0.001$)	0.043 ($p = 0.006$)	0.13 ($p = 0.40$)	0.108 ($p = 0.21$)	N/A [b]	0.57 ($p = 1.0$)	1.78 ($p = 1.0$)
After QAC + Drying	0.005 ($p < 0.001$)	0.046 ($p < 0.001$)	0.076 ($p = 0.001$)	0.24 ($p = 0.54$)	0.19 ($p = 0.26$)	1.75 ($p = 1.0$)	N/A [b]	3.13 ($p = 0.98$)
After Chlorocresol + Drying	0.002 ($p < 0.001$)	0.015 ($p = 0.001$)	0.024 ($p = 0.007$)	0.08 ($p = 0.28$)	0.06 ($p = 0.15$)	0.56 ($p = 1.0$)	0.32 ($p = 0.98$)	N/A [b]

[a] Odds ratios < 1 indicate that the cleaning and disinfection protocols listed in the left column are more efficient in removing *Salmonella* than those given in the top row. Odds ratios > 1 indicate that the cleaning and disinfection protocols listed in the top row are more efficient in removing *Salmonella* than those given in the left column.

[b] N/A = not applicable, as the cleaning and disinfection protocols being compared are the same.

Table 4.4.4. The effect of different cleaning and disinfection steps on *Enterobacteriaceae* counts in 12 lairage pens in a commercial pig abattoir

Cleaning and Disinfection Protocols	Mean *Enterobacteriaceae* Count [1] (Log_{10} CFU/cm^2)	sem
Before Power Wash	5.29	N/A [f]
(P1) After Power Wash	4.12 [a]	0.10
(P2) After QAC Disinfectant	3.26 [b]	0.13
(P3) After Chlorocresol Disinfectant	< LOD [e]	0.13
(P4) After Detergent	2.30 [c]	0.08
(P5) After Detergent + QAC Disinfectant	3.53 [b]	0.13
(P6) After Detergent + Chlorocresol Disinfectant	< LOD [e]	0.13
(P7) After QAC + Drying	1.23 [d]	0.13
(P8) After Chlorocresol + Drying	< LOD [e]	0.13

[1] Mean *Enterobacteriaceae* counts from floor swabs from all 12 pens sampled on 2-3 occasions.

[a, b, c, d] Protocols sharing the same superscript are not significantly different ($p < 0.05$).

[e] LOD – Limit of Detection.

[f] N/A - indicates not applicable, as data from 'Before Power Wash' were used as a covariate in the analysis.

Table 4.4.5. Serotypes, antimicrobial resistance (AMR) profiles and biofilm forming ability of *Salmonella* isolates recovered after different cleaning and disinfection protocols were applied in 12 lairage pens in a commercial pig abattoir

Cleaning and Disinfection Protocols [a]	*Salmonella* Serotype (No. per Serotype)	AMR Profiles (No. per Serotype) [b]	Biofilm Category [c] (No. per Serotype)	
			15ºC	37ºC
After QAC Disinfectant	*S.* 4,[5],12:i:- (6)	AMP STR TET (3); STR TET (2)	None (1); Weak (2)	None (3)
	S. Brandenburg (2)	No Resistance (2)	- [d]	-
	S. Bredeney (1)	STR SXT (1)	-	-
	S. Derby (5)	No Resistance (4); STR (1)	-	-
	S. Panama (5)	AMP STR SXT (1); AMP STR SXT TET (4)	-	-
	S. Typhimurium (26)	No Resistance (2); AMP CHL STR (1); AMP CHL STR TET (2); AMP GEN STR TET (1); AMP STR TET (3); STR (16); STR TET (1)	None (2); Weak (2); Moderate (8); Strong (2)	None (2); Weak (2); Moderate (11)
After Chlorocresol Disinfectant	*S.* 4,[5],12:i:- (2)	No Resistance (2)	None (2)	None (2)
	S. Bredeney (1)	No Resistance (1)	Weak (1)	Weak (1)
	S. Derby (8)	No Resistance (5); AMP (1); AMP SXT (2)	None (6)	None (5); Weak (1)
	S. Panama (2)	No Resistance (2)	None (2)	None (1); Weak (1)
After Detergent	*S.* 4,[5],12:i:- (19)	No Resistance (2); AMP STR TET (12); STR TET (3); TET (1)	Moderate (1)	Weak (1)
	S. Brandenburg (7)	No Resistance (4); AMP STR SXT TET (1); STR (1); TET (1)	-	-
	S. Bredeney (1)	No Resistance (1)	-	-
	S. Derby (13)	No Resistance (6); AMP FOX STR TET (3); AMP STR (1); AMP STR SXT TET(1); STR (2)	-	-
	S. Panama (5)	AMP STR SXT (1); AMP STR SXT TET (4)	-	-
	S. Typhimurium (14)	AMP CHL STR (2); AMP CHL STR TET (3); AMP GEN STR TET (1); AMP STR SXT TET (1); AMP STR TET (4); STR (2); STR TET (1)	-	-
After Detergent + QAC Disinfectant	*S.* 4,[5],12:i:- (8)	AMP STR TET (4); STR (1); STR TET (3)	None (4); Weak (1)	None (2); Weak (2); Moderate (1)
	S. Bredeney (1)	No Resistance (1)	Moderate (1)	Moderate (1)
	S. Derby (1)	No Resistance (1)	-	-
	S. Typhimurium (10)	No Resistance (1); AMP GEN STR TET (1); AMP STR SXT (1); AMP STR SXT TET (1); AMP STR TET (3); STR (3)	None (3); Weak (5); Moderate (1)	None (1); Weak (5); Moderate (3)
After Detergent + Chlorocresol Disinfectant	*S.* 4,[5],12:i:- (1)	AMP STR SXT TET (1)	None (1)	None (1)
After QAC + Drying	*S.* 4,[5],12:i:- (1)	AMP STR TET (1)	None (1)	None (1)
	S. Brandenburg (1)	STR TET (1)	Moderate (1)	Moderate (1)

[a] *Salmonella* was not recovered from any pen After Chlorocresol + Drying protocol.

[b] Ampicillin (AMP), cefoxitin (FOX), chloramphenicol (CHL), gentamicin (GEN), streptomycin (STR), tetracycline (TET), and trimethoprim/sulfamethoxazole (SXT).

[c] Isolates were classified on the basis of biofilm formation as follows: None (OD isolate </= OD cut-off), Weak (OD cut-off < OD isolate </= 2 x OD cut-off), Moderate (2 x OD cut-off < OD isolate </= 4 x OD cut-off), and Strong (4 x OD cut-off < OD isolate). The OD cut-off was defined as three standard deviations above the mean OD_{595nm} of the negative control wells. Biofilm formation was only performed on selected *Salmonella* isolates, i.e. those with unique AMR profiles, selected serotypes, and/or those that were recovered after cleaning with the disinfectants alone, after cleaning with detergent plus the disinfectants, and after drying. This is the reason why numbers presented in the two columns do not add up to the total number of isolates found.

[d] '-' indicates that biofilm formation work was not carried out as per above.

CHAPTER 5: The efficacy of disinfectant misting in the lairage of a pig abattoir to reduce *Salmonella* and *Enterobacteriaceae* on pigs before slaughter

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5.1 Abstract

Water misting/showers are used in abattoir lairages to improve meat quality, and to cool and calm pigs after transport and during hot weather. One novel approach, which has not been investigated to date, is to add a disinfectant to the misting water as a means of topically reducing *Salmonella* on pigs prior to slaughter, thereby potentially controlling this organism in the abattoir. The objective of this study was therefore to evaluate misting with water or with Virkon® S (an approved disinfectant for use in the presence of animals), for their ability to topically reduce *Salmonella* on high seroprevalence pig herds before stunning and to reduce *Enterobacteriaceae*.

Three experimental groups were investigated: control group (i.e., no misting); water group (misting with cold, 15-17 ºC, water, herein referred to as water); and a disinfectant group (misting with 0.5% Virkon® S). Each group was randomly assigned to 3 lairage pens that were separated by a non-trial pen. As pigs entered the abattoir, each animal was swabbed along its back before being allocated to its experimental group. After 30 minutes of misting with water or disinfectant, pigs were moved to the stunning area, where each pig was again swabbed, as above. Swabs were analyzed for the presence of *Salmonella* and enumeration of *Enterobacteriaceae*.

Before misting, *Salmonella* prevalence was 79.0%, 72.1% and 83.6% for the control, water and disinfectant groups, respectively. After misting, the *Salmonella* prevalence increased to 94.3%; whereas for the disinfectant group, the prevalence increased marginally to 85.9%. No change in *Salmonella* prevalence was detected for the control group. In line with the *Salmonella* results, no significant differences were observed in *Enterobacteriaceae* counts in the control group at either time point (4.37 and 5.01 $\log_{10}$ CFU/cm^2, respectively) or in the disinfectant group before and after misting (4.02 and 4.26 $\log_{10}$ CFU/cm^2, respectively). However, a 2.3 $\log_{10}$ CFU/cm^2 increase in *Enterobacteriaceae* was recorded for the water group after misting as compared to before misting ($p < 0.05$).

Since misting with water alone increased topical *Salmonella* contamination on pigs before slaughter, a risk assessment based on known *Salmonella* data, meat quality and welfare is recommended to determine whether its use is justifiable. On the other hand, the findings from this study suggest that misting with Virkon® S at 0.5% could have a role in topical antisepsis for pigs contaminated with *Salmonella* prior to slaughter and as such this warrants further investigation.

Keywords: Lairage; Misting; Virkon® S; Antisepsis; Topical decontamination

5.2 Introduction

Holding pigs in lairage pens before slaughter, presents challenges for *Salmonella* control. Numerous studies have shown that lairage pens are highly contaminated with *Salmonella,* which not only poses a risk to incoming naïve pigs but also for cross contamination along the slaughter line (Boughton et al., 2007; Duggan et al., 2010; Mannion et al., 2012; Rostango et al., 2003; Swanenburg et al., 2001; Walia et al., 2016). One approach to limit *Salmonella* contamination of pigs is to use misting devices or sprinklers already present in the lairage. During lairage holding, these devices mist or shower pigs with water, which aims to cool and calm the animals after transport, and especially during hot weather. Primarily, misting/showering is used as a means of reducing stress in order to improve meat quality with an added benefit of removing gross fecal matter on the animal before slaughter (Warriss, 2003). As a result, misting pigs may offer a means of topically reducing *Salmonella* on live pigs prior to slaughter and in the lairage environment, and as such, may be a potential strategy for *Salmonella* control in the abattoir, especially if a disinfectant can be added to the misting water. This is a novel approach which has not been investigated to date, although topical antisepsis, at weaning, of pigs previously exposed to shedding dams has proved successful in reducing subsequent *Salmonella* shedding (Patchanee et al., 2007). Previous research in cattle has shown that washing hides immediately before slaughter with water or bromide compounds can reduce carcass contamination with *Escherichia coli* O157:H7 (Byrne et al., 2000; Schmidt et al., 2012). Given that potable hot water is the only method currently approved for decontaminating pig carcasses in the EU (Regulation (EC) No 853/2004, 2004), and that carcass contamination rates in Ireland have still not declined below the 20% level found in the EU baseline survey (DAFM, personal communication; European Food Safety Authority, 2008), disinfectant misting is another possible approach to topically decontaminate infected pigs prior to slaughter. This novel misting strategy could complement the existing decontamination activities that occur after stunning (i.e., logistical slaughter, scalding, singeing), in the overall hurdle approach to control *Salmonella* in the abattoir.

Compounds such as chlorine and organic acids may be suitable for addition to the misting water, as they are effective in reducing *Salmonella* on pig skin, *in vitro,* when added to water (Kich et al., 2011). However, the challenge with topical antisepsis of pigs prior to slaughter is to ensure the use of compounds that are registered for use in

the presence of live animals. Virkon® S is one such product (Chemours, 2015; Antec International Ltd, personal communication) and was chosen for the present study. It is a commercially available broad-spectrum disinfectant that is widely used against microorganisms, including *Salmonella,* in the pig and poultry industry via boot dips, cold and thermal fogging, and misting or aerial spraying (Block, 2001). It is a stable oxidizing agent (a peroxymonosulphate) that generally denatures proteins, disrupts cell wall permeability, and oxidizes sulphydryl and sulphur bonds in proteins, enzymes, and other metabolites, ultimately leading to cell lysis and death (Block, 2001; Dunowska et al., 2005).

Since the recommended minimum contact time for Virkon® S against *Salmonella* is 10 minutes, it was hypothesized that 30 minutes of constant misting with the disinfectant, at the recommended dilution rate for use in the presence of live animals, would reduce the prevalence of *Salmonella*, topically, in pig herds with high *Salmonella* seroprevalence (i.e., > 80% *Salmonella* seroprevalence, as determined by the Irish National Pig *Salmonella* Control Programme, NPSCP). Therefore, the objectives of this study were to evaluate misting with water alone or with water containing Virkon® S disinfectant for their ability to: (1) topically reduce *Salmonella* on high seroprevalence pigs prior to slaughter, and (2) reduce *Enterobacteriaceae,* used as a measure of overall hygiene, both topically on the pigs as well as in the lairage environment.

5.3 Materials and Methods

5.3.1 In *Vitro* Pig Skin Tests

Prior to conducting the trial on live pigs in the lairage, laboratory tests were conducted on pig skin, *in vitro*, to determine the efficacy of the Virkon® S disinfectant (a blend of potassium peroxymonosulfate, sulfamic acid, and sodium chloride; Antec International Limited, Sudbury, Suffolk, UK) in reducing *Salmonella* based on the method used by Kich et al. (2011) with modifications. Briefly, 18 pig skin samples, each taken from the neck of pig carcasses before chilling, were obtained from the study abattoir and each was cut uniformly to measure 18 cm x 10 cm. Skin samples were artificially inoculated with each of three suspensions of nalidixic acid resistant *Salmonella* Typhimurium containing 10^4, 10^5, or 10^6 CFU/mL, with 6 skin samples used for each inoculum. Briefly, the nalidixic acid resistant *S.* Typhimurium strain was

grown overnight on plate count agar (PCA, Oxoid Limited, Hampshire, UK), and a single colony was inoculated into 90 mL of tryptone soya broth (TSB, Oxoid Limited), incubated overnight at 37 ºC and then re-suspended in 10 mL of maximum recovery diluent (MRD, Oxoid Limited) to achieve suspensions containing 10^4, 10^5, 10^6 CFU/mL, respectively. Spread plate counts were performed on PCA to confirm the *Salmonella* concentration in each inoculum. A volume of 1 mL of *Salmonella* suspension was pipetted onto each skin sample and spread uniformly using a plate spreader. After 2 hours, at room temperature, each of the skin samples were swabbed with sterile 100 cm^2 sponges pre-moistened with MRD (Technical Services Consultants Ltd, Lancashire, UK) before being allocated to either a control group (no treatment), water group, or disinfectant group (i.e., 2 skin samples per group, per inoculum). A fine spray of either water or 0.5% Virkon® S disinfectant was applied to the surface of each skin sample in the water and disinfectant groups, respectively at 1 minute intervals (i.e., 1 minute spraying, 1 minute not spraying) for 30 minutes, mimicking misting in the lairage as far as possible. After spraying, each skin sample was again swabbed, as above, using the MRD sponges for the water and control groups or a 50 cm^2 sponge pre-moistened with neutralizing buffer (Technical Service Consultants Ltd) for the disinfectant group. Each swab was homogenized in 90 mL of buffered peptone water (BPW; Oxoid Limited), in a stomacher for 2 minutes. One hundred microliters of the homogenate was spread-plated on xylose lysine deoxycholate (XLD; Oxoid Limited) agar containing 30 µL/mL nalidixic acid (Sigma-Aldrich Ireland Limited) made up in 100% chloroform (Sigma-Aldrich Ireland Limited), in order to enumerate the nalidixic acid resistant *Salmonella*. The plates were incubated at 37 ºC for 24 hours.

5.3.2 Lairage Trial

5.3.2.1 Experimental Design

One commercial pig abattoir in the Republic of Ireland participated in this study. This abattoir routinely operates a Monday to Friday schedule, slaughtering approximately 2000 pigs per day from herds across the country. The lairage area (Figure 5.3.1) consisted of 12 main pens with solid concrete floors and walls. Each pen is 2.05 m x 14.95 m, with a capacity for holding 65 pigs during the day and 45 pigs overnight. A 12.7 mm diameter pipe hangs above the length of each pen and provides a mist of cold (15-17 ºC) water (herein referred to as water) at a pressure of 2 bars (29

psi), delivering 1.4 L of water per minute over the entire lairage pen. One dosatron (0.2% to 2% dose rate; Hingerose Limited, Northamptonshire, UK) was fixed to the water pipes in each of the lairage pens 3, 6 and 9 (Figure 5.3.1) according to the manufacturer instructions, in order to facilitate disinfectant addition to the misting water.

The experimental groups were as follows: (1) control group, in which no misting was performed; (2) water misting; and (3) disinfectant misting, in which misting with 0.5% Virkon® S was performed using a dosatron for Virkon® S addition. Pig herds with a *Salmonella* seroprevalence greater than 80% (based on data extracted from the Irish NPSCP) were used in the study.

The 3 experimental groups were randomly assigned to 3 lairage pens, ensuring that each pen was separated by one non-trial pen (Figure 5.3.1). As the pigs entered the lairage area of the abattoir each animal was swabbed with one sterile MRD sponge, as used for the *in vitro* experiment outlined in Section 5.3.1, before being allocated to either the control, water or disinfectant group. The average length (from head to tail) of a finisher pig at market weight was determined to be ~160 cm. Therefore, each swab covered an area of ~10 cm x 160 cm along the length of each pig. In addition, 3 swabs from each of the trial lairage pens (2 floor swabs and 1 wall swab, each covering an area of 40 cm x 40 cm) were taken prior to entry of the pigs, also using the MRD sponges. Once pigs were in the lairage pens, the water or disinfectant misting was applied for 30 minutes continuously. Afterwards, the misting devices were switched off and as the pigs were moved into the stunning area, but prior to being stunned, each pig in the water or control group was again swabbed with sterile MRD sponges, as outlined above, or with sponges containing neutralizing buffer for the disinfectant misting group, as outline in the *in vitro* experiment in Section 5.3.1. Three additional swabs of each of the trial lairage pens (2 floor and 1 wall) were collected for each group after the pigs had exited, either using the sterile sponges with MRD for the control and water misting groups or the sponges with neutralizing buffer for the disinfectant misting group, as above. All swabs were collected aseptically and were kept at 4 ºC and processed within 24 hours.

The entire lairage experiment as outlined above was performed in triplicate, i.e., on 3 separate days with 3 different pig herds. Different pens were used each day to avoid any potential pen effect.

5.3.2.2 Microbiological Analysis of Lairage Trial Samples

Each sponge was homogenized in 90 mL of BPW in a stomacher for 2 minutes. This homogenate was then tested for the presence of *Salmonella* in accordance with International Organization for Standardization (ISO) 6579:2007 (Amendment 1: Annex D) method (International Organization for Standardization, 2007). All media were obtained from Oxoid Limited. Briefly, BPW homogenates were incubated at 37 ºC for 19 hours, after which 100 µL of each sample was inoculated onto modified semi-solid rappaport-vassiliadis (MSRV) agar plates and incubated at 42 ºC for 24 hours. If the MSRV plate was negative, it was incubated for a further 24 hours. Presumptive *Salmonella* growth was then streaked onto XLD and brilliant green agar (BGA) plates and incubated at 37 ºC for 24 hours. Suspect colonies from XLD or BGA plates were then streaked onto PCA plates, and incubated at 37 ºC for 24 hours. Urea agar slants and *Salmonella* chromogenic agar plates were then inoculated with colonies from the PCA plates and incubated at 37 ºC for 24 hours. Serological confirmation of colonies from the PCA plates was performed using a *Salmonella* latex agglutination kit (Oxoid).

In addition, *Enterobacteriaceae* counts were obtained from 5 pigs per group, both before and after treatment and from 2 floor swabs from each trial lairage pen before and after treatment, as follows: 10-fold serial dilutions of the BPW homogenates were performed in MRD and appropriate dilutions pour-plated on violet red bile glucose agar (VRBGA, Oxoid). Plates were overlaid with VRBGA and incubated at 37 ºC for 24 hours. The limit of detection was 10 CFU/cm^2.

5.3.2.3 Serotyping and Antimicrobial Resistance Determination of Salmonella Isolates

All presumptive *Salmonella* isolates recovered from both the pigs and the pens before and after misting were first screened using a real-time polymerase chain reaction (PCR) assay for the identification and differentiation of *Salmonella enterica* serotype Typhimurium and *S.* 4,[5],12:i:- as described by Prendergast et al. (2013). Isolates not identified as *S.* Typhimurium or its monophasic variant were then serotyped according to the White-Kauffmann-Le Minor scheme (Grimont & Weill, 2007) using commercial antisera (Pro-Lab Diagnostics, Cheshire, UK; SIFIN Institute, Berlin, Germany; Statens Serum Institute, Copenhagen, Denmark).

The antimicrobial resistance (AMR) profile of each *Salmonella* isolate was determined using the Sensititre™ Gram negative NARMS plate (Thermo Scientific, Serosep Ltd, Limerick, Ireland). The following antimicrobials were tested: amoxicillin-

clavulanic acid (AUG), ampicillin (AMP), azithromycin (AZI), cefoxitin (FOX), ceftiofur (XNL), ceftriaxone (AXO), chloramphenicol (CHL), ciprofloxacin (CIP), gentamicin (GEN), nalidixic acid (NAL), streptomycin (STR), sulfisoxazole (FIS), tetracycline (TET), and trimethoprim/sulfamethoxazole (SXT). Minimal inhibitory concentrations (MICs) were interpreted using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) epidemiological cut-off values.

5.3.3 Statistical Analysis

For *Salmonella* prevalence, the binary data were analyzed using the GLIMMIX procedure in Statistical Analyses System (SAS, V9.3, 2011). The differences between the groups were evaluated using the Tukey-Kramer least squares means adjustment for multiple comparisons, with the proportions of positive results in each of the groups before treatment (i.e., 'Control Before', 'Water Before Misting', 'Disinfectant Before Misting') used as a covariate in the model.

For *Enterobacteriaceae*, counts were log-transformed after which the data were analyzed as normally distributed data using the GLIMMIX procedure in SAS, with date as a blocking factor. Tukey-Kramer grouping for treatment least square means was performed for differences between group means.

Residual checks were made to ensure that the assumptions of the analyses were met. For all analyses, statistical significance was established at $\alpha = 0.05$.

5.4 Results

5.4.1 Enumeration of *Salmonella* from Laboratory Pig Skin Samples, *In Vitro*

Mean counts of nalidixic acid resistant *S.* Typhimurium obtained from the pig skin samples before and after treatment are shown in Figure 5.4.1. The *Salmonella* counts for the control (untreated) group remained the same for the three inocula used. After spraying with disinfectant, a 1.8, 1.3 and 1.7 $\log_{10}$ CFU/cm^2 reduction of the nalidixic acid resistant *S.* Typhimurium was achieved, at the three different inocula, respectively. This is compared to spraying with water where 1.9 and 1.0 $\log_{10}$ CFU/cm^2 reductions were achieved at the higher inocula, while a 0.9 $\log_{10}$ CFU/cm^2 increase was observed with the lower inoculum.

5.4.2 *Salmonella* Prevalence from Lairage Trial Samples

Mean prevalence of *Salmonella* before and after misting with either water or disinfectant or no treatment (i.e. control) are shown in Figure 5.4.2. In total 124, 122 and 128 pigs were swabbed for the control, water and disinfectant groups, respectively. Before pigs were allocated to the lairage pens (i.e. before misting) the *Salmonella* prevalence for each group, was 79.0% (98/124), 72.1% (88/122) and 83.6% (107/128), respectively (Figure 5.4.2). After misting, the *Salmonella* prevalence increased by 30.7% in the water group to 94.3% (115/122), whereas for the disinfectant group, the prevalence increased by a marginal 2.7% to 85.9% (110/128, Figure 5.4.2). No change in *Salmonella* prevalence was detected for the control group (Figure 5.4.2).

When the data were analysed statistically, the disinfectant was better at preventing an increase in the probability of detecting *Salmonella* on the pig skin than misting with water alone (84.9% versus 96.3%, respectively, $p < 0.05$, Table 5.4.1). Likewise, not misting (i.e., control group) was also better at preventing an increase in the probability of detecting *Salmonella* than misting with water alone (80.5% versus 96.3%, respectively, $p < 0.05$, Table 5.4.1). On the other hand, no significant differences were observed between not misting versus misting with the disinfectant on the probability of detecting *Salmonella* (80.5% versus 84.9%, respectively, $p > 0.05$, Table 5.4.1).

In terms of *Salmonella* prevalence within the trial lairage pens, before pigs were allocated to the 3 treatment pens, all pen swabs were *Salmonella*-positive for each treatment group. After water misting all pen swabs were *Salmonella*-positive (100%), while 89% (8/9) of swabs taken after disinfectant misting were *Salmonella*-positive and 7/9 (78%) of swabs taken from the control pen were *Salmonella*-positive.

5.4.3 Enumeration of *Enterobacteriaceae* from Lairage Trial Samples

The overall effect of 'no misting', water misting or disinfectant misting on topical *Enterobacteriaceae* counts on the live pigs is presented in Table 5.4.2. Similar to the *Salmonella* results, no significant differences were seen in *Enterobacteriaceae* counts for pigs in the control group before compared to after 'no misting' or in the disinfectant group before and after misting (Table 5.4.2). However, a 2.3 $\log_{10}$ CFU/cm^2 increase in *Enterobacteriaceae* was observed for the water group after misting as compared to before misting ($p < 0.05$, Table 5.4.2).

In terms of mean *Enterobacteriaceae* counts from the environmental swabs of the lairage pens, no differences were observed in the control and water misting groups after versus before treatment (6.04 versus 5.93 log_{10} CFU/cm^2 and 5.80 versus 5.67 log_{10} CFU/cm^2, respectively). On the other hand, a slight decline in *Enterobacteriaceae* counts was observed in the disinfectant group after misting compared to before misting (5.60 versus 5.95 log_{10} CFU/cm^2).

5.4.4 Serotyping and Antimicrobial Resistance Profiling of *Salmonella* Isolates from Lairage Trial Samples

The *Salmonella* serotypes and antimicrobial resistance (AMR) profiles of the 90 isolates recovered from the pigs before and after misting with water, disinfectant or no misting are detailed in Table 5.4.3. Overall, two serotypes, *S.* 4,[5],12:i:-, and *S.* Typhimurium, were detected on the pigs; and the same serotypes were also isolated from the trial lairage pens. Of the AMR profiles found for the pig isolates, 12 different profiles were detected for the *S.* 4,[5],12:i:- isolates, while 6 profiles were detected for the *S.* Typhimurium. Most of the isolates were multidrug resistant, demonstrating resistance to ampicillin, chloramphenicol, streptomycin, trimethoprim/sulfamethoxazole and tetracycline. However, a relatively small proportion of the 90 isolates showed additional resistance to: ceftiofur (1 isolate), ciprofloxacin (1 isolate), and gentamicin (15 isolates). Generally, after misting, more AMR profiles emerged on account of an increase in the recovery of *Salmonella* isolates. In addition, AMR profiles tended to include gentamicin as compared to before misting.

5.5 Discussion

In the present study, a disinfectant in the form of Virkon® S, added to the misting water at a commercial abattoir, was investigated as a novel means of reducing both environmental and skin contamination of *Salmonella* from high prevalence pig herds prior to slaughter.

Results from preliminary *in vitro* work demonstrated that application of Virkon® S, at 1 minute intervals for 30 minutes, was successful in reducing the level of *Salmonella* contamination on pig skin, demonstrating the potential of topical antisepsis strategies. This finding is similar to that of Kich et al. (2011) who found that 10 seconds of disinfectant treatment was effective at reducing *Salmonella* on artificially

contaminated pig skin, albeit the agents used (chlorine and organic acids) differed from those employed in the present study.

On the other hand, when applied in the lairage of a commercial abattoir, our results showed that misting with Virkon® S for 30 minutes on high *Salmonella* prevalence pig herds had little effect on reducing the organism, topically, prior to slaughter. However, *Salmonella* prevalence remained stable, as it did in the control group, which had no misting throughout the trial, while interestingly, misting with water alone increased the prevalence of *Salmonella*. This finding is in contrast to that of a study conducted in cattle where pressure washing with water for 3 minutes, although different from the low pressure longer duration misting used in the current study, significantly reduced *E. coli* O157:H7 from swabbed areas before animals were slaughtered (Byrne et al., 2000).

To our knowledge, this is the first study to investigate the effect of water misting in the lairage on *Salmonella* prevalence on live pigs. Interestingly, our results suggest that low pressure misting with water facilitates acquisition of *Salmonella* by pigs, probably from both the environment and from other pigs, during lairage holding. It is likely that the water droplets aid *Salmonella* dispersal or that the humid environment created by misting favours growth of the organism. Moreover, although no reductions in *Salmonella* prevalence were observed in the control or disinfectant groups, the prevalence remained constant, suggesting that misting with Virkon® S or not misting at all were better in terms of limiting *Salmonella* contamination than the current practice of misting animals with water. However, as the *Salmonella* prevalence on the animals in this study was extremely high, i.e., > 80%, the biological significance of these results in relation to *Salmonella* control is questionable and necessitates additional research. For example, studies should be performed in herds with a lower *Salmonella* prevalence (but still high enough to warrant the use of control measures), as it is possible that the effects of disinfectant misting might be more pronounced in these, as the topical *Salmonella* load would be lower. In addition, had *Salmonella* been enumerated on the animals, we may have seen greater effects on *Salmonella* reduction. Future work should also investigate carcass contamination post-slaughter, as it was outside the scope of the present study to investigate the effects of disinfectant misting on this. Furthermore, the fact that we observed a decrease in *Salmonella* contamination, *in vitro*, under controlled laboratory conditions with the disinfectant, but not on live pigs may be because *Salmonella* counts were performed *in vitro* but not *in vivo*. Nonetheless, our

lack of correlation between *in vitro* and *in vivo* findings is similar to findings of an earlier study by Mies et al. (2004). The authors found that spray wash treatments of water, lactic acid, or chlorine on cattle pre-slaughter were unsuccessful in decreasing *Salmonella,* whereas when applied at higher concentrations to cattle hides, *in vitro*, a decrease in *Salmonella* counts was observed. This highlights the importance of field trials when evaluating any *Salmonella* control measure for use in the abattoir. Additionally, since all trial pens contained at least one *Salmonella*-positive sample before the trial commenced and after pigs were removed from the pens, this suggests that misting with or without disinfectant is not effective in reducing *Salmonella* in the lairage pens.

Although *Salmonella* counts were not performed on the animals, as outlined above, we performed *Enterobacteriaceae* counts as an indicator of the overall contamination of the pigs and lairage pens by enteric organisms. These data allowed for an indirect estimate of the effect of the treatments on *Salmonella* skin and pen contamination. In agreement with the *Salmonella* results, topical *Enterobacteriaceae* counts increased after water misting but not with disinfectant misting or when no treatment was applied. This, together with the fact that there was essentially no impact on *Enterobacteriaceae* counts in the lairage pens, suggests that water misting, as currently practiced at commercial abattoirs, will not reduce the level of *Enterobacteriaceae,* either in the lairage pens or on the animal, at least in high prevalence herds, and in fact has the opposite effect where the latter was concerned. This finding is supported by several studies, two of which were conducted by the authors, which found that power washing with water alone did not reduce *Enterobacteriaceae* counts either in lairage pens (Walia et al., 2016) or in transport trucks after unloading pigs in the abattoir (Mannion et al., 2008). Additionally, Mies et al. (2004) showed that spray washing water on cattle increased aerobic plate counts (APC), coliforms or *E. coli* before slaughter as compared to using a lactic acid solution, or chlorine. Likewise, Bell (1997) and Ellerboek et al. (1993) showed that spray washing water on cattle and sheep carcasses, respectively, was ineffective at reducing APC and *E. coli* contamination. While there were differences in the studies above as compared to our study (i.e., high-pressure water, cattle and sheep, carcasses, lactic acid and chlorine), they nonetheless support our finding that washing with water alone does not decrease *Enterobacteriaceae* counts.

In the abattoir used in this study, pigs are normally misted with water for between 30 minutes and 2 hours, depending on the waiting time to stunning. Although 30 minutes of intermittently misting or showering pigs with water, is generally accepted as a means to cool, calm, and reduce aggression of pigs in lairage pens (Faucitano, 2010; Warriss, 2003; Weeding et al., 1993), there is no agreement on the optimum duration of misting. The present study was conducted in a commercial abattoir where pigs are rested for a minimum of 30 minutes in the lairage pens prior to slaughter. Therefore, 30 minutes was chosen as the contact time for the water and Virkon® S misting groups and was standardised across replicates. Earlier research has also shown that removing organic matter, albeit on non-skin surfaces, prior to disinfection, increases the efficacy of various disinfectants, including oxidizing agents, against *Salmonella* (Gradel et al., 2004; Stringfellow et al., 2009). Therefore, removing visible organic matter on pigs prior to entry into the lairage pens, which is not current practice in the study abattoir, and increasing the disinfectant contact time, as well as examining different disinfection agents are possibilities for future research. It is also possible that the water pressure and flow rate exerted from the misting device used in the present study was inadequate for decontamination. While the animals looked visibly clean, they still had a high prevalence of *Salmonella*. As such, increasing the pressure and flow rate of the misting device to perhaps a minimum of 100 psi and 7.5 L per minute, respectively, as recommended by Pordesimo et al. (2002) for the reduction of microbial contamination on meat and carcasses may reduce *Salmonella* contamination. However, any adverse effects on animal welfare and/or meat quality would require prior investigation.

5.6 Conclusions

Results from the present study show, for the first time, that misting pigs in the lairage with water alone, as is the current practice in a number of commercial abattoirs, increases topical *Salmonella* contamination prior to slaughter. This suggests that a risk assessment should therefore be completed in abattoirs based on known *Salmonella* data, meat quality and welfare considerations as to whether its use should be avoided for high *Salmonella* prevalence herds. On the other hand, the findings from this study suggest that the addition of Virkon® S to the misting water can limit this contamination and may therefore have a role in topical antisepsis of pigs contaminated with *Salmonella* prior to slaughter in abattoirs that wish to use misting.

5.7 Acknowledgments

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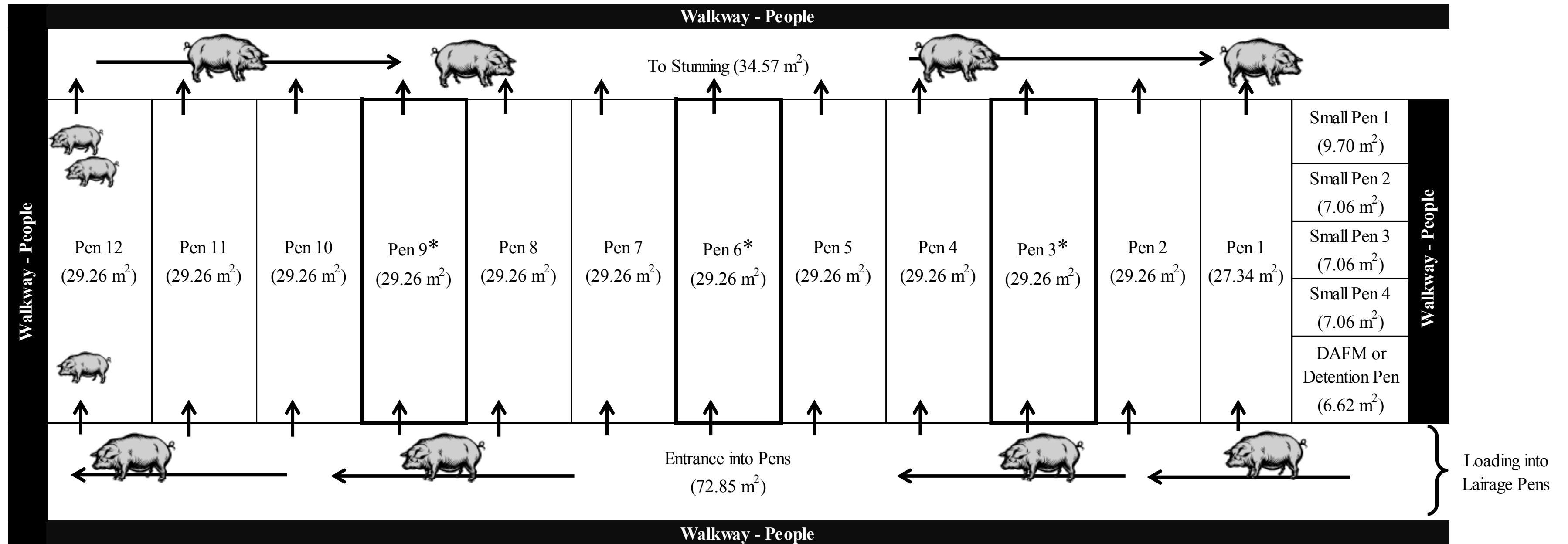
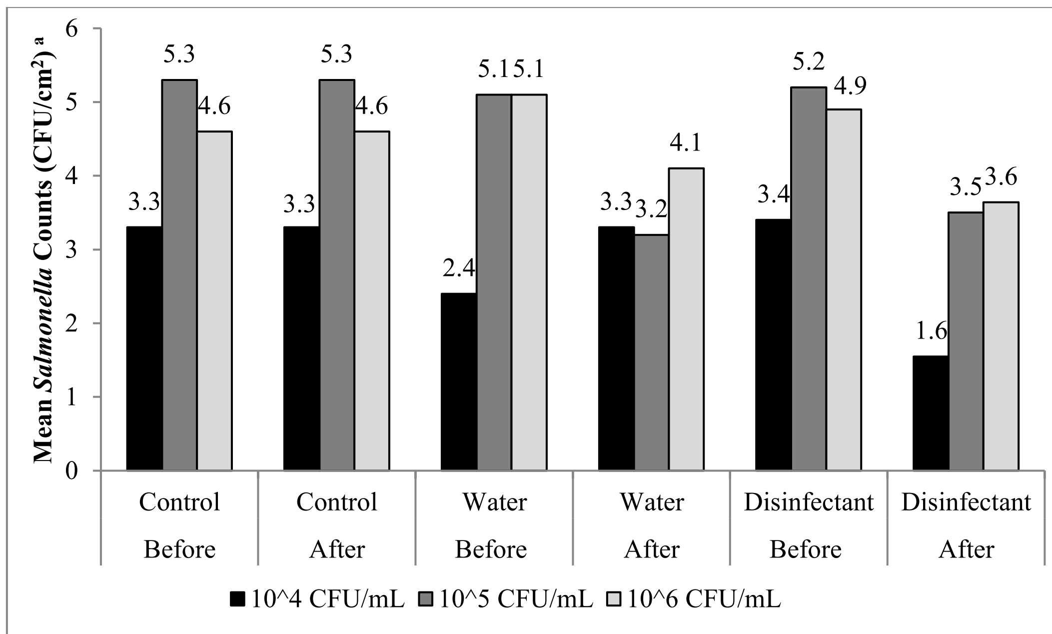


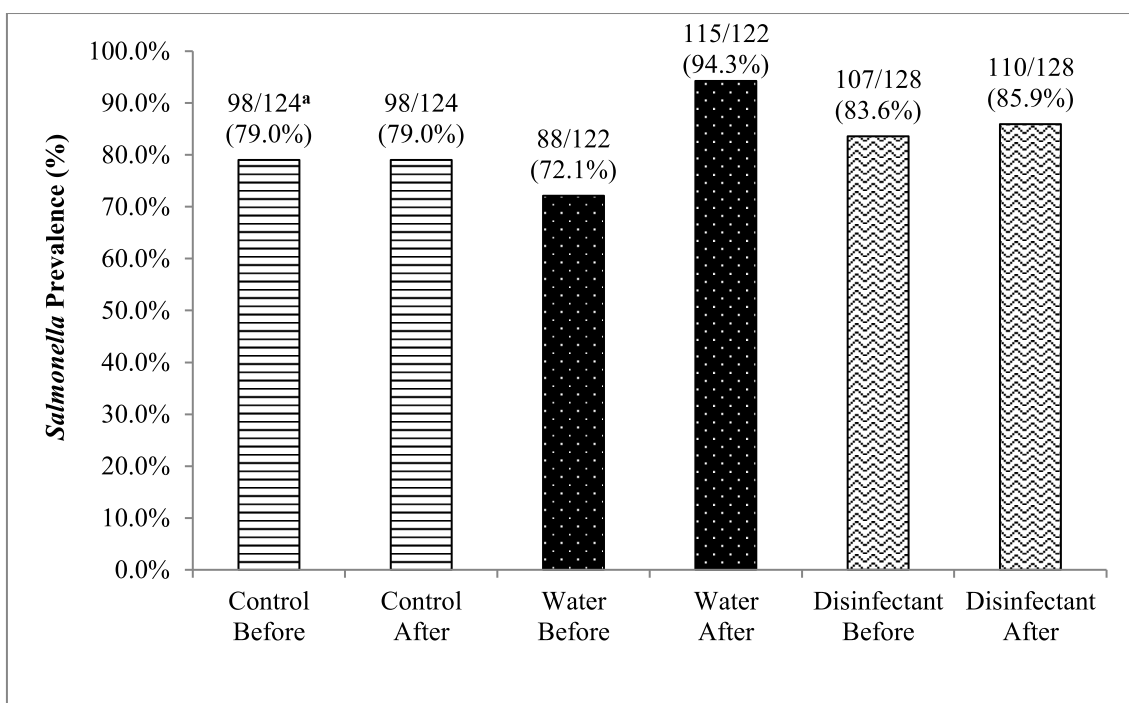
*Lairage pens 3, 6 and 9 were used in the experiment. One dosatron was fixed to the water pipes in each of these three pens according to the manufacturer instructions, in order to facilitate disinfectant (Virkon ® S) addition to the misting water. Each pen was used on a different sampling day to avoid a potential pen effect

Figure 5.3.1. Lairage pen set-up.



[a] Mean *Salmonella* counts from 2 skins samples per group, per inoculated concentration. Each skin sample was 18 cm x 10 cm.

Figure 5.4.1. Mean *Salmonella* counts (log_{10} CFU/cm^2) from samples of pig skin artificially inoculated with three different concentrations of *Salmonella* Typhimurium. Counts are shown before and after spraying with either water or disinfectant, or no treatment (i.e., control group)



[a] *Salmonella* prevalence was calculated from No. *Salmonella*-positive swabs/No. swabs taken.

Figure 5.4.2. Prevalence of *Salmonella* from live pigs swabbed before and after misting with either water or disinfectant, or no misting (i.e., control group) in a commercial pig abattoir

Table 5.4.1. Effect of misting with water or disinfectant, or no misting on the probability of detecting *Salmonella* on the skin of pigs in a commercial pig abattoir

Group	Probability (%)[a]	p-value
Control After[b] vs. Disinfectant After Misting[c]	80.5 vs. 84.9	0.65
Control After vs. Water After Misting[d]	80.5 vs. 96.3	0.001
Disinfectant After Misting vs. Water After Misting	84.9 vs. 96.3	0.01

[a] Values presented are the probability of detecting *Salmonella* as calculated from mean *Salmonella* prevalence data. The values for the ‘before’ groups were used to construct a baseline for the ‘after’ groups, by using the proportions of positive results in each of the groups befodre treatment as covariates in the statistical model.

[b] sem for ‘control after’ group was 0.037.

[c] sem for ‘disinfectant after misting’ group was 0.033.

[d] sem for ‘water after misting’ group was 0.016.

Table 5.4.2. The effect of no misting, water misting or disinfectant misting on topical *Enterobacteriaceae* counts from live pigs in a commercial pig abattoir

Group	Mean *Enterobacteriaceae* counts[c] (Log_{10} CFU/cm^2)
Control Before	4.37^{ab}
Control After	5.01^{ab}
Water Before Misting	3.31^{b}
Water After Misting	5.62^{a}
Disinfectant Before Misting	4.02^{ab}
Disinfectant After Misting	4.26^{ab}
Pooled sem	0.56

[a,b] Values within a group without a common superscript are significantly different ($p < 0.05$).

[c] Mean *Enterobacteriaceae* counts from 5 pigs per group, sampled on 3 occasions.

Table 5.4.3. *Salmonella* serotypes and antimicrobial resistance (AMR) profiles of isolates recovered from pigs, before and after water misting, disinfectant misting, or no misting (i.e., control group) were applied to live pigs in a commercial pig abattoir

Group	*Salmonella* serotype (No. per serotype)	AMR profiles (No. per serotype)[a]
Control Before	*S.* 4,[5],12:i:- (5)	AMP CHL GEN STR SXT TET (1); AMP GEN STR TET (1); AMP GEN STR SXT TET (2)
	S. Typhimurium (9)	No Resistance (2); AMP CHL STR SXT TET (4); AMP STR TET (1); STR (1); SXT (1)
Control After	*S.* 4,[5],12:i:- (5)	No Resistance (1); AMP GEN STR SXT TET (1); AMP STR SXT TET (1); STR TET (2)
	S. Typhimurium (11)	AMP CHL STR SXT TET (4); AMP STR SXT TET (2); STR (5)
Water Before Misting	*S.* 4,[5],12:i:- (5)	AMP CIP STR TET (1); AMP STR TET (1); AMP STR SXT TET (3)
	S. Typhimurium (9)	AMP CHL STR SXT TET (4); AMP STR SXT TET (2); STR (2); STR SXT (1)
Water After Misting	*S.* 4,[5],12:i:- (5)	AMP CHL SXT TET (1); AMP GEN SXT TET (1); AMP GEN STR SXT TET (1); AMP; STR SXT TET (1); AMP SXT TET (1)
	S. Typhimurium (10)	No Resistance (1); AMP CHL STR SXT TET (2); AMP STR SXT TET (3); STR (4)
Disinfectant Before Misting	*S.* 4,[5],12:i:- (5)	AMP GEN STR TET (4); AMP GEN STR SXT TET (1)
	S. Typhimurium (10)	AMP CHL STR SXT TET (3); AMP STR SXT TET (2); STR (5)
Disinfectant After Misting	*S.* 4,[5],12:i:- (6)	AMP GEN STR TET (1); AMP GEN SXT TET (1) AMP GEN STR SXT TET (1); AMP STR SXT TET (2); AMP STR TET XNL (1)
	S. Typhimurium (11)	AMP CHL STR SXT TET (3); AM STR TET (2); AMP STR SXT TET (1); STR SXT (1); STR (4)

[a] Antimicrobials consisted of: ampicillin (AMP), ceftiofur (XNL), chloramphenicol (CHL), ciprofloxacin (CIP), gentamicin (GEN), streptomycin (STR), tetracycline (TET), and trimethoprim/sulfamethoxazole (SXT).

CHAPTER 6: Case study of dietary supplementation with sodium butyrate in conjunction with cleaning and disinfection of finisher pens to control *Salmonella* shedding and seroprevalence in finishing pigs

6.1 Abstract

This case study was undertaken following previous research in Chapters 2 and 4, which showed that duration of treatment and concomitant infections are key factors in the efficacy of pre-harvest *Salmonella* control measures in finishing pigs. This reflects the situation that many farmers are faced with, wherein multiple diseases are common among pigs; posing challenges for *Salmonella* control. This study investigated the efficacy of three interventions: (1) cleaning and disinfecting finisher pens with a peroxygen agent and a chlorocresol-based disinfectant followed by a drying step, and (2) supplementing finisher feed with sodium butyrate (3 kg/t) for 63 days prior to slaughter, or (3) supplementing finisher feed with sodium butyrate (3 kg/t) for 28 days prior to slaughter on *Salmonella* shedding and seroprevalence on a commercial farm with a history of high *Salmonella* seroprevalence and secondary infections.

Two trials were conducted to investigate the three interventions. In Trial A, interventions 1 and 2 above were investigated, in combination, while Trial B investigated intervention 3 alone. Trial A consisted of 80 pigs, aged 12/13 weeks at the start of the trial, housed in 4 pens (20 pigs/pen), and Trial B consisted of 73 pigs, aged 36 weeks, housed in 5 pens (6-20 pigs/pen). Pen swabs and faeces was collected from 6-10 pigs/pen on days -7, 0, 28, 52 and 63 and examined for *Salmonella* while at slaughter on days 28 and 63, blood was collected from all pigs and tested for serology. Pooled faeces were collected from each pen on the same days and examined for the presence of secondary infections such as Rotavirus, *Lawsonia intracellularis* and *Brachyspira*. When pigs reached their target slaughter weight they were transported to a commercial abattoir and carcass quality parameters were recorded.

Both trials showed infection with Rotavirus throughout the study. In Trial A, all pen swabs were *Salmonella*-positive on days 28 and 52, while only half were *Salmonella*-positive on day 63. The *Salmonella* prevalence increased from day 28, with 17.5% faecal samples being *Salmonella*-positive, to 22.5% and 30.0% on days 52 and 63, respectively. On the other hand, in Trial B, only 2% of faecal samples and 20% of pens swabs were *Salmonella*-positive on day 28. All isolates recovered from both trials were identified as *Salmonella* Typhimurium. In line with the *Salmonella* results, for Trial A, the seroprevalence at slaughter was higher at 55.0% using the 40% OD cut-off value used in the Irish *Salmonella* control programme, while for Trial B the seroprevalence was 41.1%.

Numerical improvements in live weight before slaughter and carcass weight (4.8% and 4.2% increase, respectively) were observed for pigs supplemented with sodium butyrate for 63 days rather than 28 days.

Overall, dietary supplementation with sodium butyrate for 63 days was not successful in reducing faecal shedding of *Salmonella* or *Salmonella* seroprevalence. The secondary Rotavirus infection might have resulted in the lack of effect observed with the feed additive for this trial, despite a thorough initial cleaning, disinfection and drying of the finisher pens. However, this result does not suggest that organic acid feed additives will not work when concomitant infections are present, and as such further research is necessary.

Keywords: Swine, Organic acid, Chlorocresol, Virkon® S, Pig farm

6.2 Introduction

It is generally accepted that finishing pigs are a significant source of *Salmonella* in the abattoir (Duggan et al., 2010; Argüello et al., 2013a). For this reason, numerous studies have investigated strategies to control this organism at farm-level. Common approaches, have included dietary supplementation with organic acids and/or their salts alone (Canibe et al., 2005; Creus et al., 2007; Boyen et al., 2008; De Busser et al., 2009; Visscher et al., 2009; Gebru et al., 2010; Willamil et al., 2011; Calveyra et al., 2012; Michiels et al., 2012; Ahmed et al., 2013; Argüello et al., 2013b; Lynch et al., 2016; Rasschaert et al., 2016; Walia et al., 2016; Walia et al., 2017a), or in combination with essential oils, or sole use of essential oils (Michiels et al., 2012; Ahmed et al., 2013; Rasschaert et al., 2016; Walia et al., 2017a), use of vaccines alone or in combination with organic acids (Friendship et al., 2006; Farzan and Friendship et al., 2010; De Ridder et al., 2011; Leyman et al., 2011; Argüello et al., 2013d; De Ridder et al., 2013; De Ridder et al., 2014), and appropriate cleaning and disinfection of pens combining detergent with disinfectants in addition to high pressure washing alone (Friendship et al., 2006; Mannion et al., 2007; Walia et al., 2017b).

The work presented in this study follows previous work by Lynch et al. (2016) and Walia et al. (2016) (i.e., Chapter 2), who found that dietary supplementation with sodium butyrate for 28 days decreased *Salmonella* prevalence in weaned and finisher pigs but only in the absence of a co-infection. Since these studies did not find a reduction in seroprevalence to below the cut-off used for high-risk herds in Ireland, i.e., > 50%, with the 28-day treatment duration, the present study, instead, increased the sodium butyrate supplementation period to the full finishing period of ~2 months. Moreover, earlier research found that a minimum of 7 weeks duration was successful in decreasing the seroprevalence of *Salmonella* to below the cut-off used for high-risk herds from serology-based control programmes in Europe (Creus et al., 2007; Visscher et al., 2009; Argüello et al., 2013b). As such, this strategy aimed to determine the duration of sodium butyrate that would decrease the seroprevalence to levels that would be considered low-risk in Ireland.

In addition, since concomitant diseases may impact the efficacy of sodium butyrate in controlling *Salmonella* (Walia et al., 2016; Chapter 2), laboratory diagnostic investigations were implemented in order to determine if other common infections such as Rotavirus, *Lawsonia intracellularis* and *Brachyspira*, were present on the farm and

perhaps hindering the control strategy implemented. As a result, a second intervention was also investigated in the present study. This was based on prior research by Walia et al. (2017b) (i.e., Chapter 4) who found that cleaning and disinfection of lairage pens with a chlorocresol-based disinfectant and allowing pens to dry prior to loading pigs was successful in eliminating *Salmonella.* Likewise, since oxidizing agents have been recommended to kill Rotavirus (Kitis, 2004; Chandler-Bostock and Mellits, 2015), this second intervention combined disinfectants containing both chlorocresol and an oxidizing agent with a drying step to ensure pen hygiene prior to the introduction of pigs to the finisher section. The goal of this strategy was to utilize best practices to reduce the carryover of *Salmonella* and other co-infections in pens between batches of pigs.

Overall, this case study focused on finishing pigs on a farm with high *Salmonella* seroprevalence, i.e., > 50% according to the Irish National Pig *Salmonella* Control Programme (NPSCP) and builds on the aforementioned research. It reflects the situation that many farmers are faced with, wherein multiple diseases are common among pigs; posing challenges for *Salmonella* control. This study aimed to determine the efficacy of cleaning and disinfecting finisher pens with a combination of oxidizing and chlorocresol-based disinfectants and dietary supplementation with sodium butyrate for 63 days prior to slaughter on *Salmonella* shedding and seroprevalence along with their influence on secondary infections, such as Rotavirus. The effect of these combined interventions was compared to that where pigs were supplemented with sodium butyrate for just 28 days prior to slaughter and housed in finisher pens not subjected to cleaning and disinfection.

6.3 Materials and Methods

6.3.1 Animal Ethics and Experimental Licensing

This feeding trial was performed on a commercial pig farm from July 26, 2016 to September 27, 2016. Ethical approval was obtained from the University College Dublin ethics committee and an experimental license was obtained from the Irish Department of Health and Children (number B100/2982). All animals were handled in a humane manner and were slaughtered in a regulated abattoir.

6.3.2 Experimental Procedure

6.3.2.1 Trial Farm

The trial farm used was a 180 sow farrow-to-finish farm. The finisher houses in which the trials were conducted consisted of trowbridge houses. A total of 153 finisher pigs were used (6-20 pigs per pen). Pigs were housed in pens (each pen was 4.9 m x 2.7 m) with concrete slatted floors and the temperature of each room was maintained at ~ 20 ºC. Pigs were provided with ad-libitum access to dry pelleted feed via single-spaced wet-dry feeders and to water from two nipple drinkers per pen.

This farm had a historically high *Salmonella* seroprevalence, i.e., > 50% for 2016, (data extracted from the NPSCP; Department of Agriculture Food and the Marine (DAFM), 2010), and faecal shedding of *Salmonella* Typhimurium was confirmed bacteriologically prior to commencement of the trial.

6.3.2.2 Trial Pig Groups

Finisher pigs allocated to the study were separated into Trial A and Trial B. Trial A consisted of 80 pigs housed in 4 pens with 20 pigs per pen, while Trial B consisted of 73 pigs housed in 5 pens with 6-20 pigs per pen. In Trial A pigs were 12-13 weeks old at the start of the trial, i.e., ~3 months before slaughter, while Trial B pigs were 36 weeks old, i.e., ~1 month before slaughter.

Before pigs in Trial A were moved into the trial pens (day -1), each of the 4 pens were cleaned with an alkaline detergent softener (Biosolve E, Chemours, Agrihealth, Ireland) plus a high pressure cold water rinse (herein referred to as a water rinse). This was followed by application of an oxidizing disinfectant, blend of potassium peroxymonosulfate, sulfamic acid, and sodium chloride (Virkon® S; Antec International Limited, Agrihealth, Ireland) at a dilution rate of 1% with a 10 minutes contact time followed by a water rinse. Afterwards, a chlorocresol-based disinfectant (Interkokask®, Hysolv, UK) was applied at a dilution rate of 2-3% without a subsequent rinse and left to dry overnight for ~ 12 hours. Cleaning and disinfection of Trial A pens was carried out by an independent operator specializing in cleaning and disinfecting farm pens (Farm Relief Services, Parkmore, Roscrea, Ireland).

Since Trial B pigs were already housed in pens prior to commencing the trial, pens were not subjected to cleaning and disinfection before administering the trial diet.

In addition, footbaths were placed outside each trial pen. Footbaths consisted of a 1% solution of Virkon® S which was renewed once per week or daily if heavily soiled. Footbaths were used by personnel before and after entry from all trial pens.

6.3.2.3 Treatment Diet

All pigs were fed a standard finisher diet supplemented with 3 kg per tonne sodium butyrate (Adimix®, Nutriad, Kasterlee, Belgium). The composition of the trial diet is shown in Table 6.3.1. The pigs in Trial A were fed the experimental diet for 63 days, while the pigs in trial B were fed the experimental diet for 28 days. Pigs were fasted for ~18 h prior to slaughter.

6.3.2.4 Detection of Concomitant Infections

Prior to commencing the trials, faecal samples were collected from all stages of pigs including Trial A pigs only (day -70, i.e. at weaning) for concomitant infection with Rotavirus, *Lawsonia intracellularis* and *Brachyspira.* Pooled faeces (~5 g) per pen per production stage were collected into 90 mL sterile bottles (Sarstedt, Nümbrecht, Germany) and sent to the Central Veterinary Research Laboratory (CVRL, Department of Agriculture Food and the Marine Laboratories, Backweston, Celbridge, Co. Kildare, Ireland) for analysis of Rotavirus, and to the veterinary service at Scotland's Rural College (SRUC, SAC Consulting, Bush Estate, Penicuik, Scotland) for *L. intracellularis* and *Brachyspira* diagnostics.

On days 0 (the day prior to commencing the experimental diet), 28, 52, and 63, pooled faeces (~10 g) from 10 pigs per trial pen were collected, as above, and sent to the CVRL for analysis of Rotavirus.

6.3.2.5 Faecal Sampling and Pen Swabs

On day -7 (one week before the trials commenced), day 0 (the day prior to commencing the experimental diet), day 28 (approximately mid-way through treatment for Trial A and final treatment day for Trial B), day 52 (Trial A only), and day 63 (the final treatment day for Trial A only), freshly voided faeces (~25 g) was collected into 100 mL sterile bottles (Sarstedt, Nümbrecht, Germany) individually from 6-10 pigs per pen (2 pens in Trial B contained 6 and 7 pigs, respectively; the remaining pens in Trials A and B contained more than 10 pigs per pen).

On days -7, 0, 28, 52 and 63, two pen swabs per trial pen were collected using sterile sponges pre-moistened with 10 mL Maximum Recovery Diluent (MRD, 100

cm^2, Technical Services Consultants Ltd, Lancashire, UK). Since Trial A pens were cleaned, disinfected and dried prior to entry of pigs (as discussed above), the two pen swabs per trial pen were instead collected on day 0 with sterile sponges pre-moistened with 10 mL of neutralizing buffer (50 cm^2; Technical Service Consultants Ltd, Lancashire, UK). All samples were collected and handled aseptically to avoid cross-contamination.

6.3.2.6 Clinical Observations

All trial pigs were observed closely at least once daily by the farmer and biweekly by study investigators. Pigs were observed for clinical signs of diarrhea including the number of pats of diarrhea per pen, number of soft faeces per pen, number of coughs over a 5 minute period and pigs that seemed lethargic or un-well were documented. Any pig showing signs of ill health was treated as appropriate. All veterinary treatments were recorded including clinical signs, medication used, and dosage. If a death occurred, the pig was excluded from the trial.

6.3.2.7 Sampling of Trucks

Swabs were taken from the trucks used to transport the pigs from the farm to the abattoir immediately prior to loading the pigs and after unloading pigs at the abattoir on day 63 for Trial A and day 28 for Trial B. Three swabs before loading and three swab after unloading (i.e., total of 6 swabs), were taken from the floors of the trucks using sterile sponges, as above. Swabs were collected and handled aseptically to avoid cross-contamination.

6.3.2.8 Blood Sampling

Two weeks prior to commencing the trials, blood was collected from 188 non-trial finisher pigs during exsanguination at slaughter. Serological analysis from these pigs served as a baseline for *Salmonella* prevalence in the finishing pigs on the farm. On day 63 for Trial A and day 28 for Trial B, blood was also collected during exsanguination at slaughter from all trial pigs individually, i.e., 80 finisher pigs in Trial A and 73 finisher pigs in Trial B. All samples were collected using plastic tubes for whole blood (BD Vacutainer, Becton Dickinson, Oxford, UK). Serum was obtained after coagulation and centrifugation of the tubes (1500 rpm for 10 min) and stored at -20 °C until analysis.

6.3.2.9 *Carcass Measurements at Slaughter*

The internal organs and digestive tract were removed before measuring hot carcass weight and the head was left on the carcass. The hot carcass weight at harvest was multiplied by 0.98 to obtain the cold carcass weight and is the value reported in this study as carcass weight. Kill out yield was calculated by expressing cold carcass weight as a percentage of live weight at slaughter. Lean meat yield was estimated from back fat and muscle depth measurements taken using a Hennessy Grading probe according to S.I. No. 413 of 2001 (Government Publications, 2001).

6.3.3 *Salmonella* Isolation and Serotyping

All samples were kept at 4 °C and processed the same day or within 24 h for the presence or absence of *Salmonella* according to the International Organization for Standardization (ISO) 6579:2007 (Amendment 1: Annex D) method (International Organization for Standardization, 2007). All media and agar were obtained from Oxoid Limited (Hampshire, UK). Briefly, 25 g of each faecal sample was homogenized in 225 mL of buffered peptone water (BPW) while each sponge swab was suspended in 90 mL of BPW and homogenized in a stomacher for 2 minutes. Each BPW faecal and swab suspensions were incubated at 37 ºC for 19-hours, after which 100 µL of each sample was pipetted onto modified semi-solid rappaport-vassiliadis (MSRV) agar plates and incubated at 42 ºC for 24-hours. If the MSRV plate was negative, it was incubated for a further 24hours. Presumptive *Salmonella* growth was streaked onto xylose lysine deoxycholate (XLD) and brilliant green agar (BG) agar plates and incubated at 37 ºC for 24-hours. Suspect colonies from XLD or BG agar plates were then streaked onto plate count agar (PCA) and incubated at 37 ºC for 24-hours. Afterwards, urea agar slants and *Salmonella* chromogenic agar plates were inoculated with a colony/colonies from the PCA plates and incubated at 37 ºC for 24-hours. Serological confirmation of colonies from the PCA plates was performed using a *Salmonella* latex agglutination kit (Oxoid). All *Salmonella* isolates recovered were banked onto beads and stored at -80 °C for further characterization.

All presumptive *Salmonella* isolates was first tested using the real-time polymerase chain reaction (PCR) assay for the identification and differentiation of *Salmonella enterica* serotype Typhimurium and *S.* 4,[5],12:i:- as described by Prendergast et al. (2013). If isolates were not identified as *S.* Typhimurium or its monophasic variant, then serotyping was performed according to the White-Kauffmann-

Le Minor scheme (Grimont and Weill, 2007) using commercial antisera (Pro-Lab Diagnostics, Cheshire, UK; SIFIN Institute, Berlin, Germany; and Statens Serum Institute, Copenhagen, Denmark).

6.3.4 *Salmonella* Serological Analysis

Serum samples were analyzed in duplicate using an in-house indirect Enzyme-Linked Immunosorbent Assay (ELISA). Testing was performed by the Department of Agriculture Food and the Marine (Ireland) in accordance with the methods used for serological monitoring in the current NPSCP. The crude optical density (OD) values of the unknown samples were adjusted with OD values of the positive and negative controls [((sample – negative control)/(positive control – negative control)) X 100]. The mean of the adjusted OD values of tested samples were used to compare the treatment groups. Cut-offs were fixed at 40% OD, according to the NPSCP and previous studies (Nielsen et al., 1995; Argüello et al., 2013d).

6.4 Results

6.4.1 Concomitant Infections

The prevalence of Rotavirus, *L. intracellularis* and *Brachyspira* from pooled faecal samples collected on days -70, 0, 28, 52, and 63 is detailed in Table 6.4.1. Since samples were negative for *L. intracellularis* and *Brachyspira* before commencing the study, subsequent samples from trial pigs were only analyzed for Rotavirus. All pooled faecal samples from both trials were Rotavirus-positive throughout the study period.

6.4.2 Faecal Shedding of *Salmonella*

The number of *Salmonella*-positive faecal samples and pen swabs recovered and the resultant *Salmonella* prevalence calculated for each of the 5 sampling time points (days -7, 0, 28, 52, 63) is shown in Table 6.4.2, while the pen-level *Salmonella* prevalence is detailed in Table 6.4.3. Overall, for Trial A, faeces collected from pigs were *Salmonella*-negative on days -7 and 0. Interestingly, as the treatment period progressed, the shedding of *Salmonella* increased from sampling day 28, with 7/40 (17.5%) faecal samples being *Salmonella*-positive, to 22.5% (9/40) and 30.0% (12/40) faecal samples being *Salmonella*–positive on days 52 and 63, respectively (Table 6.4.2). For Trial B, all faeces collected from the pigs were also *Salmonella*-negative on day -7

and day 0, while only 1 faecal sample was *Salmonella*-positive on day 28 (1/43 or 2%, Table 6.4.2).

For Trial A, all pens were *Salmonella*-positive as all pen swabs (8/8 or 100%) collected on days 28 and 52 were *Salmonella*-positive, while only 2 pens were *Salmonella*-positive from 4/8 (50%) of the pen swabs collected on day 63 (Table 6.4.2). On the other hand, for Trial B, only 1 pen was *Salmonella*–positive from 2/10 (20%) of the pen swabs collected at the end of the trial.

The serotype recovered from all isolates was *S.* Typhimurium.

6.4.3 *Salmonella* from Truck Swabs

All swabs taken from the transport truck in Trials A and B before pigs were loaded at the farm and after pigs were unloaded at the abattoir, were *Salmonella*-negative (Table 6.4.2).

6.4.4 *Salmonella* Serology

The pig sera samples were analyzed with the 40% OD cut-off value used in the Irish NPSCP (Table 6.4.4).

The overall *Salmonella* seroprevalence for the baseline group was 42.0%. In line with the *Salmonella* results, the seroprevalence for pigs in Trial B was 41.1%; while for the pigs in Trial A the seroprevalence was higher at 55.0%

6.4.5 Production Parameters

On average, pigs in Trial A had an average carcass weight of 83.1 kg, an average lean meat yield of 57.4 %, and an average muscle and fat depth of 49.5 mm and 12.1 mm, respectively (Table 6.4.5). On the other hand, pigs in Trial B had a carcass weight of 79.7 kg, a lean meat yield of 57.5 %, and a muscle and fat depth of 11.6 mm and 47.4 mm, respectively.

6.5 Discussion

The present study was undertaken as a case study to determine if interventions shown in earlier research in Chapters 2 and 4 (Lynch et al., 2016; Walia et al., 2016; Walia et al., 2017b), could be used in combination to control *Salmonella* on a commercial farm with a history of high *Salmonella* seroprevalance and other herd health issues. Since initial screening of the study farm prior to commencing the trial

showed infection with Rotavirus amongst all production stages, the control measure implemented was a non-invasive, practical and low-cost solution that involved using a common peroxygen disinfectant (Virkon® S) following washing and prior to introduction of pigs to the pen. This disinfectant denatures the protein capsid of non-enveloped, double stranded RNA viruses, such as Rotavirus, thereby destroying them (Paul and Lyoo, 1993; Kitis, 2004; Chandler-Bostock and Mellits, 2015). Moreover, since Interkokask®, a chlorocresol-based disinfectant was previously shown to improve *Salmonella* prevalence and general hygiene in a lairage setting (Walia et al., 2017b; Chapter 4), Trial A investigated both Virkon® S and Interkokask® disinfectants in combination after cleaning with detergent to control Rotavirus and improve pen hygiene. However, despite this intensive cleaning and disinfection of the finisher pens prior to entry of Trial A pigs, the pigs remained infected with Rotavirus throughout the study period, as seen from the analysis of the pooled faecal samples. Although it is highly probable that the finishing pens themselves were negative for Rotavirus prior to entry of the pigs, as discussed by Chandler-Bostock and Mellits (2015), we were unable to determine this, as we did not test pen swabs for Rotavirus. Nonetheless, our results suggest that the initial cleaning and disinfection had no impact on eliminating the virus from the animal. Since Rotavirus is transmitted via the faecal-oral route, similar to *Salmonella*, and can survive in the farm environment and faecal matter for over 9 months (Ramos et al., 2000; Dewey et al., 2003; Estes and Kapikian, 2007), a single cleaning and disinfection event, as performed in the present study, could not be expected to completely remove the virus from the finishing pens. We recommend that if Rotavirus is detected on-farm, as was the case in the present study, all pens in all production stages should be thoroughly cleaned and disinfected prior to entry of pigs and for successive batches of pigs, to ensure its complete removal. In addition, since the pigs themselves were contaminated with faecal matter on their skin, it is possible that topically washing the animals with a Virkon® S solution prior to their introduction to disinfected pens might further reduce the presence of Rotavirus on the animal itself. These strategies would potentially help in the overall control of the virus particularly as Rotavirus is not typically seen in finisher pigs but rather in young pigs post-weaning; and the number of control strategies available to date is limited, with current vaccines shown to be ineffective (Chandler-Bostock and Mellits, 2015). Further research in this area is warranted especially considering that previous work by the study authors found that misting with 0.5% Virkon® S might have a role in topical antisepsis of pigs

contaminated with *Salmonella* prior to slaughter (Walia et al., 2017c; Chapter 5) and as such this may possibly be an avenue to explore for control of Rotavirus as well.

On the other hand, pens in both trials were *Salmonella*-negative even prior to cleaning and disinfection. Nonetheless, before Trial A commenced, the 4 trial pens were cleaned and disinfected with a chlorocresol-based disinfectant and dried, as recommended by Walia et al. (2017b) (i.e., Chapter 4) for lairage pens, to ensure that the pigs were housed in a *Salmonella*-free environment at the start of the finishing period. The 4 trial pens remained *Salmonella* free before pigs were introduced (i.e., day 0); however, as the study progressed to 28 days, *Salmonella* was detected in all pens in Trial A but only one pen in Trial B. Since *Salmonella* was not detected in Trial B pens until the end of the study period (day 28), and the pens for this trial did not undergo an initial cleaning and disinfection with the chlorocresol-based disinfectant, the results suggest that *Salmonella* was present in the pen environment but remained undetected by the pen swabs collected. Likewise, for pigs in Trial A, an increase in *Salmonella* prevalence in pen swabs from day 28 to the end of the study (day 63) was observed despite being housed in cleaned, disinfected, and dried pens where *Salmonella* was not detected. As such, these findings suggest that while cleaning with detergent and application of the chlorocresol-based disinfectant followed by a drying step could have prevented *Salmonella* from growing in the pens initially, *Salmonella* might have also remained undetected from the pen swabs on those two occasions (days -7 and 0). It is also possible that the pigs in Trial A were infected with *Salmonella* in advance of the finishing period and contaminated the trial pens. However, results from day -7 and day 0 showed that the pigs in this group were *Salmonella* negative in collected faecal samples, albeit not all pigs were sampled so it is possible that some pigs were shedding *Salmonella* but were missed in the sampling.

Additionally, although the *Salmonella* prevalence in both trials was low throughout the study, the increase observed from day 28 to the end of Trial A, suggests that dietary supplementation with sodium butyrate for just over 2 months was not successful in reducing faecal shedding of the pathogen. This finding is similar to the results reported by Rasschaert et al. (2016), who found that supplementing the diet of finishing pigs with butyric acid for a duration of 4.5 months (from an initial weight of 25 kg to slaughter weight of 90-100 kg) had no effect on *Salmonella* reduction in faeces. The secondary infection with Rotavirus observed in the present trial might have resulted in the lack of effect seen with diet acidification with sodium butyrate.

However, since it was not possible to collect caecal digesta or lymph nodes in this study, we cannot conclusively say that dietary supplementation with sodium butyrate for > 2 months did not inhibit intestinal colonization with *Salmonella.* Therefore, future research in this area is needed where supplementation with sodium butyrate for the full finishing period is performed in order to definitively conclude whether or not it inhibits *Salmonella* colonization, thereby reducing faecal shedding of the pathogen and recovery in caecal contents and lymph nodes. Moreover, since concomitant infections are highly common on many pig farms, future research is also needed to definitively state that the effect of sodium butyrate will be diminished in the presence of secondary infections.

The acquisition of *Salmonella* observed for Trial A pigs from day 28 to day 63 potentially reflects a breakdown in internal biosecurity and/or an increase in shedding by previously undetected *Salmonella*-positive pigs. Moreover, the ongoing Rotavirus infection may have increased the susceptibility of *Salmonella* infection and shedding. However, for Trial B pigs, the shedding levels were lower and as such, this might suggest a clustering of infection or perhaps an increase in prevalence over time, especially considering the one positive sample detected on day 28.

Despite the Irish NPSCP indicating that the study farm had a naturally high *Salmonella* seroprevalence > 50% for 2016, which was the main reason for conducting the case study on this farm, the seroprevalence of non-trial pigs sampled 2 weeks prior to starting the study showed an overall seroprevalence of 42% using the 40% OD cut-off value. Given the history of the farm in relation to high *Salmonella* prevalence in faeces and serology, and with concomitant infections over the last 2 years (2014-2016), we set to investigate strategies that could help reduce *Salmonella* prevelance on this farm.

This study also aimed to determine the duration of sodium butyrate feeding needed to decrease the *Salmonella* seroprevalence in pigs to levels that would be considered low-risk. In pigs supplemented with sodium butyrate for 63-days, the seroprevalence did not decrease to below the 50% level, which is used as a cut-off value in the NPSCP; in fact, at 55%, it was higher than in the baseline group of pigs. This finding is in line with the *Salmonella* shedding results and suggests that supplementation with sodium butyrate for 63-days was not effective in reducing *Salmonella* seroprevalence. On the other hand, when supplemented with the feed additive for 28 days, the seroprevalence was 41%, which is below the NPSCP threshold. This finding is in contrast to that by Walia et al. (2016) (i.e., Chapter 2) who found that

supplementation with sodium butyrate for 28-days, on the same farm, did not reduce the seroprevalence to below the 50% threshold. However, it should be borne in mind that the seroprevalence results indicate that 41% of the pigs supplemented with sodium butyrate for 28-days were infected with *Salmonella* at one point during their growth. It does not reflect the current shedding status of the animals or indicate if they were infected with the pathogen at slaughter (EFSA, 2008). It is possible that given the low (i.e., 2%) shedding rate of the pigs in this trial, that most of the animals were not shedding *Salmonella* after 28-days of supplementation with sodium butyrate. However, since lymph nodes were not obtained from these animals at slaughter we cannot conclude if they were infected with *Salmonella* at slaughter but not currently shedding, or if they were no longer infected but still possessed *Salmonella* antibodies. As such, this is a limitation of the present study. It also highlights the issue of serological analysis versus bacteriological analysis of *Salmonella* as discussed in several previous studies (Nollet et al., 2005; EFSA, 2008; Ball et al., 2011; Rostango et al., 2012). Bacteriological testing of faeces from individual pigs provides a good measurement of the current *Salmonella* shedding status of the animals/herds on the farm and is considered the current gold standard in determining the *Salmonella* status prior to slaughter (Ball et al., 2011). Nollet and colleagues (2005) showed that pig herds that were serologically negative could be bacteriologically positive when jejunal and colonic digesta and mesenteric lymph nodes were tested. This observation was further corroborated by Rostango et al. (2012) who found wide variations in bacteriologic and serologic *Salmonella* prevalence (12.9% and 35.4%, respectively) in the same group of finishing pigs, repeatedly sampled every 1-2 weeks for 12-weeks.

In relation to *Salmonella* control, only two studies to date have shown numerical improvements in average live weight before slaughter and carcass weights in weaner and finisher pigs fed a diet supplemented with sodium butyrate for 28 days as compared to an un-supplemented diet (Lynch et al., 2016; Walia et al., 2016; Chapter 2). As such, the present study was a follow-on from these earlier studies to determine what effect a > 2 months supplementation period would have on the growth performance of finishing pigs. On average, the finishing pigs that were fed the feed additive for > 2 months had a 4.8% increase in live weight before slaughter, a 4.2% increase in carcass weight and fat depth, and a 4.3% increase in muscle depth compared to the pigs fed the sodium butyrate supplemented feed for 28 days. However, since we do not know if the pigs in both groups were the same age when sent to slaughter, the numerical increases

mentioned above should be considered with caution. Unfortunately, a cost-benefit analysis was not possible, as the weight of the animals prior to commencing the study and the average daily feed intake of the pigs in each of the trials were not determined. Nonetheless, it appears that even though some of the pigs were infected with *Salmonella* and Rotavirus in spite of an initial cleaning and disinfection of pens, a longer dietary supplementation period with sodium butyrate increased pig growth relative to the shorter 28-day duration of feeding. Future research to investigate the cost-benefit of this improvement is warranted especially considering that both Lynch et al. (2016) and Walia et al. (2016) (i.e., Chapter 2) determined that dietary supplementation with sodium butyrate for 28 days provided a net benefit of €0.07 and €0.04 in feed cost per kg of live-weight gain, respectively.

6.6 Conclusions

Initial cleaning and disinfection of finisher pens with peroxygen compound- and chlorocresol-based disinfectants in combination with dietary sodium butyrate supplementation for 63 days was not successful in reducing faecal shedding of *Salmonella* or seroprevalence in finishing pigs. The secondary infection with Rotavirus detected throughout the study might have contributed to the lack of effect observed. However this result does not suggest that organic acid feed additives will not work when concomitant infections are present, and as such further research is necessary. Since we could not conclude that an initial cleaning and disinfection of finisher pens in addition to supplementation with sodium butyrate for 63 days did not inhibit intestinal colonization of *Salmonella,* future research in this area is also needed. Moreover, since numerical improvements in live weight before slaughter and carcass weight were observed in the finisher pigs supplemented with sodium butyrate for 63 days, further investigation to determine the cost-benefit of this is also warranted.

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Table 6.3.1. Declared composition of finisher diet used (on an air-dry basis, %)

Ingredient	
Maize	20.0
Wheat	20.0
Barley	31.7
Soya (Bean) Meal Dehulled	14.3
Pollard	-
Rapeseed Meal	8.0
Soya Hulls	-
Soya Oil	2.21
Sugar Cane Molasses	1.0
Mineral and Vitamin Premix	2.79[1]
Chemical Composition	
Dry Matter	87.9
Crude Protein	16.2
Crude Oils and Fats	3.42
Crude Fiber	4.21
Crude Ash	4.60
Lysine	1.15
Digestible Energy, MJ/kg	14.0

[1]Premix provided per kg of complete diet: vitamin A, 1000 IU; vitamin D3, 2000 IU; vitamin E, 90 IU; calcium iodate anhydrous, 2.86 mg; zinc oxide, 111 mg; sodium selenite, 6.6 mg; manganese oxide, 81 mg; ferrous sulphate monohydrate, 400 mg; cupric sulphate pentahydrate, 50 mg; endo-1.4 beta-xylanase, 10 IU; butylated hydroxyanisole, 0.45 mg; ethoxyquin, 0.45 mg; Ca, 6.5 g; NaCl, 5.5 g; P, 5.0 g; methionine, 3.4 g; threonine, 6.9 g; tryptophan, 2.0g; Phytase, 5000 FTU.

Table 6.4.1. Prevalence of Rotavirus, *Lawsonia intracellularis* and *Brachyspira* in pooled faecal samples from finisher trial pen (4 pens in Trial A; 5 pens in Trial B)

		No. Pigs Positive/No. Pigs Sampled (% Prevalence)		
		Rotavirus	***Lawsonia intracellularis***	***Brachyspira***
Day - 70	**Trial A**	4/4 (100%)[b]	0/4 (0%)	0/4 (0%)
	Trial B	-[a]	-	-
Day 0	**Trial A**	4/4 (100%)[c]	-	-
	Trial B	5/5 (100%)[c]	-	-
Day 28	**Trial A**	4/4 (100%)[d]	-	-
	Trial B	5/5 (100%)[d]	-	-
Day 52	**Trial A**	4/4 (100%)[e]	-	-
Day 63	**Trial A**	4/4 (100%)[e]	-	-

[a] '-' indicates no samples were taken.

[b] Trial A: Pens 6, 7, 8 and 9 were Rotavirus A, B, C positive.

[c] Trial A: Pens 6, 7 and 9 were Rotavirus A, B, C positive; Pen 8 was Rotavirus B, C positive. Trial B: Pen 1 was Rotavirus A and B positive; Pens 2, 4 and 5 were Rotavirus A, B, C positive; Pen 3 was Rotavirus B positive.

[d] Trial A: Pens 6 and 8 were Rotavirus A, B, C positive; Pen 7 was Rotavirus B positive; Pen 9 was Rotavirus B, C positive. Trial B: Pen 1 was Rotavirus A and B positive; Pens 2, 3, 4 and 5 were Rotavirus B positive.

[e] Trial A: Pens 6, 8 and 9 were Rotavirus A, B positive; Pen 7 was Rotavirus B positive.

Table 6.4.2. *Salmonella* prevalence in faeces, pen swabs and truck swabs collected from 10 finisher pigs (per pen) fed a diet supplemented with sodium butyrate in conjunction with an initial cleaning and disinfection of finisher trial pens (Trial A)[a] or without cleaning and disinfection (Trial B)

		No. Positive for *Salmonella*/No. Sampled (% *Salmonella* Prevalence)			
		Faeces	**Pen Swabs**	**Truck Swabs Before Loading (on Farm)**	**Truck Swabs After Unloading (at Abattoir)**
Day -7	**Trial A**	0/40 (0%)	0/8 (0%)	-	-
	Trial B	0/43 (0%)	0/10 (0%)	-[d]	-
Day 0	**Trial A**	0/40 (0%)	0/8 (0%)	-	-
	Trial B	0/43 (0%)	0/10 (0%)	-	-
Day 28	**Trial A**	7/40 (17.5%)	8/8 (100%)	-	-
	Trial B	1/43 (2%)	2/10 (20%)[b]	0/3 (0%)	0/3 (0%)
Day 52	**Trial A**	9/40 (22.5%)	8/8 (100%)	-	-
Day 63	**Trial A**	12/40 (30%)	4/8 (50%)[c]	0/3 (0%)	0/3 (0%)

[a] Trial A pens were cleaned and disinfected with Virkon® S (preoxygen disinfectant) and Interkokask® (chlorocresol-based disinfectant) and dried for ~12 hours on day -1.

[b] Only pen 5 was *Salmonella*-positive on day 28.

[c] Only 2 pens, pens 7 and 9, were *Salmonella*-positive on day 63.

[d] '-' indicates no samples were taken.

Table 6.4.3. Pen-level *Salmonella* prevalence in faeces, collected from finisher pigs fed a diet supplemented with sodium butyrate in conjunction with an initial cleaning and disinfection of finisher trial pens (Trial A) or without cleaning and disinfection (Trial B)

Trial	Pen	No. Pigs/Pen	No. Pigs Sampled	Sampling Period [a] Day -7		Day 0		Day 28		Day 52		Day 63	
				No. Pigs Positive for *Salmonella*	*Salmonella* Prevalence (%)	No. Pigs Positive for *Salmonella*	*Salmonella* Prevalence (%)	No. Pigs Positive for *Salmonella*	*Salmonella* Prevalence (%)	No. Pigs Positive for *Salmonella*	*Salmonella* Prevalence (%)	No. Pigs Positive for *Salmonella*	*Salmonella* Prevalence (%)
A	1	20	10	0	0.0	0	0.0	4	40.0	4	40.0	0	0.0
A	2	20	10	0	0.0	0	0.0	0	0.0	0	0.0	1	10.0
A	3	20	10	0	0.0	0	0.0	2	20.0	2	20.0	5	50.0
A	4	20	10	0	0.0	0	0.0	1	10.0	3	30.0	6	60.0
B	5	20	10	0	0.0	0	0.0	0	0.0	- [b]	-	-	-
B	6	7	7	0	0.0	0	0.0	0	0.0	-	-	-	-
B	7	6	6	0	0.0	0	0.0	0	0.0	-	-	-	-
B	8	20	10	0	0.0	0	0.0	1	10.0	-	-	-	-
B	9	20	10	0	0.0	0	0.0	0	0.0	-	-	-	-

[a] The *Salmonella* prevalence reported is calculated from the No. Pigs Positive for *Salmonella*/No. Pigs Sampled as only half of the animals per pen were sampled, with the exception of pens 6 and 7 in Trial B in which all pigs were sampled.

[b] '-' indicates no samples were taken.

Table 6.4.4. *Salmonella* seroprevalence at the end of the finishing period (at slaughter) in finisher pigs fed either a diet supplemented with sodium butyrate in conjunction with an initial cleaning and disinfection of finisher trial pens (Trial A) or without cleaning and disinfection (baseline group[a] and Trial B)

	OD 40
Baseline Group (non-trial pigs)[b]	
No. Positive Pigs	79
No. Negative Pigs	109
***Salmonella* Prevalence (%)**	42.02
Trial A[b]	
No. Positive Pigs	44
No. Negative Pigs	36
***Salmonella* Prevalence (%)**	55.0
Trial B[b]	
No. Positive Pigs	30
No. Negative Pigs	43
***Salmonella* Prevalence (%)**	41.1

[a] The baseline group consisted of pigs 188 non-trial finisher pigs that served as a baseline for *Salmonella* prevalence in the finishing pigs on the farm

[b] Trial A = 80 pigs; Trial B = 73 pigs.

Table 6.4.5. The effect of dietary supplementation with sodium butyrate on growth, feed efficiency, and carcass quality in finisher pigs for the full finishing period (Trial A) or 1 month (Trial B) on a commercial pig farm

	Trial A	**Trial B**
Weight - Day 28 (kg)	-	102
Weight - Day 63 (kg)	107	-[a]
Carcass Weight (kg)	83.1	79.7
Kill Out Yield (%)	77.8	78.0
Lean Meat Yield (%)	57.4	57.5
Muscle Depth (mm)	49.5	47.4
Fat Depth (mm)	12.1	11.6

[a] '-' indicates no weight was taken.

CHAPTER 7: Summary

7.1 Summary and General Discussion

The research presented herein resulted from the EU baseline survey, which showed that 20% of Irish pig carcasses were contaminated with *Salmonella* (EFSA, 2008) despite implementation of the Irish NPSCP in 2003 and its update in 2010. Since approximately 18% of Irish pig herds currently have a *Salmonella* prevalence of greater than 50% (data extracted from the 2016 NPSCP), this equates to ~ 50,000 pigs which could lose their quality status with quality assurance boards such as Bord Bia, and consequentially their market share if their prevalence remains high. As the Irish pig industry generates ~ €5.9 million per year, being able to decrease *Salmonella* in pigs and ultimately in pigmeat could increase the competitiveness of the Irish pig industry as products sold would be labeled "*Salmonella*-free". Therefore, the experimental chapters presented in this thesis provide the science to underpin low-cost risk based control for *Salmonella* in pigs. They focus on select *Salmonella* control strategies at the source – at farm level – and at a recognized secondary point in pig processing – within the lairage holding pens in the abattoir. The following two sections discuss the overall outcomes from the interventions studied within these two stages and aims to provide recommendations for industry, and for future research.

7.1.1 Control of *Salmonella* at Farm Level

The main premise behind chapters 2 and 3 was to evaluate commercial products currently on the market and which are currently used in the pig industry as a *Salmonella* control measure but for which limited efficacy data are available in the public domain. Chapters 2 and 3 did not set-out to create new products; instead practical solutions with known products were investigated. Chapter 2 (Walia et al., 2016) and chapter 3 (Walia et al., 2017a) were both the first field trials that evaluated the efficacy and determined the cost-benefit of two commercially available feed additives: (1) sodium butyrate and (2) an encapsulated blend of formic acid, citric acid and essential oils, to reduce *Salmonella* shedding and intestinal carriage in finishing pigs on farms with a history of high seroprevalence. Results from both chapters showed that both feed additives were successful in decreasing *Salmonella* shedding over a 28-day period, albeit limited effects were observed in chapter 3. Concomitant diseases and onset of infection were also shown to impact the efficacy of sodium butyrate (chapter 2) in controlling *Salmonella* but possibly with other feed additives as well. However, since the findings

in chapter 2 are novel, future research is needed to definitively state that concomitant infections, which are highly common on many pig farms, weaken the effect of sodium butyrate and other feed additives. As such, not only were laboratory diagnostic investigations recommended when clinical signs were present but determining the onset of infection was also recommended as the efficacy of the feed additive can be diminished (chapters 2 and 3). While the results from chapters 2 and 3 may question the value of on-farm interventions especially if their effects can easily be negated during transport and lairage, as observed with the potential new infections detected from transport/lairage; they also showed the value of on-farm interventions. By recommending that pig farms implement a *Salmonella* control strategy, a reduction in faecal shedding would greatly reduce the risk of acquiring the pathogen from transport or from the lairage holding pens (Berends et al., 1996). As such, on-farm interventions should be the first step in the overall hurdle approach in controlling *Salmonella* (Goldbach and Alban, 2006; Ojha and Kostrzynska, 2007).

In chapter 6, an additional case study was performed as a result of the findings from chapters 2 and 3, which suggested that a longer duration of treatment might reduce the *Salmonella* seroprevalence to below the high seroprevalence threshold in Ireland (i.e., > 50%). Unfortunately, the findings from chapter 6 did not show a reduction in seroprevalence to below the 50% cut-off after 63-days of dietary supplementation. Interestingly, the findings in chapter 6 were in contrast to that of chapter 2 wherein pigs supplemented with sodium butyrate for 28-days showed a reduction in seroprevalence. However, in chapter 6, the *Salmonella* shedding rates of the pigs in the 28-day supplementation group was low, with only 2% of faecal samples being *Salmonella*-positive. Therefore, the seroprevalence of 41% does not reflect the current shedding status of the animals at slaughter nor indicate if they were infected with the pathogen (EFSA, 2008). This fact also highlights the issue of serological versus bacteriological analysis discussed in chapters 1 and 6. Furthermore, chapter 6 also implemented the recommendation from chapter 2 regarding laboratory diagnostic investigations when clinical signs were present. All pigs in the case study were infected with Rotavirus and although non-invasive measures were taken to control the virus, through initial intensive cleaning and disinfection of finisher pens with Virkon® S, eradication of the virus was not achieved. It was possible that, despite the low *Salmonella* shedding prevalence during the study period; the lack of effect observed from dietary supplementation with sodium butyrate was in part affected by the infection with Rotavirus. Moreover, as

discussed in chapter 6, it was also possible that due to the long survival time of Rotavirus, a one off cleaning and disinfection procedure was not appropriate. As such, in addition to laboratory diagnostic investigations when clinical signs are present, continual cleaning and disinfection of all pens for successive generations of batches of pigs was recommended to ensure elimination of Rotavirus from the environment.

Given that the literature to date is limited in terms of the cost-benefit of dietary supplementation in relation to *Salmonella* control, chapters 2, 3 and 6 set-out to investigate this aspect as well. Overall, dietary supplementation with sodium butyrate for 28-days provided the farmer with a net benefit of €0.04 per kg of live-weight gain, while supplementing the diet with an encapsulated blend of formic acid, citric acid, and essential oils, increased the feed cost by €0.84 per kg live-weight gain. As such, the latter might gain from an increase in treatment duration but an additional cost-benefit would be required. For 63-days of dietary supplementation with sodium butyrate, a cost-benefit could not be determined. Since chapter 6 implemented specific recommendations from chapter 2, the increased supplementation with sodium butyrate provided slight improvements in terms of live weights before slaughter and carcass weights at slaughter. These weights were similar amongst both chapters with slightly higher weights observed in chapter 2 than in chapter 6. Therefore, the results presented herein show that 28-days of diet supplementation with the feed additive is the minimum length needed for a net gain to the primary producer.

As discussed above and in chapters 2, 3 and 6, controlling *Salmonella* in pigs at primary production is a complex issue with no ‘quick’ fixes. While good biosecurity goes a long way in limiting the transmission of the pathogen, especially between stages, factors such as the type of feed additive, its mode of action, dose and duration of treatment, the onset of *Salmonella* infection and the presence of other underlying infections, such as *L. intracellularis* and Rotavirus, are also critical in determining the success of control strategies. While it is simple to say that farmers need a control programme, it is difficult to change behaviours and poor habits. If complacency and general managerial practices are not taken into consideration, then success of any implemented control strategy will not be achieved. By addressing these issues, the persistence of *Salmonella* on farms can be better managed.

7.1.2 Control of *Salmonella* in the Lairage

The lairage holding pens in the abattoir are a well-known point in pig processing where pigs are at risk of acquiring *Salmonella* if the environment is contaminated. As a result, chapters 4 (Walia et al., 2017b) and 5 (Walia et al., 2017c) investigated potential approaches to limit the risk that this environment posed on pigs entering the abattoir, especially for naïve, "*Salmonella*-free" pigs.

In chapter 4, several cleaning and disinfection protocols were evaluated. Overall, results showed that simply washing pens with a high-pressure cold water (15-17 ºC) wash, as is the current practice in many abattoirs, was not conducive to reducing *Salmonella* prevalence in the lairage pens. This finding can also be applied to pens on the farm. On the other hand, the type of disinfectant was one of two important factors in reducing the prevalence of *Salmonella*. Results showed that applying a chlorocresol-based disinfectant after pens were washed with high-pressure cold water was more effective at killing *Salmonella* and *Enterobacteriaceae* than applying a QAC-based disinfectant after high-pressure cold water washing. The second key factor, and a critical step in eradicating *Salmonella* from the lairage pens, was drying. Results showed that by ensuring that the lairage pens were dried for a minimum of 24 hours after intensive cleaning (combining a high-pressure cold water wash with a detergent) followed by disinfection with a chlorocresol-based disinfectant was the most effective hygiene routine to eliminate *Salmonella* and reduce *Enterobacteriaceae* counts. Since *Salmonella* can survive in harsh environments through forming biofilms, a known phenomenon, the results presented in chapter 4 demonstrate that the ability to form biofilms within the lairage pen depended on temperature and the intensity of the cleaning protocol. As such, similar to *Salmonella* control at farm level, there are also complexities in controlling the pathogen in the abattoir. Despite the fact that all parts of the pen should be cleaned to the same standard, with attention being paid to particular problem areas such as holes and cracks, the key factors that need consideration when *Salmonella* control measures are implemented are: temperature, type of disinfect, intensity of the cleaning and disinfection protocol (combining the use of detergent and a pressure wash followed by application of a disinfectant), and drying.

In addition to cleaning and disinfection of lairage pens, chapter 5 evaluated a novel approach in lairage wherein pigs were misted with a disinfectant to topically reduce the surface contamination of *Salmonella* on the animals prior to slaughter. Similar to the other strategies discussed in chapters 2, 3, 4, and 6, this chapter presented

another practical strategy that utilized existing infrastructure in the abattoir as a means to control this pathogen. Overall, results showed that adding Virkon® S at 0.5% to the misting water might have a role in topical antisepsis of pigs contaminated with *Salmonella* prior to slaughter. However, simply misting with water alone increased the topical contamination prevalence of *Salmonella*, on the animals before slaughter. This was unexpected and since the group of animals not misted or misted with 0.5% Virkon® S were un-affected, chapter 5 discussed the possibility that low pressure misting with water alone aided the acquisition of *Salmonella*. As such, a risk assessment was also recommended for abattoirs as to whether misting with water alone should be avoided for high *Salmonella* prevalence herds. Farms that use or are contemplating installing such misting systems should consider the findings discussed in this chapter, as they are also of relevance.

7.2 Recommendations

As briefly mentioned above, the conclusions presented in chapters 4 and 5, pertaining to the lairage, can also be applied to the farm. Since pigs spend all their time housed in pens on the farm, in addition to determining interventions to control *Salmonella* through diet (chapters 2, 3, 6), solutions to improve common practices such as cleaning and disinfection are also necessary. However, simply improving current practices whether via the introduction of new feed additives or new disinfectants or improving biosecurity plans or housing conditions can reduce *Salmonella* shedding on the farm, the research presented in chapter 6 shows that there is no 'quick' fix for *Salmonella* control. Since combining several strategies, such as the ones studied in the aforementioned chapter, did not change the prevalence of *Salmonella* on the farm, a more comprehensive approach would be to focus on the whole health of the herd rather than on pin-pointing individual problems. By evaluating the health status of the herd and focusing on a multifactorial approach to good herd health, diseases such as *Salmonella*, Rotavirus, *L. intracellularis* and others can become a nonissue. The recommendations listed below are based on the research findings summarized above and discussed at length in chapters 2-6, and are aimed towards the key stakeholders in the pig industry: farmers, abattoirs and government.

- Before implementing a *Salmonella* control strategy: (1) address issues of complacency and poor managerial practices; (2) determine the onset of *Salmonella* infection; and (3) perform laboratory diagnostic investigations on concomitant infections when clinical signs are present, or suspicion of other infections.
- Twenty-eight days of dietary supplementation with a sodium butyrate feed additive is the minimum length needed to decrease *Salmonella* shedding and provide a net gain to the primary producer.
- Using seroprevalence alone to classify pig herds as high or low risk of *Salmonella* infection should not be the definitive standard; bacteriological monitoring is also necessary. By incorporating both the serological and bacteriological results, a comprehensive picture of the *Salmonella* status on the farm is provided.
- Allocate high seroprevalence pig herds, i.e., > 50%, to specific lairage pens in the abattoir.
- Applying a rigorous 4-step protocol combining the use of a detergent and a chlorocresol-based disinfectant with a subsequent drying step for 24 hours will remove *Salmonella* from the lairage pens and possibly also from pens on the farm.
- Use heaters or other means of artificially drying lairage (and farm) pens after cleaning and disinfection between batches of animals. Not only will this shorten the drying time, but it can potentially provide an additive benefit of using heat to kill *Salmonella* and potentially other pathogens as well.
- Pay particular attention to cleaning and disinfecting pens, focusing on problem areas in the pen including cracks and holes in the floors and walls.
- Clean and disinfect the walls and ceiling to the same standard as the floors.
- A risk assessment based on known *Salmonella* data, meat quality and welfare considerations is required to determine whether misting with water alone should be avoided for high *Salmonella* prevalence herds.

7.3 Future Direction

As a result of the studies presented herein, the questions and statements listed below are proposed for future work for the pre-harvest control of *Salmonella*.

- Why do certain *Salmonella* serotypes survive well in the farm environment versus the lairage environment? What are the underlying causes? And are there serotype-specific genetic factors leading to its survival or is it host adaptive characteristics?
- Since, essential oils have mechanistic actions that could inhibit *Salmonella* growth and invasion *in vivo*, investigating their potential as a pre-harvest control measure is needed especially considering the lack of field trials available to date.
- What is the underlying mechanism that allowed *Salmonella* to survive in dry conditions following application of detergent and the QAC-based disinfectant?
- Additional field trials are also needed on the effectiveness of misting and/or washing animals prior to slaughter in the overall control of *Salmonella* in the abattoir, using a different approved/registered disinfectant, or using Virkon® S but applying it at a higher pressure/flow rate.
- What is the efficacy of removing visible organic matter on pigs prior to entry into lariage pens, and increasing the misting contact time of the Virkon® S disinfectant on *Salmonella* contamination?
- What effect would an increase in pressure and flow rate of the misting device to perhaps a minimum of 100 psi and 7.5 litres per minute have on, topically, reducing *Salmonella* on live animals? And what adverse effects, if any, are produced as a result of this increase in pressure?
- What effect does improving the overall herd health have on the persistence and transmission of *Salmonella* on the farm?

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APPENDICES

APPENDIX A: BOX PLOTS OF BIOFILM FORMATION AT 15ºC AND 37ºC FOR SELECTED ISOLATES FROM CHAPTER 4

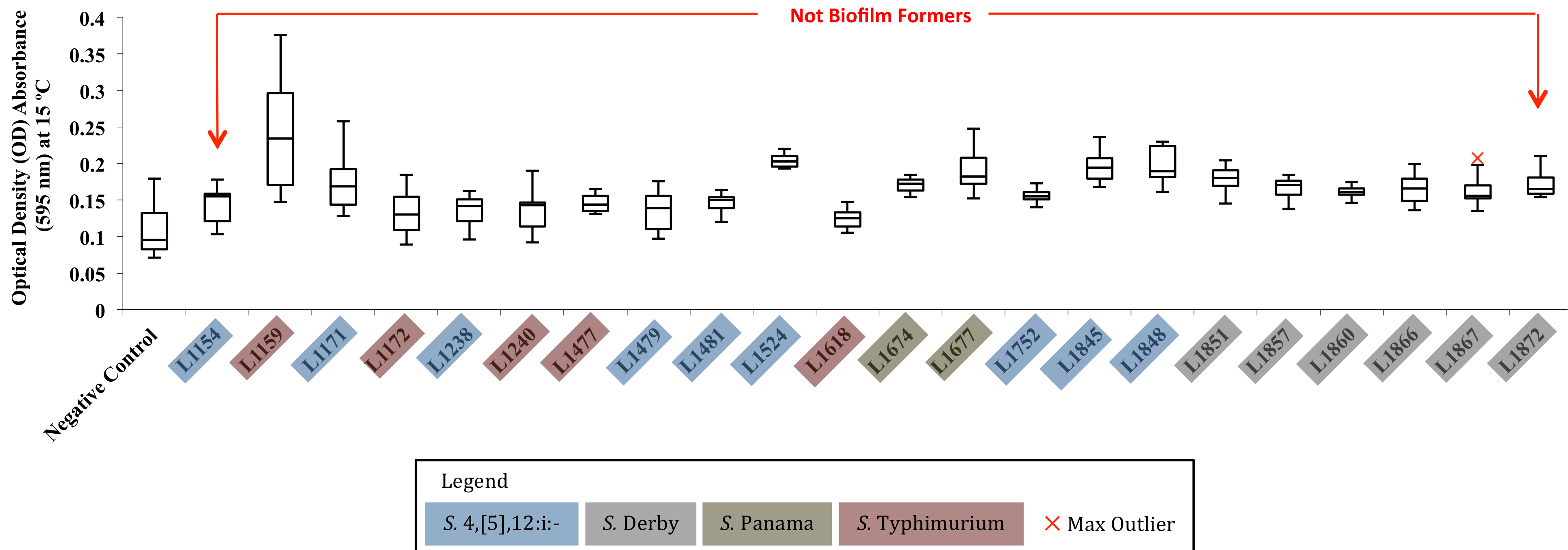

Figure A.1. Box plots of *Salmonella* isolates classified as not biofilm formers (OD isolate </= OD cut-off) at 15 ºC. The OD cut-off was defined as three standard deviations above the mean OD_{595nm} of the negative control wells. Biofilm formation was only performed on selected *Salmonella* isolates, i.e. those with unique AMR profiles, selected serotypes, and/or those that were recovered after cleaning with the disinfectants alone, after cleaning with detergent plus the disinfectants, and after drying.

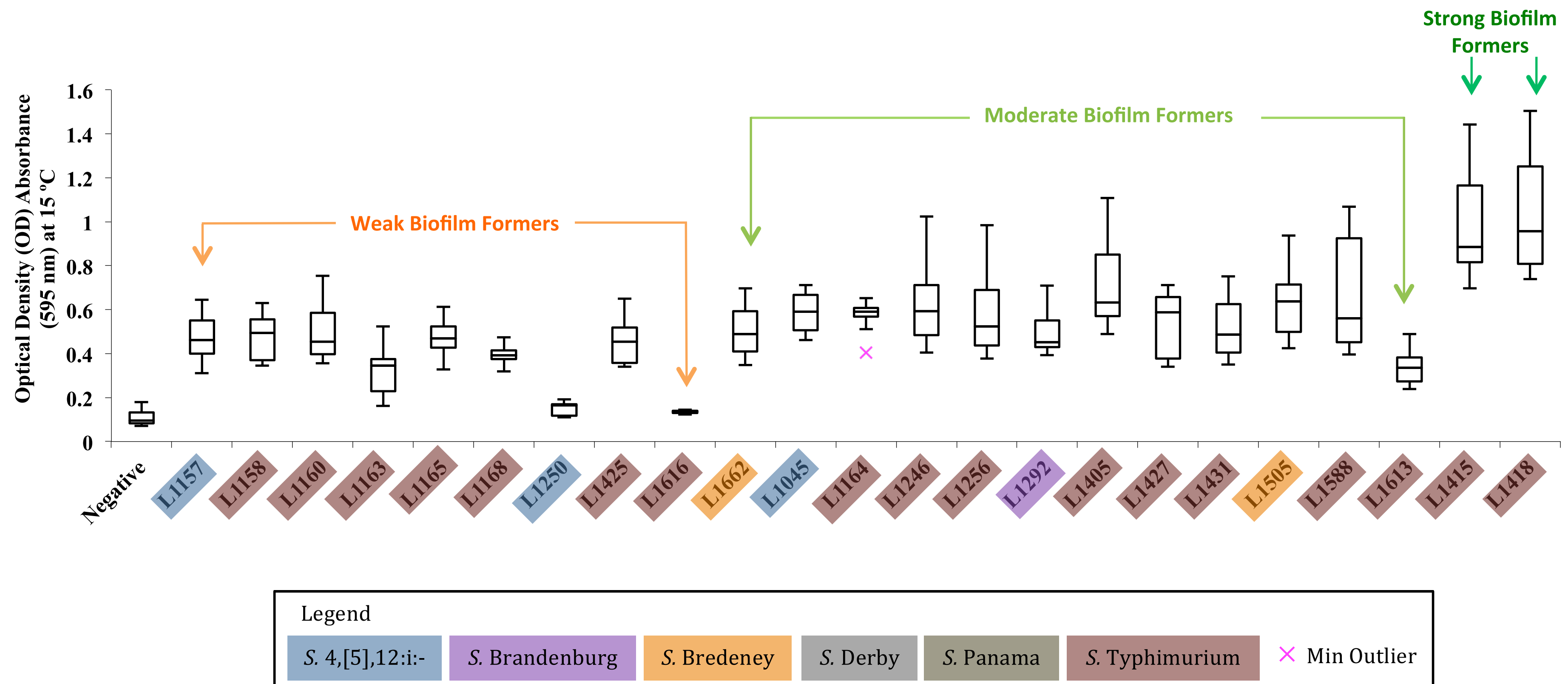

Figure A.2. Box plots of *Salmonella* isolates classified as weak (OD cut-off < OD isolate </= 2 x OD cut-off), moderate (2 x OD cut-off < OD isolate </= 4 x OD cut-off), or strong (4 x OD cut-off < OD isolate) biofilm formers at 15 ºC. The OD cut-off was defined as three standard deviations above the mean OD_{595nm} of the negative control wells. Biofilm formation was only performed on selected *Salmonella* isolates, i.e. those with unique AMR profiles, selected serotypes, and/or those that were recovered after cleaning with the disinfectants alone, after cleaning with detergent plus the disinfectants, and after drying.

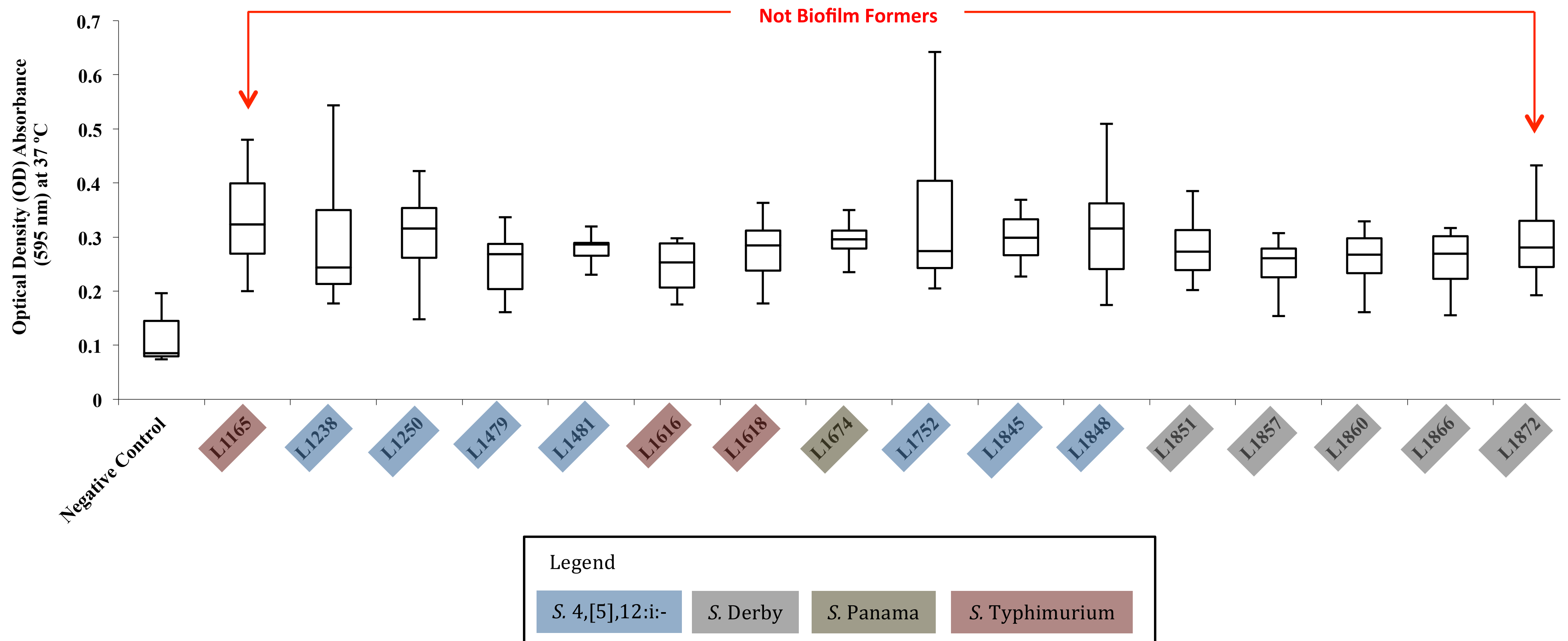

Figure A.3. Box plots of *Salmonella* isolates classified as not biofilm formers (OD isolate </= OD cut-off) at 37 ºC. The OD cut-off was defined as three standard deviations above the mean OD_{595nm} of the negative control wells. Biofilm formation was only performed on selected *Salmonella* isolates, i.e. those with unique AMR profiles, selected serotypes, and/or those that were recovered after cleaning with the disinfectants alone, after cleaning with detergent plus the disinfectants, and after drying.

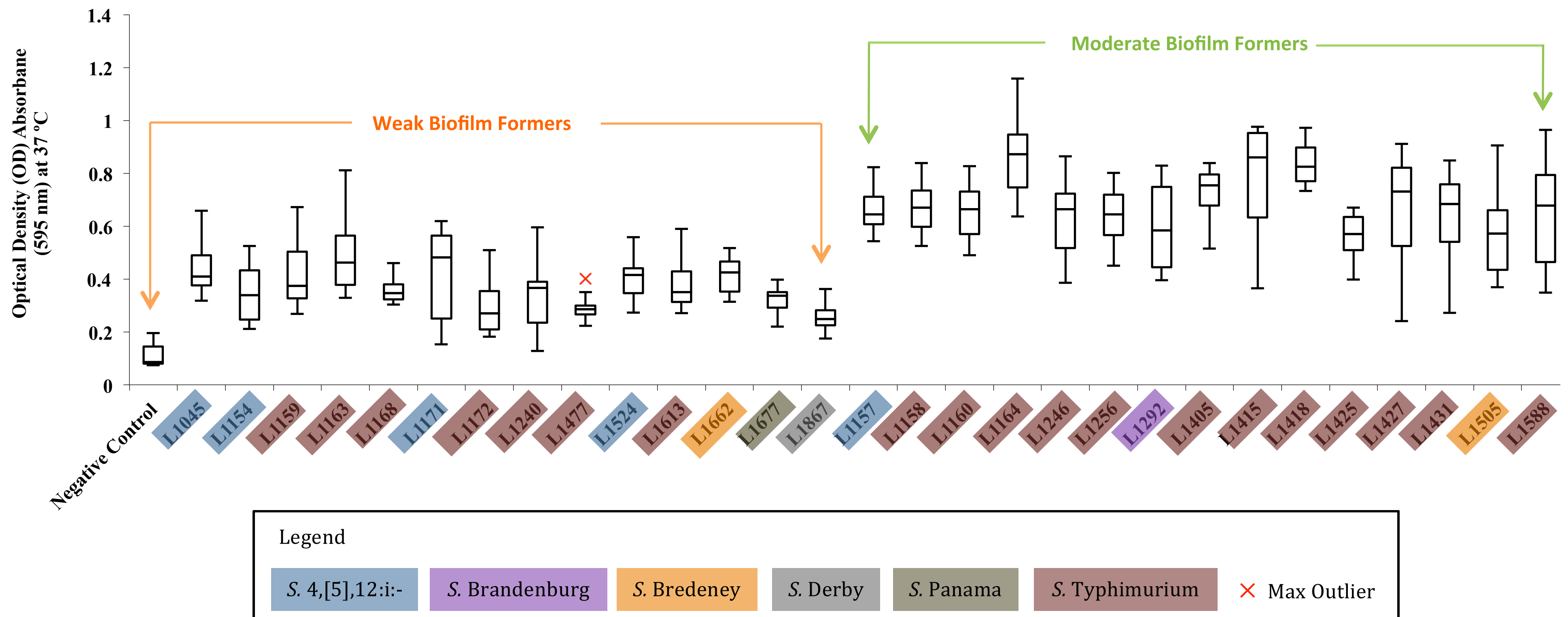

Figure A.4. **Box plots of *Salmonella* isolates classified as weak (OD cut-off < OD isolate </= 2 x OD cut-off), moderate (2 x OD cut-off < OD isolate </= 4 x OD cut-off), or strong (4 x OD cut-off < OD isolate) biofilm formers at 37 ºC. The OD cut-off was defined as three standard deviations above the mean OD_{595nm} of the negative control wells. Biofilm formation was only performed on selected *Salmonella* isolates, i.e. those with unique AMR profiles, selected serotypes, and/or those that were recovered after cleaning with the disinfectants alone, after cleaning with detergent plus the disinfectants, and after drying.**

APPENDIX B: LIST OF PUBLICATIONS, PRESENTATIONS, AND CONFERENCE ATTENDANCE

First Author Publications:

Walia, K., Argüello, H., Lynch, H., Leonard, F.C., Grant, J., Yearsley, D., Kelly, S., Duffy, G., Gardiner, G.E., Lawlor, P.G., 2016a. Evaluating the effectiveness of a sodium butyrate feed additive for the control of *Salmonella* carriage in finishing pigs. Prev. Vet. Med. 131, 79-86.

Walia, K., Argüello, H., Lynch, H., Leonard, F.C., Duffy, G., Gardiner, G.E., Lawlor, P.G. 2017a. Effect of strategic administration of an encapsulated blend of formic acid, citric acid, and essential oils on *Salmonella* carriage, seroprevalence, and growth of finishing pigs. Prev. Vet. Med. 137, 28-35.

Walia, K., Lynch, H., Argüello, H., Grant, J., Leonard, F.C., Lawlor, P.G., Gardiner, G.E., Duffy, G. 2017b. The efficacy of different cleaning and disinfection procedures to reduce *Salmonella* and *Enterobacteriaceae* in the lairage environment of a pig abattoir. Int. J. Food. Microbiol. 246, 64-71.

Walia, K., Lynch, H., Grant, J., Duffy, G., Leonard, F.C., Lawlor, P.G., Gardiner, G.E. 2017c. The efficacy of disinfectant misting in the lairage of a pig abattoir to reduce *Salmonella* and *Enterobacteriaceae* on pigs prior to slaughter. Food Control. 75, 55-61.

Named Author Publications:

Argüello, H., Lynch, H., Gardiner, G.E., Egan, J., **Walia, K.**, Duffy, G., Edgar, G.M., Lawlor, P.G., Leonard, F.C. 2016. Surveillance data highlights feed form, biosecurity and disease control as significant factors associated with *Salmonella* infection on farrow-to-finish pig farms. Prev. Vet. Med. (Accepted with Revisions).

Lynch, H., Argüello, H., **Walia, K.**, Lawlor, P.G., Duffy, G., Gardiner, G.E., Leonard, F.C. 2016a. Evaluation of an alternative experimental infection method which closely mimics the natural route of transmission of monophasic *Salmonella* Typhimurium (mST) in pigs. Foodborne Path. Dis. 14,1-6.

Lynch, H., Leonard, F.C., **Walia, K.**, Lawlor, P.G., Duffy, G., Fanning, S., Markey, B.K., Brady, C., Gardiner, G.E., Argüello, H. 2017a. Investigation of in-feed organic acids as a low cost strategy to combat *Salmonella* in grower pigs. Prev. Vet. Med. (In Press).

Lynch, H., **Walia, K.**, Argüello, H, Leonard, F.C., Lawlor, P.G., Manzanilla, E.G., Grant, J., Duffy, G., Gardiner, G.E., Cormican, M., King, J., Markey, B.K., Fanning, S. 2017b. *Salmonella* in breeding pigs: Shedding pattern and transmission of the infection to the progeny in Irish farrow-to-finish herds. Vet. Microbiol. (Submitted).

Oral Presentations:

How safe is your chicken and pork products? Science Week, 25th November 2013, Teagasc Food Research Centre, Ashtown, Dublin 15, Ireland.

Evaluating the effectiveness of a sodium butyrate feed additive for the control of *Salmonella* carriage in finishing pigs. Safefood *Salmonella* Knowledge Network Annual Conference, 16th April 2015, DAFM Laboratories, Backweston, Celbridge, Ireland.

Tracking and management of *Salmonella* in Irish pigs. Safefood *Salmonella* Knowledge Network Seminar Series, 22nd March 2016, Radisson Blu St. Helen's Hotel, Stillorgan Road, Blackrock, Co. Dublin, Ireland.

The effect of cleaning and disinfection to reduce *Salmonella* in the lairage environment of a pig abattoir. Waterford Institute of Technology Research Day 2016, 4th May 2016, Waterford Institute of Technology, Cork Road, Waterford.

Effect of strategic administration of sodium butyrate in the late finishing period on *Salmonella* control. 24th International Pig Veterinary Society Congress (IPVS)/8th European Symposium of Porcine Health Management (ESPHM), 8th June 2016, Royal Dublin Society (RDS), Dublin, Ireland.

Targeted low cost solutions for control of *Salmonella* in pig production. Stakeholders Information Day, 6th December 2016, Teagasc, Conference and Training Centre, Ashtown, Dublin 15, Ireland.

Conference Posters and Conference Proceedings:

Walia, K., Argüello, H., Lynch, H., Lawlor, P.G., Leonard, F.C., Duffy, G. and Gardiner, G.E. 2015. Evaluating the effectiveness of a sodium butyrate feed additive for the control of *Salmonella* carriage in finishing pigs. SafePork 2015 Conference, 7th-10th September 2015, Porto, Portugal.

Walia, K., Argüello, H., Lynch, H., Leonard, F.C., Grant, J., Yearsley, D., Kelly, S., Duffy, G., Gardiner, G.E., Lawlor, P.G. 2016. The effect of a sodium butyrate fed to finishing pigs on *Salmonella* carriage, seroprevalence and growth. Teagasc Pig Research Dissemination Day 2016, 27th-28th April 2016, Cavan Crystal Hotel, Cavan, Ireland and Horse and Jockey Hotel, Tipperary, Ireland.

Lynch, H., **Walia, K.**, Leonard, F.C., Manzanilla, E.G., Lawlor, P.G., Duffy, G., Gardiner, G.E., Argüello, H. 2016. *Salmonella* in breeding pigs: Shedding pattern and transmission of infection to progeny in farrow-to finish herds. In: 24th International Pig Veterinary Society Congress (IPVS)/8th European Symposium of Porcine Health Management (ESPHM), 08th June 2016, Royal Dublin Society (RDS), Dublin, Ireland.

Lynch, H., Argüello, H., **Walia, K.**, Lawlor, P.G., Duffy, G., Gardiner, G.E., Leonard, F.C. 2016. The effect of organic acids on *Salmonella* shedding and growth performance in weaned pigs. In: 25th International Committee on Food Microbiology and Hygiene (ICFMH) Conference, Food Micro 2016, 19th-22nd July 2016, University College Dublin, Ireland.

Walia, K., Argüello, H., Lynch, H., Leonard, F.C., Grant, J., Yearsley, D., Kelly, S., Duffy, G., Gardiner, G.E., Lawlor, P.G. 2016. The efficacy of feeding sodium butyrate during the late finishing period on *Salmonella* shedding, seroprevalence and growth of finishing pigs. In: 25th International Committee on Food Microbiology and Hygiene (ICFMH) Conference, Food Micro 2016, 19th-22nd July 2016, University College Dublin, Ireland.

Walia, K., Lynch, H., Argüello, H., Grant, J., Leonard, F.C., Lawlor, P.G., Gardiner, G.E., Duffy, G. 2016. The efficacy of different cleaning and disinfection procedures to reduce *Salmonella* and *Enterobacteriaceae* in the lairage environment of a pig abattoir.

In: 25th International Committee on Food Microbiology and Hygiene (ICFMH) Conference, Food Micro 2016, 19th-22nd July 2016, University College Dublin, Ireland.

APPENDIX C: PUBLISHED PAPERS

Preventive Veterinary Medicine 131 (2016) 79–86

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Effect of feeding sodium butyrate in the late finishing period on *Salmonella* carriage, seroprevalence, and growth of finishing pigs

Kavita Walia[a,b,e], Hector Argüello[a], Helen Lynch[a,c,e], Finola C. Leonard[c], Jim Grant[a], Dermot Yearsley[d], Sinead Kelly[d], Geraldine Duffy[a], Gillian E. Gardiner[b,*], Peadar G. Lawlor[e]

[a] Teagasc, Food Research Centre, Ashtown, Dublin 15, Ireland
[b] Department of Science, Waterford Institute of Technology, Waterford, Ireland
[c] School of Veterinary Medicine, University College Dublin, Dublin, Ireland
[d] Department of Agriculture, Food and the Marine, Central Veterinary Research Laboratory, Backweston, Ireland
[e] Teagasc, Pig Development Department, Animal and Grassland Research and Innovation Centre, Moorepark, Fermoy, Co. Cork, Ireland

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ABSTRACT

Pork is an important source of human salmonellosis and low-cost on-farm control measures may provide a useful element in reducing the prevalence of this pathogen in food. This study investigated the effectiveness of dietary supplementation with sodium butyrate administered to finisher pigs for ~4-weeks prior to slaughter to control *Salmonella* shedding on highly contaminated farms.

Two trials (A and B) were conducted on two commercial pig farms, which had a history of high *Salmonella* seroprevalence. In both trials, pens (14 pens of 12 pigs/pen in Trial A and 12 pens of 12–17 pigs/pen in Trial B) were randomly assigned to a control (finisher feed without additive) or a treatment group (the same feed with 3 kg sodium butyrate/t) for 24–28 days, depending on the trial. Faeces were collected from each pig on days 0, 12 and 24/28, and blood, caecal digesta and ileocaecal/mesenteric lymph nodes were collected from the slaughterhouse. Pigs were weighed at the start and end of the trials, feed intake was recorded, and carcass quality parameters were recorded at slaughter.

In Trial A, *Salmonella* shedding was reduced in the treatment compared to the control group at the end of the trial (30% versus 57% probability of detecting *Salmonella* in faeces, respectively; $p < 0.001$). This reflected the serology results, with detection of a lower seroprevalence in the treatment compared to the control group using the 20% optical density cut-off (69.5% versus 89%; $p = 0.001$). However, no effect on faecal shedding or seroprevalance was observed in Trial B, which may be explained by the detection of a concomitant infection with *Lawsonia intracellularis*. No significant differences in *Salmonella* recovery rates were observed in the caecal digesta or lymph nodes in either trial. Furthermore, feed intake, weight gain, and feed conversion efficiency (FCE) did not differ between groups ($p > 0.05$) in either trial. Numerical improvements in weight gain and FCE were found with sodium butyrate treatment, which gave a cost benefit of €0.04/kg of live-weight gain.

Overall, results suggest that strategic feeding of sodium butyrate, at 3 kg/t of feed, to finishing pigs for 24–28 days prior to slaughter was effective in reducing *Salmonella* shedding and seroprevalance but perhaps only in the absence of co-infection with other pathogens. However, sodium butyrate supplementation at this rate did not influence intestinal carriage, nor did it reduce seroprevalence to below the cut-off used for the high *Salmonella* risk category in Ireland (50%), or significantly improve growth performance.

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1. Introduction

Asymptomatic intestinal carriage of *Salmonella* in pigs presented for slaughter can result in pork carcass contamination. An EU baseline survey conducted in 2006–2007, showed that Ireland had a high prevalence of *Salmonella* contamination on pork

Abbreviations: NPSC, national pig *Salmonella* control program; ILN, ileocaecal lymph nodes; MLN, mesenteric lymph nodes; ADFI, average daily feed intake; ADG, average daily gain; FCE, feed conversion efficiency.

* Corresponding author.
E-mail address: ggardiner@wit.ie (G.E. Gardiner).

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carcasses (20%) (EFSA, 2008). This can be linked to the relatively high prevalence of *Salmonella* in some Irish pig herds (McCarthy et al., 2013; Burns et al., 2015). In an attempt to reduce this prevalence, the National Pig *Salmonella* Control Program (NPSCP) was updated in 2010 (Department of Agriculture Food and the Marine (DAFM), 2010). Despite this, *Salmonella* herd prevalence has not declined (DAFM personal communication). This highlights a need to find low-cost control measures to reduce *Salmonella* shedding in pigs at primary production, especially finishing pigs (35–100 kg), as carriage rates are high during this stage of production (Burns et al., 2015) and finishers are a significant source of *Salmonella* in the abattoir (Duggan et al., 2010; Argüello et al., 2013a).

Dietary supplementation with organic acids or their salts is a potential strategy for the control of *Salmonella* in finishing pigs (Creus et al., 2007; Wales et al., 2010). Organic acids can decrease gastrointestinal pH, thus creating an environment, which is hostile to *Salmonella* while favouring the growth of beneficial bacteria such as lactobacilli. The un-dissociated form of various acids can also freely cross the bacterial cell membrane and enter the bacterial cell, causing cell death (Van Immerseel et al., 2006). In addition, some organic acids (e.g., butyric acid and propionic acid) also down regulate the expression of invasion genes (e.g., *hilA*) in *Salmonella*, thereby suppressing its ability to invade intestinal epithelial cells (Boyen et al., 2008).

Dietary supplementation with sodium butyrate has previously been shown to reduce *Salmonella* shedding and intestinal colonization in weaner pigs which were deliberately infected with *Salmonella* (Boyen et al., 2008). However, to our knowledge, no field trial has evaluated the effectiveness of sodium butyrate as a *Salmonella* control measure in finishing pigs on farms with historically high levels of the pathogen. In addition, despite the number of field trials that have evaluated organic acids for the control of *Salmonella* in pigs, few have investigated their use for a short targeted period prior to slaughter and the cost-benefit associated with their use (Gálfi and Bokori, 1990; Creus et al., 2007). Therefore, the objectives of the present study were to conduct a field study on two selected farms with a high *Salmonella* seroprevalence, to investigate the ability of dietary supplementation with sodium butyrate during the last month of growth pre-slaughter to: (1) reduce faecal shedding and intestinal carriage of *Salmonella*, and (2) impact growth performance in finisher pigs. Based on the findings, a cost-benefit analysis was also conducted.

2. Materials and methods

2.1. Animal ethics and experimental licensing

Two separate feeding trials (Trial A and Trial B) were performed on two commercial pig farms in the last quarter of 2014 and the first quarter of 2015. Ethical approval was obtained from the Waterford Institute of Technology ethics committee and an experimental license was obtained from the Irish Department of Health and Children (number B100/2982). All animals were handled in a humane manner and were slaughtered in a regulated abattoir.

2.2. Experimental procedure

2.2.1. Trial A farm

Trial A was conducted on a 90 sow farrow-to-finish farm. The finisher house in which the trial was conducted consisted of a barn with 14 pens. A total of 169 finisher pigs were used (72 males and 97 females; 12 pigs per pen). Each pig was ear tagged with a unique number for identification purposes. Pigs were housed in pens (each pen was 4.5 m × 2.8 m) with concrete slatted floors and provided with ad-libitum access to water from 2 nipple drinkers per pen. The temperature of the barn was maintained at ~20 °C. Ad-libitum access was provided to dry pelleted feed via single-spaced wet-dry feeders.

This herd had a historically high *Salmonella* seroprevalence (data extracted from the NPSCP); however, the prevalence of the batch of finishing pigs immediately prior to this trial declined to 0%. As a result, pens in the finishing house were artificially contaminated with a monophasic *Salmonella* Typhimurium (*S.* 4,[5],12:i:-) strain with an antimicrobial resistance (AMR) pattern of ASSuT, which had previously been isolated from sows in the same herd. Briefly, a single colony of *S.* 4,[5],12:i:- was inoculated into 90 mL of Tryptone Soya Broth (TSB, Oxoid, Basingstoke, UK), incubated overnight at 37 °C and then diluted in Phosphate Buffered Saline (PBS, Oxoid) to a final concentration of $\sim 5 \times 10^3$ CFU/mL. Five 25 mL vials per pen (each containing $\sim 10^3$ CFU/mL of *Salmonella*) were spread at five points: 3 in the defecation area, and 2 near the feeder. The final concentration of *Salmonella* at each inoculation point was 2.5×10^4 CFU. Contamination of the pens was performed 7 days before commencing the trial.

2.2.2. Trial B farm

Trial B was conducted on a 180 sow farrow-to-finish farm. The finisher house in which the trial was conducted consisted of a 2-room barn, each with 6 pens per room. A total of 177 finisher pigs were used (86 males and 91 females; 12–17 pigs per pen). Each pig was ear tagged with a unique number for identification purposes. Pigs were housed in pens (each pen was 3.2 m × 3.4 m) with concrete slatted floors and provided with ad-libitum access to water from 2 nipple drinkers per pen. The temperature of each room was maintained at ~20 °C. Ad-libitum access was provided to dry pelleted feed via single-spaced wet-dry feeders.

This farm had a historically high *Salmonella* seroprevalence (i.e. > 50% for 2014), and faecal shedding of *Salmonella* Typhimurium had been confirmed bacteriologically prior to commencement of the trial.

2.2.3. Treatment groups

Approximately 4 weeks before the target slaughter date, pigs in both trials A and B were blocked by sex and weight and randomly assigned to one of two diet groups: a standard finisher feed with no feed additive (control group) or the same finisher feed supplemented with 3 kg per tonne sodium butyrate (Adimix®, Nutriad, Kasterlee, Belgium; treatment group). The composition of the trial diets is shown in Table S-1 in the Supplementary material. In Trial A, the pigs were fed the experimental diets for 28 days and in Trial B, for 24 days. Pigs in both trials were fasted for ~18 h prior to slaughter.

2.2.4. Blood and faecal sampling and measurement of production parameters

For serological analysis, blood was collected during two occasions: (1) by jugular venipuncture, prior to feeding the experimental diets, and (2) during exsanguination at slaughter. All samples were collected using plastic tubes for whole blood (BD Vacutainer, Becton Dickinson, Oxford, UK). Serum was obtained after coagulation and centrifugation of the tubes (1500 rpm for 10 min) and stored at −20 °C until analysis.

On day 0 (the day prior to commencing experimental treatments), day 12 and either day 28 (Trial A) or day 24 (Trial B) (i.e., the final treatment day), faeces (~25 g) was collected into 100 mL sterile bottles (Sarstedt, Nümbrecht, Germany) from each pig by digital rectal stimulation. All samples were collected and handled aseptically to avoid cross-contamination.

Feed intake was recorded throughout each trial and individual body weights were recorded on day 0 and day 28 (Trial A) or day 24 (Trial B). These weights were used to calculate average daily feed

intake (ADFI), average daily gain (ADG), and feed conversion efficiency (FCE). Pigs were observed closely at least twice daily. Any pig showing signs of ill health was treated as appropriate. All veterinary treatments were recorded including identity of pig, clinical signs, medication used, and dosage. If a death occurred or antibiotics were administered, the pig(s) were weighed and subsequently removed from the pen(s) and excluded from the trial.

2.2.5. *Sampling of trucks and lairage*

Swabs were taken from the trucks immediately prior to loading the pigs, in each trial. Two swabs samples were taken from the floors of the trucks using sterile sponges pre-soaked with Maximum Recovery Diluent (MRD, Oxoid). Pigs from the treatment group in each trial were compartmentalized on the upper floor of the truck while the control group were on the lower level. Upon arrival at the abattoir, two lairage pens (one for each diet group) were swabbed with sponges, as above, prior to unloading the pigs from Trial B (3 swabs per pen i.e. front, middle and back of each pen). Lairage swabs were not collected in Trial A.

2.2.6. *Sampling and carcass measurements at slaughter*

For each trial, caecal digesta, ileocaecal lymph nodes (ILN), and mesenteric lymph nodes (MLN) were collected from the gastrointestinal tract (GIT) of 88 pigs (45 from the control group and 43 from the treatment group). Caecal digesta (~10 g) was collected via puncture of the blind end of the caecum; while ILN and MLN (≥10 g) were removed from the viscera and pooled for each animal. All samples were collected aseptically to avoid cross-contamination.

The internal organs and digestive tract were removed before measuring hot carcass weight and the head was left on the carcass. The hot carcass weight at harvest was multiplied by 0.98 to obtain the cold carcass weight and is the value reported in this study as carcass weight. Kill out yield was calculated by expressing cold carcass weight as a percentage of live weight at slaughter. In Trial B, lean meat yield was estimated from back fat and muscle depth measurements taken using a Hennessy Grading probe according to S.I. No. 413 of 2001 (Government Publications, 2001).

Half of the trial pigs (41 control and 47 treatment) in Trial B were sent for slaughter six days after the first half and although no samples were taken from these pigs all other factory measurements were recorded and were used in the analysis of data.

2.2.7. Salmonella *isolation from faecal, digesta and intestinal samples, and truck and lairage swabs*

All samples were kept at 4 °C and processed the same day or within 24 h for the presence or absence of *Salmonella* according to the International Organization for Standardization (ISO) 6579:2007 (Amendment 1: Annex D) method (International Organization for Standardization, 2007). The ILN and MLN were first processed according to EC Regulation 668/2006 (Regulation (EC) No 668/2006 (06.10.2006), 2006) by removing the fat and capsula followed by immersion in 90% ethanol (v/v). They were then flamed to sterilize the outer surface, and cut into small pieces using sterile scissors to an approximate weight of 10 g.

All *Salmonella* isolates recovered were banked onto beads and stored at −80 °C for further characterization.

2.3. *Serotyping and antimicrobial resistance determination of* Salmonella *isolates*

All presumptive *Salmonella* isolates were first tested using the real-time polymerase chain reaction (PCR) assay for the identification and differentiation of *Salmonella enterica* serotype Typhimurium and S. 4,[5],12:i:- as described by Prendergast et al. (2013). If isolates were not identified as *S.* Typhimurium or its monophasic variant, then serotyping was performed according to the White-Kauffmann-Le Minor scheme (Grimont and Weill, 2007) using commercial antisera (Pro-Lab Diagnostics, Cheshire, UK; SIFIN Institute, Berlin, Germany; and Statens Serum Institute, Copenhagen, Denmark).

The antimicrobial resistance (AMR) pattern of each isolate was determined using the Sensititre™ Gram Negative NARMS Plate (Thermo Scientific, Waltham, MA, USA). The following antimicrobials were tested: amoxicillin-clavulanic acid (AUG), ampicillin (AMP), azithromycin (AZI), cefoxitin (FOX), ceftiofur (XNL), ceftriaxone (AXO), chloramphenicol (CHL), ciprofloxacin (CIP), gentamicin (GEN), nalidixic acid (NAL), streptomycin (STR), sulfisoxazole (FIS), tetracycline (TET), and trimethoprim/sulfamethoxazole (SXT). Minimal Inhibitory Concentrations (MICs) were interpreted using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) epidemiological cut-off values.

2.4. Salmonella *serological analysis*

Serum samples were analyzed in duplicate using an in-house indirect Enzyme-Linked Immunosorbent Assay (ELISA). Testing was performed by the Department of Agriculture Food and the Marine (Ireland) in accordance with the methods used for serological monitoring in the current NPSCP. The crude optical density (OD) values of the unknown samples were adjusted with OD values of the positive and negative controls [(sample − negative control/positive control − negative control) × 100)]. The mean of the adjusted OD values of tested samples were used to compare the control and treatment groups. Cut-offs were fixed at ODs of 20% and 40%, according to previous studies (Nielsen et al., 1995; Argüello et al., 2013c).

2.5. *Statistical analysis*

Pens of pigs were blocked on sex and weight. Entire males or gilts and feed with or without sodium butyrate supplementation were used in a 2 × 2 factorial arrangement with seven pen replicates per diet group in Trial A and six pen replicates per diet group in Trial B.

For *Salmonella* prevalence and serology, data were analyzed using the GLIMMIX procedure in Statistical Analyses System (SAS, V9.3, 2011). The pig was the experimental unit. To avoid potential clustering and effects of over-dispersion, pen was included as a random effect in the model. Means for *Salmonella* prevalence and serology were separated using the Tukey-Kramer least square means adjustment for multiple comparisons and evaluated as the probability of detecting *Salmonella* in faeces, caeca, ILN/MLN, or *Salmonella* antibodies in serum. The *Salmonella* prevalence on day 0 (before control and treatment diets were administered) was used as a covariate in the Tukey-Kramer least square means adjustment.

For growth performance, data were analyzed using the mixed models procedure in SAS. The experimental unit was the pen of pigs. Fixed effects were diet group, sex and day and block was included as a random effect. Where significant in the model initial weight or carcass weight was included as a covariate in the analysis.

Residual checks were made to ensure that the assumptions of the analyses were met. For all analyses, significance in difference was established at $\alpha = 0.05$.

3. Results

3.1. *Faecal shedding of* Salmonella

For Trial A, one female pig was removed from the treatment group following the administration of penicillin. The probability of detecting *Salmonella* was similar in control and treatment groups on day 12. By the end of the trial (day 28), the administration of

Table 1
The effect of dietary supplementation with sodium butyrate on the probability of detecting *Salmonella* in faeces from finisher pigs on day 12 and day 24/28 for Trials A and B on two commercial pig farms (LS means +/– sem).

	Trial A				Trial B			
	Day 12	Day 28	sem	p-value	Day 12	Day 24[a]	sem	p-value
Control (%)	50	57	0.063	0.80	30	29	0.053	1.00
Treatment (%)	66	30	0.059	< 0.001	23	23	0.049	1.00
sem	0.062	0.060			0.052	0.050		
p-value	0.26	0.018			0.81	0.86		

[a] A sex by treatment by day effect ($p < 0.001$) was observed for the probability of finding *Salmonella* in faeces. On day 24, the probability of finding *Salmonella* in faeces was reduced in males when sodium butyrate was added to feed; whereas in females the probability increased.

Table 2
Salmonella prevalence in faeces, caecum and pooled ileocaecal and mesenteric lymph nodes (ILN/MLN), collected from finisher pigs fed either a control diet or a diet supplemented with sodium butyrate on days 0, 12, 24/28 (on farm) and days 26/29 (slaughter) for Trials A and B on two commercial pig farms.

			No. Pigs Positive for *Salmonella*/No. Pigs Sampled (% *Salmonella* Prevalence)		
			Faeces	Caecum	ILN/MLN
Trial A	Day 0	Control	15/80 (18.8)	–[a]	–
		Treatment	35/79 (44.3)	–	–
	Day 12	Control	43/83 (51.8)	–	–
		Treatment	53/81 (65.4)	–	–
	Day 28	Control	47/81 (58)	–	–
		Treatment	28/78 (35.9)	–	–
	Day 29	Control	–	37/45 (82.2)	20/45 (44.4)
		Treatment	–	35/43 (81.4)	17/43 (39.5)
Trial B	Day 0	Control	17/83 (20.5)	–	–
		Treatment	5/88 (5.7)	–	–
	Day 12	Control	27/82 (32.9)	–	–
		Treatment	20/87 (23)	–	–
	Day 24	Control	28/86 (32.6)	–	–
		Treatment	20/88 (22.7)	–	–
	Day 26	Control	–	41/45 (91.1)	19/45 (42.2)
		Treatment	–	36/43 (83.7)	12/43 (27.9)

[a] Indicates no samples were taken.

sodium butyrate to finishing pigs had resulted in a decline in the probability of detecting *Salmonella* compared to the control group (30% versus 57%, respectively; $p < 0.05$; Table 1). Table 2 details the number of *Salmonella*-positive faecal samples recovered and the resultant *Salmonella* prevalence was calculated for each of the 3 sampling time points (days 0, 12, and 28). Between day 12 and day 28, supplementation with sodium butyrate reduced the probability of detecting *Salmonella* shedding from 66% to 30%, ($p < 0.001$; Table 1). However, the probability of detecting *Salmonella* was 50% and 57%, on day 12 and day 28, respectively, for the control group ($p > 0.05$; Table 1).

For Trial B, no effect of sodium butyrate treatment was observed either on day 12 or 24 ($p > 0.05$); neither was there an effect of treatment over time ($p > 0.05$; Table 1).

The *Salmonella* serotype recovered from pigs in Trial A was S. 4,[5],12:i:-; while in Trial B, all of the isolates were typed as S. Typhimurium.

3.2. *Salmonella* serology

The pig sera samples were analyzed with 20% and 40% OD cut-off values, which are commonly used in *Salmonella* control programmes (Table 3).

In Trial A, all of the pigs (82 per group) were seronegative at the beginning of the trial. In agreement with the reduction in *Salmonella* prevalence observed in faecal samples, significantly lower seroprevalence was detected in the sodium butyrate-treated group at slaughter using the 20% OD cut-off as compared to the control group (69.5% versus 89.0%, respectively; $p < 0.01$). When using the 40% OD cut-off, no significant difference in seroprevalence was detected between groups ($p > 0.05$). When adjusted OD values from the control (mean OD value 65.1; sem = 4.31) and treatment group (47.7 ; sem = 4.67) pigs were compared, significant differences were detected between groups ($p < 0.05$).

In Trial B, blood was collected from 40 control pigs and 36 sodium butyrate-treated pigs at the start of the finishing period. Half of the pigs from the control group had seroconverted at this stage and the serological prevalence (or OD values) were higher in the control group compared to the sodium butyrate- treated group ($p < 0.01$). At the end of the trial, most pigs had seroconverted, indicating the presence of infection in both groups. During this second sampling, no differences in prevalence were detected between groups with either cut-off value (20% OD $p > 0.05$; and 40% OD $p > 0.05$), or when the mean of the adjusted OD values of the control group (mean OD 66.7; sem = 7.13) was compared with that of the sodium butyrate-treated group (mean OD 87.0; sem = 9.27) ($p > 0.05$).

3.3. *Salmonella from truck and lairage swabs*

For both Trials A and B, the truck swabs taken prior to loading of pigs were *Salmonella* negative. Swabbing of the lairage pens to which pigs from Trial B were allocated prior to slaughter showed *Salmonella* in both pens (one sample of the three taken per pen was positive). The serotype recovered from these two pens was S. 4,[5],12:i:.

3.4. *Salmonella in intestinal samples*

Table 2 details the number of *Salmonella*-positive caecal and ILN/MLN samples recovered and the resultant *Salmonella* prevalence calculated for each trial. Overall, no differences in the probability of detecting *Salmonella* in the caecal digesta were observed between control and treated pigs for Trial A (85% versus

Table 3
Salmonella seroprevalence at the start of the finishing period and at the end (at slaughter) in finisher pigs fed either a control diet or a diet supplemented with sodium butyrate[1]

	Entry to finisher house				Slaughter			
	OD 20		OD 40		OD 20		OD 40	
	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment
Trial A								
No. Positive Pigs	0	0	0	0	73	57	54	43
No. Negative Pigs	82	82	82	82	9	25	28	39
Salmonella Prevalence (%)	0	0	0	0	89.0^{a}	69.5^{b}	65.9	52.4
Trial B								
No. Positive Pigs	20	3	16	1	39	30	33	25
No. Negative Pigs	20	33	24	35	1	6	7	11
Salmonella Prevalence (%)	50.0^{a}	9.1^{b}	66.7^{x}	2.9^{y}	97.5	83.3	82.5	69.4

^{a,b,x,y}Within a row for the same OD value and same stage of sampling, values with different superscripts are significantly different ($p < 0.01$).
[1] Trial A = 82 pigs per group; Trial B = 40 pigs in the control group, 36 pigs in the treatment group.

84%, respectively; sem = 0.060; $p > 0.05$) and Trial B (91% versus 83%, respectively, sem = 0.052; $p > 0.05$). Similarly for the pooled ILN/MLN samples, no differences in the probability of detecting *Salmonella* were observed between control and treated pigs in Trial A (35% versus 36%, respectively; sem = 0.14; $p > 0.05$) and Trial B (42% versus 28%, respectively; sem = 0.073; $p > 0.05$).

In Trial A, the *Salmonella* serotype recovered from all *Salmonella*-positive caecal digesta and ILN/MLN samples was *S.* 4,[5],12:i:-.

In Trial B, isolates recovered were identified as both *S.* Typhimurium and *S.* 4,[5],12:i:-. However, only a small number of pigs in the control group (7 pigs) and treatment group (4 pigs) were positive for *S.* 4,[5],12:i:-. Five samples of caecal digesta, from 3 control pigs and 2 treatment pigs, and 6 samples of lymph nodes, from 4 control pigs and 2 treatment pigs, were positive for *S.* 4,[5],12:i:-. The *S.* 4,[5],12:i:- isolates when recovered from the caecal digesta were not recovered from the lymph nodes of the same pig and vice-versa.

3.5. Production parameters

No significant differences between groups were observed for ADFI, ADG or FCE in either trial (Table 4). Despite this, pigs in the sodium butyrate-treated group in both trials had numerically higher ADG than those in the control group (Table 4). A numerical increase of 7% and 2.6% in ADG was found in Trials A and B, respectively as a result of feeding sodium butyrate. Moreover, the FCE for pigs in both trials was numerically better in the sodium butyrate-treated group as compared to the control group. A numerical improvement of 8.5% and 4.3% in FCE was found in Trials A and B, respectively as a result of feeding sodium butyrate. Although the growth performance of pigs in either trial was not significantly affected by treatment, the numerical differences observed, particularly in Trial A, were of biological importance. For this reason the cost-benefit of supplementing the diet with sodium butyrate was determined by considering the increased feed cost associated with incorporating sodium butyrate into the diet and the feed efficiency of pigs during the trial period (Table 4). From Table 5, the final feed cost per kg live-weight gain during Trial A was €0.89 and €0.85 for the control and treatment groups, respectively; and for Trial B it was €0.91 and €0.92 for the control and treatment groups, respectively.

4. Discussion

Decreasing *Salmonella* at farm-level can be considered an initial step in any overall control strategy to limit its spread throughout the pig production cycle (Goldbach and Alban, 2006; Ojha and Kostrzynska, 2007). Control strategies at farm-level not only decrease the infection pressure during production but the resultant reduction in *Salmonella* carriage can also lessen cross contamination in transport vehicles and lairage – two points at which pigs are prone to acquiring *Salmonella* (Berends et al., 1996). This should also result in a reduction in pork carcass contamination at slaughter.

Organic acids and/or their salts, as a potential *Salmonella* control measure, have been tested in challenge and non-challenge trials in pigs at various stages of growth (Canibe et al., 2005; Creus et al., 2007; Boyen et al., 2008; Visscher et al., 2009; Gebru et al., 2010; Willamil et al., 2011; Calveyra et al., 2012; Argüello et al., 2013b). However, results from these studies are inconclusive and the success of the interventions depends on the product used, its concentration and the duration of administration (Creus et al., 2007; Argüello et al., 2013b). The dietary supplement evaluated in the present study was a commercially available sodium butyrate feed additive used at the manufacturer's recommended inclusion rate with a relatively short treatment period i.e. approximately the last four weeks prior to slaughter. As feed intake is

Table 4
The effect of dietary supplementation with sodium butyrate on growth, feed efficiency, and carcass quality in finisher pigs on Trials A and B conducted on two commercial pig farms.

	Trial A[a]				Trial B[a]			
	Control	Treatment	sem	p-value	Control	Treatment	sem	p-value
Weight - Day 0 (kg)	86.2	88.8	5.58	0.43	78.5	75.4	1.92	0.14
Weight - Day 24 (kg)	N/A	N/A	N/A	N/A	101	101	0.7	0.96
Weight - Day 28 (kg)	113	115	1.5	0.3	N/A	N/A	N/A	N/A
Average Daily Feed Intake (g)	2781	2832	80.61	0.66	2718	2666	79.1	0.65
Average Daily Gain (g)	919	984	62.7	0.29	840	862	24.1	0.5
Feed Conversion Efficiency (g/g)	3.15	2.88	0.11	0.12	3.24	3.1	0.08	0.25
Carcass Weight (kg)	86.2	87.4	1.24	0.52	80.2	79.6	0.58	0.54
Kill Out Yield (%)	76.2	74.9	0.66	0.14	78.7	78.3	0.32	0.35
Lean Meat Yield (%)	N/A	N/A	N/A	N/A	57.1	56.7	0.26	0.39

[a] N/A – indicates not applicable.

Table 5
Cost-benefit analysis of dietary supplementation of finisher pigs with sodium butyrate on Trials A and B conducted on two commercial pig farms.

	Trial A		Trial B	
	Control	Treatment	Control	Treatment
Weight Gain (kg)	25.7	27.6	20.2	20.7
Feed Conversion Efficiency (kg/kg)	3.15	2.88	3.24	3.10
Cost of Sodium Butyrate (€/kg)	–	5	–	5
Inclusion Rate of Sodium Butyrate (kg/t)	–	3	–	3
Total Cost of Sodium Butyrate (€/t)	–	15	–	15
Cost of Sodium Butyrate (€/pig)	–	1.19	–	0.96
Finisher Feed Price in Ireland for July 2015 (€/t)	281	281	281	281
Finisher Feed Price with/without added Sodium Butyrate (€/t)	281	296	281	296
Total Feed Intake (kg/pig)	81.1	79.3	65.3	64.1
Finisher Feed Cost (€/kg)	0.281	0.296	0.281	0.296
Finisher Feed Cost per pig (€/pig)	22.78	23.49	18.35	18.98
Total Finisher Feed Cost per kg Live Weight Gain (€/kg live weight gain)	0.89	0.85	0.91	0.92

high during the finisher period and diet acidification is expensive, the latter was done to evaluate the efficacy of sodium butyrate while minimizing its impact on feed costs. This particular additive was chosen as it is in a coated form, which ensures delivery to the lower GIT. In addition, there is good evidence for its mechanism of action in terms of reducing *Salmonella* (Van Immerseel et al., 2006). Butyric acid is a short-chain-fatty-acid (SCFA), which down regulates the expression of several *Salmonella* invasion genes including *hilA*, and *invF*,leading to reduced invasion of intestinal epithelial cells (Gantois et al., 2006). As a result, *Salmonella* uptake into the cytosol of epithelial cells is diminished along with caecal colonization (Gantois et al., 2006; Van Immerseel et al., 2006). However, scientific literature on the usefulness of butyric acid and/or its salts for *Salmonella* control in livestock animals, specifically pigs, is scarce, with only one trial reported, in which weaner pigs were supplemented with coated butyrate (Boyen et al., 2008).

The present study is the first on-farm trial to evaluate the efficacy of sodium butyrate as a control measure to reduce *Salmonella* shedding and intestinal carriage in finishing pigs on farms with a history of high seroprevalence. Results showed that the additive was successful in decreasing *Salmonella* shedding over a 28-day period on a highly contaminated farm (Trial A) in the absence of a secondary infection. This is in agreement with previous research, which showed that 12 days of dietary supplementation with coated butyrate tended to reduce *Salmonella* shedding for 3 days post-infection in weaner pigs deliberately infected with *Salmonella* (Boyen et al., 2008). However, the fact that *Salmonella* prevalence increased in the first half of the treatment period in the first trial, provides clear evidence that supplementation with sodium butyrate is not a 'quick fix' in terms of controlling *Salmonella* at farm-level. Similar to earlier studies, a reduction in prevalence was observed only after several weeks of treatment (Creus et al., 2007; Argüello et al., 2013b), which supports the idea that the duration of administration is one of the key factors affecting the success of *Salmonella* control using in-feed organic acids or their salts. Cost is also an important issue for the primary producer and increasing the treatment period increases feed costs. Therefore, it is necessary to identify the minimum period for efficacy of any feed additive for it to be commercially viable.

However, in the second trial, sodium butyrate did not reduce faecal shedding of *Salmonella*. As diarrhoea was common in pigs during Trial B, laboratory analysis was performed on faecal samples to investigate the presence of other intestinal pathogens, i.e. *Brachyspira hyodisenteriae*, *Brachyspira pilosicoli* and *Lawsonia intracellularis*. Interestingly, *L. intracellularis* was detected in these samples (data not shown). Infection with multiple pathogens is commonly observed as part of the porcine intestinal disease complex (Brockmeier et al., 2002) and may help to explain why sodium butyrate had no effect on *Salmonella* shedding during the second trial. This suggests that factors such as concomitant diseases may impact the efficacy of sodium butyrate used to control *Salmonella*. Laboratory diagnostic investigations should therefore be performed during feeding trials, when clinical signs are present. Moreover, in this second trial, animals were already infected with *Salmonella* at the start of the finisher period, whereas pigs were artificially exposed to the organism one week before the study commenced in the first trial. This difference in infection onset may have also contributed to the lack of effect from sodium butyrate administration in the second trial.

All faecal, caecal and ILN/MLN isolates obtained from the first trial were identified as *S.* 4,[5],12:i:-, a monophasic variant of *S.* Typhimurium. This was expected, as all finisher pens in this trial had been artificially contaminated with this serotype to mimic natural environmental contamination. This serotype has become widespread in pigs in recent years (Mueller-Doblies et al., 2013; Argüello et al., 2014; Burns et al., 2015) and is increasingly associated with human salmonellosis (EFSA, 2010). In the second trial, the *Salmonella* isolates recovered from the caecal digesta and the ILN/MLN were identified as both *S.* Typhimurium and *S.* 4,[5],12:i:-, although the former predominated. Interestingly, *S.* 4,[5],12:i:- was not recovered from faecal samples on the farm but was recovered from the two lairage pens at the abattoir, prior to entry of the pigs. This suggests that the pigs may have acquired a new infection in the lairage.

The serological results confirmed the successful establishment of *Salmonella* infection in both trials. All pigs in the first trial were seronegative before the trial commenced, which was why artificial environmental contamination was performed. Significant differences in seroprevalence were detected at the end of the trial using a 20% OD cut-off. This is one of two cut-off values most commonly used in the *Salmonella* control programme in Ireland and Denmark (Alban et al., 2002; Department of Agriculture Food and the Marine (DAFM), 2010). While a lower seroprevalence was observed in the sodium butyrate-treated group, in agreement with faecal shedding data, pigs in this group would still be considered high seroprevalence, i.e. > 50%. In the second trial the serology data revealed that half, or more than half, of the control pigs and only 1–3 pigs in the sodium butyrate group were already infected by *Salmonella* at the beginning of the finishing period when using the 20% and 40% OD cut-off values. The fact that most of the pigs had seroconverted by the end of the trial indicates that the infection pressure was similar in both groups and is in agreement with the bacteriological results obtained. As in the first trial, the pigs fed sodium butyrate were high seroprevalence, i.e. > 50% at the end of the trial. Such high values mean a number of restrictions during slaughter would apply to pigs from this herd according to the Irish NPSCP regulations. These findings are in contrast to those of other studies, which showed that dietary supplementation with organic acids and/or

their salts reduces seroprevalence to below the cut-off for high risk herds used in serology-based control programmes in Europe (Creus et al., 2007; Visscher et al., 2009; Argüello et al., 2013b). Creus et al. (2007) and Argüello et al. (2013b) showed that finishing pigs supplemented with a combination of lactic and formic acid in feed for 14 weeks and potassium diformate in feed for 7 weeks, respectively, resulted in a reduction of *Salmonella* prevalence. It is possible that a longer duration of treatment in the present study would have reduced *Salmonella* seroprevalence to below the high seroprevalence threshold; however, this would certainly have increased the financial cost of the intervention.

Ideally, control measures used on-farm should reduce *Salmonella* carriage in pigs at slaughter. Numerous studies have shown a reduction of *Salmonella* in caecal digesta and/or lymph nodes when acidified feed is used (Creus et al., 2007; Boyen et al., 2008; Visscher et al., 2009; Willamil et al., 2011; Argüello et al., 2013b); however, there are others that failed to show a significant effect (De Busser et al., 2009; Michiels et al., 2012; Argüello et al., 2013b). The fact that no significant differences in *Salmonella* detection in the caecal digesta or ILN/MLN were observed for either trial in the present study adds to the inconclusive nature of the evidence. Factors such as, stress, the period of feed withdrawal and mixing of pigs, which can lead to contamination during transport and lairage can suppress effects seen at farm-level (Argüello et al., 2012; Mannion et al., 2012). The fact that a new serotype was isolated from some of the pigs post-slaughter and that this serotype was also isolated from the lairage holding pens prior to entry of the trial pigs supports this suggestion.

In addition to evaluating the efficacy of in-feed sodium butyrate as a *Salmonella* control measure, effects on growth performance and an associated cost-benefit analysis were also investigated. Many studies have shown that dietary supplementation with organic acids and/or their salts is beneficial to the growth performance of pigs (Gálfi and Bokori, 1990; Partanen and Mroz, 1999; Øverland et al., 2000; Mroz et al., 2002; Partanen et al., 2002; Lawlor et al., 2005, 2006; Creus et al., 2007; Walsh et al., 2007; Øverland et al., 2009; Gebru et al., 2010; Htoo and Molares, 2012; Upadhaya et al., 2014). Few studies, however, have investigated the cost-benefit of diet acidification (Gálfi and Bokori, 1990; Creus et al., 2007). Goldbach and Alban (2006) provided an economic analysis of four different *Salmonella* control strategies in Denmark, including the use of acidified feed for slaughter pigs. While the authors noted that acidified feed reduced *Salmonella* prevalence, they determined that it did so at a net financial cost to the primary producer (Goldbach and Alban, 2006). These authors, however, failed to perform a cost-benefit analysis which considered improvements in growth and feed efficiency as a result of diet acidification. Gálfi and Bokori (1990) had earlier showed that sodium butyrate was an effective growth promoter in pigs between weaning and slaughter when it reduced feed costs by 9% and increased sales receipts by 13% (Gálfi and Bokori, 1990). While the present study had a much shorter treatment period, a 7% increase in growth rate and an 8% improvement in FCE were found during the 28 day trial in response to feeding sodium butyrate in Trial A. Although sodium butyrate supplementation added €0.71 to the feed cost of a pig during the trial, when growth and FCE are considered, strategic dietary supplementation with sodium butyrate for approximately 28 days prior to slaughter reduced feed cost per kg live weight gain by €0.04. As the numerical improvements in ADG and FCE were less in Trial B, supplementation with sodium butyrate increased feed cost per kg live weight gain during the trial by a marginal €0.01. The reason for the latter was most likely a consequence of the *Lawsonia* infection detected in pigs during this trial.

5. Conclusions

Overall, strategic feeding of sodium butyrate to finishing pigs for a relatively short period of time (< 30 days) immediately prior to slaughter was effective in reducing *Salmonella* shedding and seroprevalance in one of two trials. Lack of efficacy in the second trial may be explained by a concomitant infection with *L. intracellularis*. Sodium butyrate supplementation did not reduce intestinal carriage, nor did it reduce seroprevalence to below the cut-off used for the high *Salmonella* risk category in Ireland (50%). Although it did not significantly improve growth performance, the numerical improvements found, for both growth rate and FCE, were sufficient to reduce feed cost by €0.04 per kg of live-weight gain in the absence of concomitant enteric infections.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.prevetmed.2016.07.009.

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Original Article

Evaluation of an Alternative Experimental Infection Method, Which Closely Mimics the Natural Route of Transmission of Monophasic *Salmonella* Typhimurium in Pigs

Helen Lynch,[1,2] Hector Argüello,[1] Kavita Walia,[1,3] Peadar G. Lawlor,[4] Geraldine Duffy,[1]
Gillian E. Gardiner,[3] and Finola C. Leonard[2]

Abstract

Salmonella carriage in pigs is a significant food safety issue. This study describes a new protocol of *Salmonella* infection based on exposure to an artificially contaminated environment that closely mimics natural exposure to the organism. The aim of the study was to develop and evaluate the effectiveness of this protocol, which could then be used as a tool in the investigation of control measures. In addition, *Salmonella* shedding pattern and growth performance of the pigs were examined. Trial pigs ($n = 10$) were placed in a pen that had been previously contaminated by housing two pigs experimentally challenged with a monophasic *Salmonella* Typhimurium (mST). A further 10 pigs were placed in a *Salmonella*-free pen. Pigs were weighed on days 0 and 28. Feces was collected on days 0, 2, 3, 5, 7, 14, 21, and 28 and examined for the presence and quantity of *Salmonella*. The trial was replicated once. All pigs in the contaminated pens shed *Salmonella* within the first 2 days of exposure with values ranging from 10^0 to 10^4 CFU/g. The noninfected pigs had significantly higher final body weights on day 28 than those exposed to the *Salmonella* contaminated environment in both replicates. The pigs in the *Salmonella*-free pen had significantly higher average daily weight gain over the 28-day period compared to the infected animals ($p < 0.001$). Although not significant, numerical improvements in average daily feed intake and feed conversion efficiency were observed in the *Salmonella*-free pigs when compared to the contaminated pigs. The approach used was successful in infecting pigs with *Salmonella* without the need for direct inoculation or exposure to seeder pigs. This "natural" method of infection in which pigs are exposed to low levels of environmental contamination with *Salmonella* may be an effective tool that could be utilized when investigating control measures.

Keywords: infection protocol, pig, *Salmonella*, monophasic *Salmonella* Typhimurium.

Introduction

Salmonella is one of the most important foodborne pathogens. In 2013, 8.9% of European human cases of salmonellosis were linked to the consumption of pork or pork products (EFSA, 2015). Recently, monophasic *Salmonella* Typhimurium (mST) has emerged and is now one of the most commonly isolated serovars from humans in many EU countries (Mandilara *et al.*, 2013). Confirmed cases of human nontyphoidal salmonellosis linked to mST increased by 68% between 2011 and 2013, with four additional EU member states reporting this variant in 2013 compared to 2011 (EFSA, 2015). In addition, this variant has a high rate of multidrug resistance associated with it (EFSA, 2010; Argüello *et al.*, 2014; Yang *et al.*, 2015). A clone that is resistant to ampicillin, streptomycin, sulfonamides, and tetracycline (R-type ASSuT) has been implicated in many outbreaks (Mossong *et al.*, 2007; Bone *et al.*, 2010; Barco *et al.*, 2014).

This is a major public health concern and highlights the need for control measures, often including interventions at the primary production level (Boyen *et al.*, 2008). Previous studies have examined the efficacy of control measures through experimental inoculation or field trials. Experimental inoculation studies typically challenge the pigs with high doses of up to 10^9 CFU of *Salmonella* (Fedorka-cray *et al.*, 1995; Hurd *et al.*, 2003; Boughton *et al.*, 2007; Casey *et al.*,

[1]Food Safety Department, Teagasc Food Research Centre Ashtown, Dublin, Ireland.
[2]UCD Veterinary Sciences Centre, University College Dublin, Dublin, Ireland.
[3]Department of Science, Waterford Institute of Technology, Waterford, Ireland.
[4]Teagasc, Pig Development Department, Moorepark, Ireland.

2007) or alternatively use seeder pigs shedding high numbers of organisms to transmit infection to their pen mates (Michiels *et al.* 2012). Neither of these approaches closely mimics natural infection as it occurs on most farms. The high doses of *Salmonella* used in these models often result in clinical signs and fecal excretion of high numbers of bacteria, thereby causing the infection pressure to become excessive for the realistic evaluation of control strategies.

Field trials are useful in that they use the natural *Salmonella* infection already present on-farm, but due to the multifactorial nature of *Salmonella* infection on farms, results vary greatly between herds and between studies (Wales *et al.*, 2011), making it difficult to fully assess the effectiveness of the implemented interventions. The present study describes a new protocol for experimental *Salmonella* infection based on exposure to an artificially contaminated environment that closely mimics natural exposure to the organism. The aim of the study was to develop and evaluate the feasibility and reproducibility of this protocol as well as to examine the *Salmonella* shedding pattern and growth performance of the infected pigs compared to noninfected batch mates.

Materials and Methods

Animal ethics and experimental licensing

Ethical approval was obtained from the University College Dublin Animal Research Ethics Committee (Approval number: AREC 15 09), and an experimental authorization was obtained from the Irish Health Products Regulatory Authority (Authorization number: AE19113/P005).

Animals and facilities

Seven-week-old [PIC337 × (Landrace × Large-White)] pigs with a mean weight of 14.7 kg were loose-housed in boxes adapted for holding pigs. Each box included a clean area and a pen (12 m^2) with a solid concrete floor. Pig density complied with Irish and EU legislation (EU Directive 2010/63). The pen floor, apart from the dunging area, was covered with a deep layer of sawdust. A feed hopper was provided in each pen, together with a nipple drinker. Average temperature was maintained at 23°C with a relative humidity of 88%. Pigs were sourced from a commercial herd with 0% seroprevalence levels according to the Irish National *Salmonella* Control Programme (NPSCP) classification level for the 3 months before the commencement of the trial. Fecal samples were collected from the herd 2 weeks before the purchase of the pigs and tested for the presence of *Salmonella* to ensure the pigs were not shedding the organism in their feces.

Environmental contamination

A field isolate of *Salmonella enterica* subspecies *enterica* serovar 4,[5],12:i:-, phage type DT193 with multiple drug resistance pattern ASSuT (Ampicillin [A], streptomycin [S], sulfonamide [Su], and tetracycline [T]) was streaked to extinction on Plate count agar (PCA) to achieve single colonies. This was incubated at 37°C for 24 h. Two colonies from the PCA plate were inoculated into 10 mL of Tryptone soya broth (TSB) and incubated at 37°C for 24 h. This pure broth culture with an expected concentration of 10^8 CFU/mL was diluted in phosphate-buffered saline (PBS) to obtain a concentration of 10^7 CFU/mL. This culture was then further diluted 1:10 in PBS to achieve a final concentration of 10^6 CFU/mL. Two female 7-week-old pigs were orally inoculated with 5 mL (10^6 CFU/mL) of this inoculum by oral gavage. These pigs were placed in one of the pens where they were provided with *ad libitum* access to feed and water.

The animals were allowed to shed *Salmonella* in their feces for 5 days at which point they were removed from the pen and euthanized. Following their removal, the feces in the defecating area of the pen was mixed to ensure more even distribution of contamination levels before entry of the trial pigs into the contaminated environment. A pooled fecal sample (10 g) was collected from five points in the defecating area, and the level of *Salmonella* was found to be 2.51×10^3 CFU/g of feces in the first replicate and 7.94×10^3 CFU/g of feces in the second replicate.

Pen swabs were collected from a negative control pen also to confirm the absence of *Salmonella*. Trial pigs ($n = 10$), confirmed as *Salmonella* free, were then placed in the *Salmonella*-contaminated pen. A further 10 pigs were placed in the *Salmonella*-free pen. A compulsory biosecurity protocol was followed by all personnel moving between the *Salmonella*-free pen and the *Salmonella*-contaminated pen that included use of different boots, boot covers, and overalls. The trial was replicated once, with the second replicate performed following full cleaning and disinfection of the pens and challenge of another two pigs as for the first replicate.

Sampling

Feces were collected from each pig by digital rectal stimulation on days 0, 2, 3, 5, 7, 14, 21, and 28, with day 0 being the day of entry to the trial pens. All samples were collected and handled aseptically to avoid cross-contamination.

Blood was collected from each pig by jugular venepuncture for serological analysis on day 0 to ensure pigs were *Salmonella* free and day 28 of the experiment. Samples were collected using plastic tubes for whole blood (BD Vacutainer, Becton Dickinson, United Kingdom). Sera was obtained after coagulation and centrifugation of the tubes (1500 rpm for 10 min) and stored at −20°C until analysis.

Individual pig weights were recorded on days 0 and 28. Feed disappearance was recorded throughout the trial. These weights were used to calculate average daily feed intake (ADFI), average daily weight gain (ADWG), and feed conversion efficiency (FCE) (Lawlor *et al.*, 2006). Pigs were checked daily to ensure they were bright, alert, eating, and drinking normally. The incidence and severity of diarrhea were recorded by visual examination of fecal consistency and were scored on a scale of 1–4 as follows: 1—Normal formed feces, 2—soft feces, 3—liquid feces, and 4—watery feces.

Sample analyses

All samples were stored at 4°C and analyzed within 24 h. Feces (10 g) were screened for the presence/absence of *Salmonella* using the International Standard Organization 6579:2007 (Amendment 1: Annex D) method (ISO 6579:2002/Amd.1:2007). *Salmonella* was also enumerated in the feces using a miniaturized most probable number (MPN) technique following the ISO 6579-2:2012 standard. The number of wells giving a positive confirmed reaction for each dilution was recorded and used to calculate the MPN using relevant software (Jarvis *et al.*, 2010).

Serum samples were analyzed in duplicate by the Department of Agriculture Food and Marine (Ireland) using an in-house indirect Enzyme-Linked Immunosorbent Assay (ELISA) (Nielsen *et al.*, 1995). Testing was performed in accordance with the methods used for serological monitoring in the current National *Salmonella* Control Programme. The crude optical density (OD) values of the unknown samples were adjusted with OD values of the positive and negative controls [(sample−negative control/positive control−negative control)×100]. Two different cutoffs were fixed at optical densities of 10% and 20% to elucidate the seroconversion and seroprevalence.

Statistical analyses

All statistical analyses were performed using SAS 9.3 (Cary, NC). Statistical differences among the number of shedders at each sampling point and the concentration of *Salmonella* in feces were estimated by a chi-square test and the mixed model procedure, respectively. For growth performance, data were analyzed using mixed models and initial pig weight was used as a covariate for the analysis of pig weight at day 28, feed intake, average daily gain, and FCE. Means for serology were separated using the Tukey–Kramer least square means adjustment for multiple comparisons and evaluated as the presence of *Salmonella* antibodies in serum. Significant differences were established at $p \leq 0.05$.

Results

Pig health

Two of four of the orally challenged pigs developed diarrhea (score 4) 5 days postinoculation. Eight of 20 pigs exposed to contaminated pens developed transitory diarrhea (score 3) during week 1 of the trial. One of the 20 control pigs also showed mild diarrhea on a single day during the first week, but this was not linked to *Salmonella* (negative feces in the analysis). All animals remained bright and alert and continued to eat and drink throughout the study.

Salmonella *shedding*

Salmonella was not detected in any fecal samples taken before introduction of the pigs into the contaminated environment or in the *Salmonella*-free pen at any stage. All pigs in the contaminated pen shed *Salmonella* in their feces within the first 2 days of exposure, with values ranging from 10^0 to 10^4 CFU/g (Fig. 1). Although the feces of all pigs, except two, were positive in both replicates up to day 14 (Table 1), marked differences were detected on day 21 ($p < 0.001$) when all feces from replicate 1 pigs were negative. Three pigs from this replicate shed *Salmonella* again 7 days later (Table 1). This abrupt cessation of shedding was not observed in the last two samplings for replicate 2. The levels of *Salmonella* in the feces reduced after day 7, as did the variability in the quantity of *Salmonella* shed by pen mates (Fig. 1). In the first replicate, a significant decrease was observed in the mean concentration of *Salmonella* detected in feces between day 7 and 21 ($p < 0.001$) and levels reduced to as low as 10^0 CFU/g on day 28.

Serology

The serological results were based on 10% OD and 20% OD cutoff points. On day 0, before introduction of pigs to the contaminated environment, all pigs were seronegative. The negative control group remained seronegative throughout the trial. By day 28 most of the pigs in the *Salmonella*-positive group (17 pigs out of 20) had seroconverted using both the 10% OD and 20% cutoff points

Growth parameters

The noninfected pigs had significantly higher ($p < 0.001$) final body weights on day 28 than those exposed to the contaminated pens in both replicates (Table 2). The pigs in the *Salmonella*-free pen had significantly higher ADWG over the 28-day period compared to the infected animals ($p < 0.001$). Although not significant, numerical improvements in ADFI and FCE were observed in the *Salmonella*-free pigs when compared to the contaminated pigs.

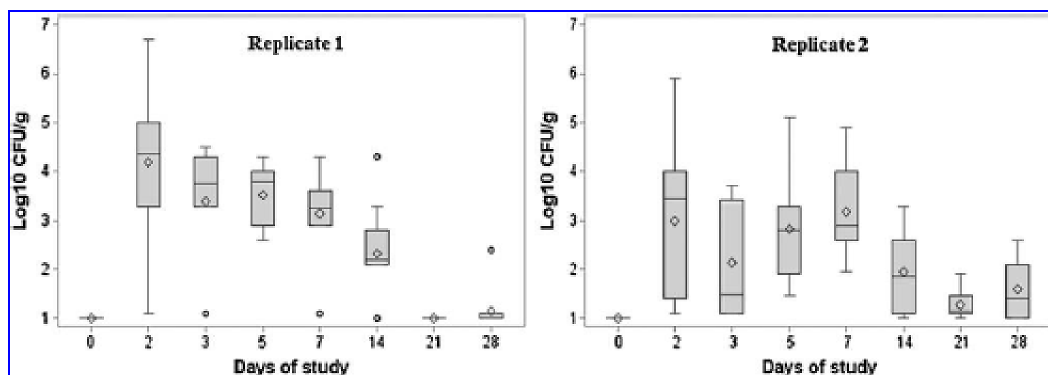

FIG. 1. Concentration of *Salmonella* in feces from 10 pigs in each of two replicates after their introduction to a contaminated environment.

Table 1. *Salmonella* Detection in Feces Collected from Pigs Introduced to an Environment Contaminated with Monophasic *Salmonella* Typhimurium During a 28-Day Monitoring Period

	No. pigs positive for Salmonella *(%* Salmonella *prevalence)*							
	Day 0	*Day 2*	*Day 3*	*Day 5*	*Day 7*	*Day 14*	*Day 21*	*Day 28*
Replicate 1								
No. positive pigs	0 (0)	10 (100)	10 (100)	10 (100)	10 (100)	8 (80)	0 (0)	3 (30)
No. negative pigs	10 (100)	0 (0)	0 (0)	0 (0)	0 (0)	2 (20)	10 (100)	7 (70)
Replicate 2								
No. positive pigs	0 (0)	10 (100)	10 (100)	9 (90)	10 (100)	8 (80)	9 (90)	6 (60)
No. negative pigs	10 (100)	0 (0)	0 (0)	1 (10)	0 (0)	2 (20)	1 (10)	4 (40)

Discussion

In this study, we aimed to develop a protocol of experimental infection that closely mimics natural on-farm exposure of pigs to *Salmonella*. We wished to simulate mild or subclinical infection rather than clinical disease, as this is what is commonly observed on commercial farms (Beloeil *et al.*, 2003). Using this protocol, where the environment was contaminated before entry of the animals, the pigs were exposed to *Salmonella* in a way that closely reflects that encountered on a commercial farm. On day 0 before the introduction of the pigs to the contaminated environment, all pigs were seronegative and *Salmonella* was not detected in any of the fecal samples showing that any *Salmonella* isolated was as a result of infection from the mST present in the pen.

Rapid infection was observed, with all pigs infected within the first 2 days of exposure, despite environmental concentrations being considerably lower than the oral doses usually used in challenge studies. Trial pigs were exposed to an environment contaminated with 10^3 CFU/g of feces. In their study of contamination levels in the lairage, Loynachan and Harris (2005) observed that at least 10^3 CFU/g of feces was required to infect pigs by a contaminated environment (Loynachan and Harris, 2005).

Mild clinical signs were observed in some (8 of 20) exposed pigs, and *Salmonella* was not isolated from the negative control group at any time. Diarrhea seen in the *Salmonella*-free pen was self-limiting and occurred during the first week. The commercial herd from which the pigs were sourced had no recorded occurrence of dysentery, intestinal spirochetosis, clinical ileitis, postweaning *Escherichia coli* diarrhea in 7–8-week-old pigs, or any infectious intestinal disorder such as porcine epidemic diarrhea. Thus, we ascribed the diarrhea in the negative control group to the change in diet and possible stress associated with the change of environment as *Salmonella* was not detected in the feces of the negative control animals.

Table 2. Growth Parameters of Growing Pigs in *Salmonella*-Infected and *Salmonella*-Free Groups, After 28 Days of Study (Mean of Two Replicates)

	Salmonella *contaminated pen*	Salmonella-*free pen*	*SEM*	p
Weight (kg)				
Day 0	14.5	14.7	0.1895	0.6002
Day 28	31.9	35.3	0.3493	0.001
ADFI (g/day)				
Day 0–28	1098	1179	42.06	0.423
ADWG (g/day)				
Day 0–28	614	733	12.47	0.001
FCE (g/g)				
Day 0–28	1.88	1.63	0.1994	0.7259

ADFI, average daily feed intake; ADWG, average daily weight gain; FCE, feed conversion efficiency.

With regard to the diarrhea in the *Salmonella* contaminated pen, other factors may have had some influence. *Salmonella* is usually a subacute subclinical infection under natural conditions, but the combination of a virulent serotype with pigs from a *Salmonella*-free herd (no maternal antibodies) may have exacerbated the infection with resultant mild clinical signs. Ideally, if resources had been unlimited, it would have been interesting to try several different contamination strategies, including lower levels of contamination with different groups of pigs. The majority of pigs shed *Salmonella* for at least 7 days at concentrations of 10^0–10^4 CFU/g. This is in contrast to other studies where pigs were experimentally infected or seeder pigs were used, in which pigs frequently shed 10^6–10^8 CFU/g in the first few days postinfection (Boyen *et al.*, 2008).

An important difference between the present protocol and studies which use "seeder" pigs is that the trial pigs are not mixed with infected animals that are shedding unknown concentrations of *Salmonella*, but instead are introduced to a contaminated environment where the *Salmonella* concentrations are quantified. In this way, the concentration of *Salmonella* to which the trial pigs are exposed is known and can be controlled at least in part to ensure that the infectious dose is lower than that given in most experimental challenge studies. Although differences in the amount of feces analyzed, methodology used, and serotype involved may explain in part the variability reported in published studies, it is likely that challenge doses of up to 10^9 CFU frequently used in experimental infection models are in excess of levels of exposure experienced during natural infection. This makes the efficacy and practical application of potential control strategies difficult to fully assess in such studies.

Nevertheless, challenge studies using direct inoculation are useful as they provide a more controlled method of experimental infection than field trials, which can be associated with many logistical and compliance problems. The protocol described in this article may provide a useful alternative method of infection that retains some of the advantages of

challenge studies while substantially removing the unpredictable aspects of field studies. During natural infection, pigs commonly exhibit intermittent shedding of low numbers of the organism in their feces after the initial acute phase of infection (Beloeil *et al*., 2003). The number of positive pigs and the concentration in feces (10^4–10^0 CFU/g) started to decrease after 2 weeks, which is in agreement with other studies (Boyen *et al*., 2009).

The intermittent nature of shedding after the initial phase of infection should be taken into account when interventions are evaluated for longer than 2 weeks postinfection. Despite the fact that pigs in both replicates of the study were exposed to the same concentration of *Salmonella* in the environment and were obtained from the same herd and at the same age, there were differences in *Salmonella* shedding between replicates; the number of positive animals was consistent between replicates up to day 14 but varied thereafter (Table 1).

In addition, greater variability was observed in replicate 2 in terms of the numbers of organisms shed by individual pigs (Fig. 1). This confirms that variability can be expected, not only among pigs in the pen but also between replicates of the study. Variation between individual animals would be expected in animals infected by other methods also, including natural infection due to individual host factors of susceptibility and immune response.

As expected, exposure to *Salmonella* affected all production parameters measured, including ADWG, similar to that found in previous studies (Bruno *et al*., 2013).

Conclusion

This design of experimental infection involving exposure to a contaminated environment was chosen as it closely reflects the natural route of transmission and exposure to *Salmonella* that is encountered on farm. Infection was successful without the need to inoculate or mix with seeder pigs. This protocol may be an effective tool, which could be utilized when investigating control measures for *Salmonella* for use on commercial farms.

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Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Helen Lynch, BSc
Food Safety Department
Teagasc Food Research Centre Ashtown
Dublin 15
Ireland

E-mail: helen.lynch@teagasc.ie

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Effect of strategic administration of an encapsulated blend of formic acid, citric acid, and essential oils on *Salmonella* carriage, seroprevalence, and growth of finishing pigs

Kavita Walia[a,b,e], Hector Argüello[a], Helen Lynch[a,c,e], Finola C. Leonard[c], Jim Grant[a], Dermot Yearsley[d], Sinead Kelly[d], Geraldine Duffy[a], Gillian E. Gardiner[b,*], Peadar G. Lawlor[e]

[a] Teagasc, Food Research Centre, Ashtown, Dublin 15, Ireland
[b] Department of Science, Waterford Institute of Technology, Waterford, Ireland
[c] School of Veterinary Medicine, University College Dublin, Dublin, Ireland
[d] Department of Agriculture, Food and the Marine, Central Veterinary Research Laboratory, Backweston, Ireland
[e] Teagasc, Pig Development Department, Animal and Grassland Research and Innovation Centre, Moorepark, Fermoy, Co. Cork, Ireland

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ABSTRACT

Controlling *Salmonella* at farm level can act as the first line of defence in reducing salmonellosis from pork. This study investigated the efficacy of an encapsulated blend of formic acid, citric acid, and essential oils (FormaXOL™) administered to finisher pigs for 28 days prior to slaughter in controlling *Salmonella* shedding on a commercial farm with a history of high *Salmonella* seroprevalence.

Fourteen pens of 8–10 pigs/pen were randomly assigned to a control (finisher diet without additive) or a treatment group (the same diet with 4 kg/t of FormaXOL™) for 28 days. Faeces were collected from each pig on days 0, 14, and 28, while on day 29 blood, caecal digesta and ileocaecal-mesenteric lymph nodes were collected at slaughter. Pigs were weighed at the start and end of the trial, feed intake was recorded, and carcass quality parameters were recorded at slaughter.

On day 14, *Salmonella* shedding was reduced in the treatment compared to the control group (27.9% versus 51.7% probability of detecting *Salmonella* in faeces, respectively; p = 0.001). However, on day 28, no reduction was observed (20.6% versus 35.9% probability of detecting *Salmonella* in faeces, respectively; p = 0.07). Interestingly, *Salmonella* shedding rates in the treated pigs remained stable throughout the trial compared to the control group. This suggests that the feed additive prevented additional pigs from acquiring the *Salmonella* infection. A lower *Salmonella* seroprevalence was detected at slaughter in the treatment compared to the control group using the 40% optical density cut-off (64.5% versus 88.5%, respectively; p = 0.01). However, no significant differences in *Salmonella* recovery rates were observed in the caecal digesta or lymph nodes between treated and control groups. Treated pigs had a lower feed intake than pigs fed the control diet (p = 0.001); however, average daily gain and feed conversion efficiency were not affected by treatment (p = 0.45 and 0.55, respectively). Consequently, supplementing the diet with FormaXOL™ for 28 days increased the feed cost per kg of live-weight gain by €0.08.

Overall, results suggest that strategic administration of an encapsulated blend of formic acid, citric acid, and essential oils, to finishing pigs for 28 days prior to slaughter has potential to prevent increased *Salmonella* shedding at certain time points as well as seroprevalence. However, this additive did not lower intestinal carriage, nor did it reduce seroprevalence to below the cut-off used for the high *Salmonella* risk category in Ireland (50%) or improve growth performance.

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Abbreviations: NPSCP, national pig *Salmonella* control program; ILN, ileocaecal lymph nodes; MLN, mesenteric lymph nodes; ADFI, average daily feed intake; ADG, average daily gain; FCE, feed conversion efficiency; SCFA, short chain fatty acid; MCFA, medium chain fatty acid; ATP, adenosine triphosphate; AMR, antimicrobial resistance; TSB, tryptone soya broth; PBS, phosphate buffered saline; GIT, gastrointestinal tract; BPW, buffered peptone water; MSRV, modified semi-solid rappaport-vassiliadis; XLD, xylose lysine deoxycholate; BG, brilliant green; PCA, plate count agar; ELISA, enzyme-linked immunosorbent assay; SAS, statistical analyses system; OD, optical density; CI, confidence interval.

* Corresponding author.
E-mail address: ggardiner@wit.ie (G.E. Gardiner).

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1. Introduction

Globally, *Salmonella* is one of the most common causes of foodborne disease in humans and pork is considered an important source of human salmonellosis (EFSA, 2008). In the latest summary report on trends and sources of foodborne outbreaks within the European Union (EU), 225 foodborne outbreaks were linked to *Salmonella* (EFSA, 2015). Of these, 9.3% were linked to the consumption of pork, the third most commonly reported food vehicle after eggs and egg products and bakery products. The non-typhoidal *Salmonella* serotypes that cause human infection are usually carried asymptomatically in pigs, causing little or no clinical signs of disease (Callaway et al., 2008). As such, pigs become reservoirs for *Salmonella* contamination along the production chain (Rodriguez et al., 2006; Ojha and Kostrzynska, 2007; Dorr et al., 2009; Duggan et al., 2010). The most recent EU survey in slaughter pigs showed that *Salmonella* prevalence in intestinal lymph node samples was 10.3% and that 8.3% of carcasses were contaminated, indicating the extent of the problem (EFSA, 2008).

Controlling the introduction, persistence, and transmission of *Salmonella* at farm level is therefore often the first line of defence in reducing human salmonellosis. Various control measures have been investigated in pigs to date, including dietary supplementation with organic acid feed additives (Berge and Wierup, 2012; De Busser et al., 2013; Walia et al., 2016). Generally, these organic acids are short- and medium-chain fatty acids (SCFA, MCFA), which, when used in an un-dissociated form ultimately disrupt vital metabolic processes within the bacterial cell, leading to cell death (Van Immerseel et al., 2006). Essential oils have also been shown to exhibit anti-*Salmonella* activity, mainly acting via membrane disruption, non-specific permeabilization of cell membranes, leakage of adenosine triphosphate (ATP) and potassium/hydrogen ions, inhibition of ATPase activity, and an increase in the fluidity of phospholipid bilayers (Burt, 2004; Oussalah et al., 2007; Bakkali et al., 2008; Barbosa et al., 2009; Berge and Wierup, 2012; Hyldgaard et al., 2012; Langeveld et al., 2014).

However, to our knowledge, only three in vivo studies to date have investigated essential oils as a dietary strategy for *Salmonella* reduction in pigs (Ahmed et al., 2013; Michiels et al., 2012; Rasschaert et al., 2016). Furthermore, despite the number of field studies that have evaluated in-feed organic acids for the control of *Salmonella* in pigs, only two of the studies above evaluated an essential oil in combination with organic acids and only one was conducted in finishers. Moreover, none of these studies performed a cost-benefit analysis. Additionally, no field trial to our knowledge, has evaluated the efficacy of an encapsulated blend of formic acid, citric acid, and essential oils as a dietary additive for *Salmonella* control in finishing pigs. Previous studies showed success in reducing *Salmonella* in finishing pigs when supplemented with various organic acid feed additives, i.e., potassium diformate, lactic-formic acid, formic-propionic acid for a minimum of 7 weeks (Creus et al., 2007; Visscher et al., 2009; Argüello et al., 2013a). Yet, few have evaluated a shorter duration of feeding (i.e., <30 days) as a low-cost approach to controlling *Salmonella* at farm level (Walia et al., 2016). Additionally, the economic value of administering a formic-citric acid and essential oil blend to finishing pigs for such a short period prior to slaughter, is absent from published literature. Therefore, given these knowledge gaps, the present study aimed to investigate the ability of targeted dietary supplementation with an encapsulated blend of formic acid, citric acid, and essential oils, during the last 28 days of the finishing period, to reduce faecal shedding, intestinal carriage, and *Salmonella* seroprevalence, together with an evaluation of its impact on growth performance.

2. Materials and methods

2.1. Animal ethics and experimental licensing

The feeding trial was performed on a commercial pig farm in the last quarter of 2015. Ethical approval was obtained from the Waterford Institute of Technology ethics committee and an experimental license was obtained from the Irish Department of Health and Children (number B100/2982). All animals were handled in a humane manner and were slaughtered in a regulated abattoir.

2.2. Experimental procedure

The feeding trial was conducted on a 90 sow farrow-to-finish farm. The finisher house in which the trial was conducted consisted of a barn with 14 pens. One hundred and twenty four finisher pigs (70 males and 54 females; in 14 pens of 8–10 same gender pigs per pen), managed as a single all-in-all-out group, were used in the experiment. Each pig was ear tagged with a unique number for identification purposes. Each pen was 4.5 m × 2.8 m with concrete slatted floors and ad-libitum access to water was provided from 2 nipple drinkers per pen. The temperature of the barn was maintained at ∼20 °C. Ad-libitum access was provided to dry pelleted feed via single-spaced wet-dry feeders.

This herd had a historically high *Salmonella* seroprevalence [data extracted from the National Pig *Salmonella* Control Programme (NPSCP)]; however, the prevalence of the batch of finishing pigs immediately prior to this trial had declined to 0%. In order to guarantee *Salmonella* carriage in the pigs, pens in the finishing house were artificially contaminated with a *S.* 4,[5],12:i:-, which had previously been isolated from sows in the same herd and had an antimicrobial resistance (AMR) profile of ASSuT. Briefly, a single colony of *S.* 4,[5],12:i:- was inoculated into 90 mL of Tryptone Soya Broth (TSB, Oxoid, Basingstoke, UK), incubated overnight at 37 °C and then diluted in phosphate buffered saline (PBS) to a final concentration of $\sim 5 \times 10^3$ CFU/mL. Five 25 mL vials (each containing $\sim 5 \times 10^3$ CFU/mL of *Salmonella*) were spread at five points in each pen: 3 in the defecation area, and 2 near the feeder. The final concentration of *Salmonella* at each inoculation point was therefore expected to be 2.5×10^4 CFU/mL. Contamination of the pens was performed 7 days before commencing the trial.

2.2.1. Diets

Approximately 4 weeks before the target slaughter date, pens of pigs were blocked (7 blocks) by sex and weight and randomly assigned within block, using a random number generator in Excel, to one of two dietary treatments: a standard finisher diet with no feed additive (control group) or the same finisher diet supplemented with 4 kg per tonne of an encapsulated blend of formic acid, citric acid, and essential oils from citrus fruit extract, cinnamon, oregano, thyme, and capsicum (FormaXOL™, Kemin Industries, Inc. Southport, Merseyside, UK). The composition of the trial diets is shown in Supplementary Table S-1. The pigs were fed the experimental diets for 28 days and were fasted for ∼18 h prior to slaughter.

2.2.2. Blood and faecal sampling and measurement of production parameters

For serological analysis, blood was collected by jugular venipuncture, prior to feeding the experimental diets, and during exsanguination at slaughter. All samples were collected using plastic vacutainers for whole blood (BD Vacutainer, Becton Dickinson, Oxford, UK). Serum was obtained after coagulation and centrifugation of the tubes (1500 rpm for 10 min) and was stored at −20 °C until analysis.

On day 0 (the day prior to commencing experimental treatments), day 14 and day 28 (i.e., the final treatment day), faeces (~25 g) was collected from each pig by digital rectal stimulation into 100 mL sterile bottles (Sarstedt, Nümbrecht, Germany). All samples were collected and handled aseptically to avoid cross-contamination.

Feed intake was recorded throughout the trial and individual live weights were recorded on day 0 and day 28. These weights were used to calculate the average daily feed intake (ADFI), average daily gain (ADG), and feed conversion efficiency (FCE). In addition, weight gain, FCE and feed intake over the 28-day feeding period, together with industry prices for the feed and feed additive were used in the cost-benefit analysis for the two experimental diets. Pigs were observed closely at least twice daily. Any pig showing signs of ill health was treated as appropriate. All veterinary treatments were recorded including identity of pig, clinical signs, medication used, and dosage. If a death occurred or antibiotics were administered, the pig(s) were weighed and subsequently removed from the pen(s) and excluded from the trial.

2.2.3. Sampling of truck floors and lairage pens

Swabs were taken from the truck used to transport pigs to the abattoir immediately prior to loading the pigs. Four swabs were taken from the floors of the truck, two from the back of the truck and two from the front of the truck, using sterile sponges pre-soaked with maximum recovery diluent (Technical Services Consultants Ltd, Lancashire, UK). Each swab covered a 40 cm × 40 cm area. Pigs from the treatment group were compartmentalised in the back of the truck, while the control group were confined in the front. Upon arrival at the abattoir, two lairage pens (one for each diet group) were swabbed with sponges, as above, prior to unloading the pigs (3 swabs per pen i.e. front, middle and back of each pen, with each swab covering a 40 cm × 40 cm area).

2.2.4. Samples and measurements collected at slaughter

Caecal digesta, ileocaecal lymph nodes (ILN), and mesenteric lymph nodes (MLN) were collected from the gastrointestinal tract (GIT) of 74 pigs (42 control pigs and 32 treated pigs). These 74 pigs were selected as they shed *Salmonella* at most of the time points throughout the trial. Caecal digesta (~25 g) was collected via puncture of the blind end of the caecum, while ILN and MLN (≥10 g) were removed from the mesentery and pooled for each animal. All samples were collected aseptically into sterile containers to avoid cross-contamination.

The internal organs and digestive tract were removed before measuring hot carcass weight (the head was left on the carcass). The hot carcass weight at harvest was multiplied by 0.98 to obtain the cold carcass weight, which is the value reported in this study as carcass weight. Kill out yield was calculated by expressing cold carcass weight as a percentage of live weight prior to slaughter. Lean meat yield was estimated from back fat and muscle depth measurements taken using a Hennessy Grading probe according to S.I. No. 413 of 2001 (Government Publications, 2001).

2.2.5. Salmonella isolation and serotyping

All samples were kept at 4 °C and tested the same day or within 24 h for the presence of *Salmonella* according to the International Organization for Standardization (ISO) 6579:2007 (Amendment 1: Annex D) method (International Organization for Standardization, 2007). All media were obtained from Oxoid.

The ILN and MLN were first processed according to EC Regulation 668/2006 (Regulation (EC) No 668/2006 (06.10.2006), 2006) by removing the fat and capsula followed by immersion in 90% ethanol (v/v). They were then flamed to sterilize the outer surface, and cut into small pieces using sterile scissors to an approximate weight of 10 g.

Briefly, 25 g of each faecal or digesta sample was homogenized in 225 mL of buffered peptone water (BPW) and 10 g of ILN/MLN was homogenized in 90 mL of BPW. All BPW suspensions were incubated at 37 °C for 19 h, after which 100 μL of each enrichment was inoculated onto modified semi-solid rappaport-vassiliadis (MSRV) agar plates and incubated at 42 °C for 24 h. If the MSRV plate was negative, it was incubated for a further 24 h. Presumptive *Salmonella* growth was then streaked onto xylose lysine deoxycholate (XLD) and brilliant green (BG) agar plates and incubated at 37 °C for 24 h. Suspect colonies from XLD or BG agar plates were then streaked onto plate count agar (PCA), and incubated at 37 °C for 24 h. Urea agar slants and *Salmonella* chromogenic agar plates were then inoculated with colonies from the PCA plates and incubated at 37 °C for 24 h. Serological confirmation of colonies from PCA was performed using a *Salmonella* latex agglutination kit (Oxoid). All presumptive *Salmonella* isolates recovered were banked onto beads and stored at −80 °C for further characterization.

All presumptive *Salmonella* isolates were first tested using the real-time polymerase chain reaction (PCR) assay for the identification and differentiation of *Salmonella enterica* serotype Typhimurium and *S.* 4,[5],12:i:- as described by Prendergast et al. (2013). If isolates were not identified as *S.* Typhimurium or its monophasic variant, then serotyping was performed according to the White-Kauffmann-Le Minor scheme (Grimont and Weill, 2007) using commercial antisera (Pro-Lab Diagnostics, Cheshire, UK; SIFIN Institute, Berlin, Germany; and Statens Serum Institute, Copenhagen, Denmark).

2.3. Salmonella serological analysis

Serum samples were analysed in duplicate using an in-house indirect Enzyme-Linked Immunosorbent Assay (ELISA) (Nielsen et al., 1995). Testing was performed by the Department of Agriculture Food and Marine (Ireland) in accordance with the methods used for serological monitoring in the current NPSCP. The crude optical density (OD) values of the unknown samples were adjusted with OD values of the positive and negative controls [(sample − negative control)/(positive control − negative control)] X 100. The mean of the adjusted OD values of tested samples were used to compare the control and treatment groups. Cut-offs were fixed at ODs of 20% and 40%, according to previous studies (Nielsen et al., 1995; Argüello et al., 2013b).

2.4. Statistical analysis

The experiment was a randomised complete block design with treatment applied at the pen level. Within block, pens of pigs were fed with or without FormaXOL™ in the diet.

For *Salmonella* prevalence and serology, data were analysed using the GLIMMIX procedure in Statistical Analyses System (SAS, V9.3, 2011), while for the adjusted OD values from sera, data were analysed using the mixed models procedure in SAS. The model assumed a binary response distribution using the logit link function, with pen being used as a random effect for correlation within pen. The pig was the experimental unit. Means for *Salmonella* prevalence and serology were separated using the Tukey-Kramer least square means adjustment for multiple comparisons and evaluated as the probability of detecting *Salmonella* in faeces, caecal digesta, ILN-MLN, or the presence of *Salmonella* antibodies in serum. The *Salmonella* prevalence on day 0 (before the diets were administered) was used as a covariate in the Tukey-Kramer least square means adjustment.

For growth performance parameters, data were analysed using the GLIMMIX model procedure in SAS. The experimental unit was the pen. Fixed effects were dietary group, sex, and day. Block was included as a random effect in the model and adjustment was also

Table 1
Salmonella prevalence in faeces, caecum and pooled ileocaecal and mesenteric lymph nodes (ILN-MLN), collected from finisher pigs fed either a control diet or a diet supplemented with an encapsulated blend of formic acid, citric acid, and essential oils.

		No. Pigs Positive for *Salmonella*/No. Pigs Sampled (% *Salmonella* Prevalence)		
		Faeces	Caecum	ILN-MLN
Day 0	Control	16/60 (26.7)	–[a]	–
	Treatment	14/61 (23.0)	–	–
Day 14	Control	31/62 (50.0)	–	–
	Treatment	17/62 (27.4)	–	–
Day 28	Control	22/61 (36.1)	–	–
	Treatment	12/60 (20.0)	–	–
Day 29	Control	–	30/42 (71.4)	11/42 (26.2)
	Treatment	–	27/32 (84.4)	6/32 (18.8)

[a] '–' indicates no samples were taken.

made for pen effect. Initial weight at day 0 was used as a covariate in the analysis of pig weight at day 28, ADFI, ADG and FCE. Carcass weight was included as a covariate in the analysis for lean meat yield, muscle depth, and fat depth.

Residual checks were made to ensure that the assumptions of the analyses were met. For all analyses, statistical significance was established at $\alpha = 0.05$ and 95% confidence intervals (CI) were reported.

3. Results

3.1. Salmonella *shedding in faeces*

On day 0, before the experimental diets were administered, faecal shedding of *Salmonella* was comparable between the control and treatment groups (26.7% versus 23.0%, respectively; Table 1). Two weeks later, *Salmonella* shedding increased in the control compared to the treatment group, with the probability of detecting *Salmonella* in faeces being 51.7% versus 27.9%, respectively (p = 0.001; Table 2). Faecal *Salmonella* prevalence decreased in both groups by day 28 compared to day 14 and there was a tendency for a decrease in the probability of detecting *Salmonella* in the faeces of the treatment group compared to the control group (20.6% versus 35.9%, respectively; p = 0.07; Table 2). When comparing the probability of detecting *Salmonella* in the faeces over time for each group, no differences were detected between day 14 and day 28 for the treatment group (27.9% versus 20.6%; p = 0.24) unlike the control group, in which the probability of detecting *Salmonella* was lower on day 14 compared to day 28 (51.7% versus 35.9%; p = 0.03; Table 2). Supplementary Table S-2 details the pen-level prevalence of *Salmonella* shedding over the three sampling days. The serotype of all of the faecal isolates recovered from the pigs was *S.* 4,[5],12:i:-.

Table 2
The effect of dietary supplementation with an encapsulated blend of formic acid, citric acid and essential oils on the probability of detecting *Salmonella* in faeces from finisher pigs on days 14 and 28 on a commercial pig farm)[a].

	Day 14[b]	Day 28[b]	p-value
Control (95% CI)	51.7% (39.0–64.2)	35.9% (24.6–49.0)	0.03
Treatment (95% CI)	27.9% (17.9–40.6)	20.6% (12.0–32.3)	0.24
p-value	0.001	0.07	

[a] The values reported are based on statistical analysis from a single model containing effects of diet, day and the interaction of diet by day. The interaction between diet and day was only statistically significant for the control group. Confidence intervals (95%) are given for the estimated probabilities.
[b] The *Salmonella* prevalence at day 0 was used as a covariate in the analysis of the probabilities for the control and treatment groups.

3.2. Salmonella *serology*

All pigs, 61 in the control group and 62 in the treatment group, were seronegative at the beginning of the trial. A lower seroprevalence was found in the treatment group compared to the control group (64.3% versus 89.2%, respectively; 95% CI = 47.1–78.6 versus 76.4–95.5, respectively; p = 0.01) at the end of the experiment using the 40% OD cut-off. However, no significant reduction was detected in the treatment group as compared to the control group (88.2% versus 98.0%, respectively, 95% CI = 66.5–96.6 versus 85.9–99.8, respectively; p = 0.13) when the 20% OD cut-off was used. When adjusted mean OD values from both groups were compared, the treatment group showed a significantly lower adjusted mean OD than the control group (62.4 versus 94.6, respectively; 95% CI = 42.0–82.9 versus 74.1–100, respectively; p = 0.03).

3.3. Salmonella *from truck and lairage swabs*

Twenty five percent (1/4) of the truck swabs taken from the floor at the back of the transport truck prior to loading the treatment pigs were positive for *Salmonella*. The remaining swabs, including 2 taken from the floor at the front of the truck, where the control pigs were carried, were *Salmonella*-negative. Swabbing of the lairage pens prior to unloading the pigs at the abattoir showed the presence of *Salmonella* in one pen (one swab of the three taken from this pen was positive). Pigs from the treatment group were randomly allocated to this pen. The serotype recovered from both the truck and lairage pen swabs was *S.* 4,[5],12:i:-.

3.4. Salmonella *in caecal digesta and lymph nodes*

The number of *Salmonella*-positive caecal and pooled ILN-MLN samples found and the calculated *Salmonella* prevalence is shown in Table 1. Overall, no difference in the probability of detecting *Salmonella* in the caecal digesta was observed between the control and treatment groups (72.5% versus 83.9%, respectively; 95% CI = 56.5-84.2 versus 66.3–93.2, respectively; p = 0.26). Likewise, for the pooled ILN-MLN, no significant differences were observed when the control and treatment groups were compared (27.5% probability of detecting *Salmonella* versus 19.4%, respectively; 95% CI = 15.8-43.5 versus 8.8–37.3, respectively; p = 0.43).

The serotype recovered from all *Salmonella*-positive pooled ILN-MLN samples and 55/57 *Salmonella*-positive caecal digesta samples was *S.* 4,[5],12:i:-. *Salmonella* Derby and *S.* Typhimurium were recovered from the two other *Salmonella*-positive caecal digesta samples; one obtained from a control pig and the other from a pig in the treatment group.

Table 3
The effect of dietary supplementation with an encapsulated blend of formic acid, citric acid, and essential oils on growth, feed efficiency, and carcass quality of finisher pigs on a commercial pig farm[a].

	Control (95% CI)	Treatment (95% CI)	p-value
Weight – Day 0 (kg)	80.5 (75.4–85.6)	76.4 (71.3–81.5)	0.04
Weight – Day 28 (kg)[b]	111 (108–114)	109 (106–112)	0.45
Average Daily Feed Intake (g)[b]	3037 (2992–3090)	2943 (2888–2985)	0.001
Average Daily Gain (g)[b]	1160 (1053–1263)	1107 (1001–121)	0.45
Feed Conversion Efficiency (g/g)[b]	2.71 (2.53–2.89)	2.78 (2.60–2.96)	0.55
Carcass Weight (kg)	81.1 (78.9–83.2)	79.4 (77.2–81.5)	0.25
Kill Out Yield (%)	73.4 (72.8–73.9)	72.672.1–73.1)	0.04
Lean Meat Yield (%)[c]	56.4 (55.8–57.0)	57.5 (56.9–58.1)	0.02
Muscle Depth (mm) [c]	50.6 (49.5–51.8)	52.6 (51.4–53.7)	0.004
Fat Depth (mm)[c]	13.37 (12.7–14.1)	12.44 (11.7–13.2)	0.07

[a] The statistical model used for comparison of the growth performance variables listed in the table included dietary group, sex, and day. Block was included as a random effect and adjustment was also made for pen effect. Confidence intervals (95% CI) are provided for each growth performance/carcass quality variable.
[b] Initial body weight at day 0 was used as a covariate in the analysis.
[c] Carcass weight was used as a covariate in the analysis.

3.5. Production parameters

No differences in ADG (p = 0.45) or FCE (p = 0.55) were detected between control and treatment groups (Table 3). However, pigs in the treatment group had lower ADFI as compared to the control group (p = 0.001), along with lower carcass weight (p = 0.25), and kill-out yield (p = 0.04). Although, pigs fed the treatment diet were leaner than those fed the control diet (p = 0.02), supplementing the diet with the organic acid-essential oil blend was not cost beneficial in this trial. The total feed cost per kg of live-weight gain for the duration of the experiment was €0.76 for the control group and €0.84 for the treatment group (Table 4).

4. Discussion

Research on the efficacy of dietary supplementation with organic acid-essential oil combinations as a pre-harvest *Salmonella* control strategy in pigs is scarce, with only two trials reported to date (Michiels et al., 2012; Rasschaert et al., 2016). Instead, much of the current literature focuses on the use of organic acids and/or their salts alone or in combination (Canibe et al., 2005; Creus et al., 2007; Boyen et al., 2008; Visscher et al., 2009; Gebru et al., 2010; Willamil et al., 2011; Calveyra et al., 2012; Rajtak et al., 2012; Walsh et al., 2012; Argüello et al., 2013a; Walia et al., 2016). The present study is the first on-farm trial to evaluate the efficacy of an encapsulated blend of formic acid, citric acid, and essential oils as a dietary treatment to reduce *Salmonella* shedding and intestinal carriage in finishing pigs. The feed additive used is a commercial product and was used at the manufacturer's recommended inclusion rate for a short treatment period (28 days) prior to slaughter. Feed accounts for ~70% of the total cost of producing a pig (Teagasc Agriculture and Food Development Authority, 2015), and as such, identifying cost-effective dietary solutions that limit the persistence and transmission of *Salmonella* during the finisher stage will increase profitability. The targeted 28-day administration period employed in the present study was chosen to evaluate the efficacy of the feed additive for *Salmonella* control while reducing its impact on feed costs.

Results demonstrated the efficacy, albeit somewhat limited, of the organic acid-essential oil treatment in controlling *Salmonella* on-farm. While inclusion of the feed additive did not prevent *Salmonella* infection per se, shedding in the treatment group was lower than in the control group, in which as many as half of the pigs were *Salmonella*-positive at one point during the trial. Interestingly, *Salmonella* shedding rates in the treated group remained stable throughout the trial, as opposed to the control group, in which *Salmonella* prevalence spiked two weeks into the trial. This suggests that the additive provided protection against *Salmonella* by preventing acquisition of infection in at least some of the pigs in the treated group. Our findings are contrary to those of a previous study which showed that 26–27 days of dietary supplementation with a formic-citric acid-essential oil combination did not reduce *Salmonella* shedding in weaner pigs when compared to an untreated control group (Michiels et al., 2012). However, a direct comparison is not possible due to the different stage of production and the fact that the pigs were deliberately infected with *Salmonella*. On the other hand, our results are supported by those of a recent study in fattening pigs from a high *Salmonella* seroprevalence farm that showed a reduction in *Salmonella* shedding on supplementation with an organic acid-essential oil blend. However, the feed additive used was different to that fed in the present study in that it contained MCFAs, lactic acid, and oregano oil (Rasschaert et al., 2016). Furthermore, *Salmonella* reductions were only seen after supplementation for the entire fattening period, with earlier

Table 4
Cost-benefit analysis of dietary supplementation with an encapsulated blend of formic acid, citric acid, and essential oils to finisher pigs on a commercial pig farm.

	Control	Treatment
Weight Gain (kg)	32.5	31.0
Feed Conversion Efficiency (kg/kg)	2.71	2.78
Cost of Formic acid, Citric acid, and Essential oils (€/kg)	–	5
Inclusion Rate of Formic acid, Citric acid, and Essential oils (kg/t)	–	4
Total Cost of Formic acid, Citric acid, and Essential oils (€/t)	–	20
Cost of Formic acid, Citric acid, and Essential oils (€/pig)	–	1.72
Finisher Feed Price in Ireland for July 2015 (€/t)	281	281
Finisher Feed Price with/without added Formic acid, Citric acid, and Essential oils (€/t)	281	301
Total Feed Intake (kg/pig)	88.0	86.2
Finisher Feed Cost (€/kg)	0.281	0.301
Finisher Feed Cost per pig (€/pig)	24.73	25.93
Total Finisher Feed Cost per kg Live Weight Gain (€/kg live weight gain)	0.76	0.84

faecal samples taken 5 weeks into treatment showing no differences in shedding between treatment and control groups. The latter highlights the fact that better efficacy may have been seen in our study had the duration of treatment been extended. As such, additional field trials with this feed additive are warranted, especially given the fact that a reduction in *Salmonella* shedding was only detected at certain time points in the current study.

Prior to commencing the present study, all pigs were seronegative, which is why the initial artificial contamination of pens was required. Significant differences in seroprevalence were detected at slaughter, using a 40% OD cut-off, which is the cut-off value used in the Irish NPSCP (Department of Agriculture Food and the Marine (DAFM), 2010). This finding correlates with the bacteriological results discussed above and demonstrates that the feed additive did reduce infection pressure. However, the lower seroprevalence observed in the treated group, would still be considered high, i.e., >50%, and therefore in the high prevalence category according to the Irish NPSCP, and as such restrictions during slaughter would apply to pigs from this herd. It is possible that the treatment duration was too short to elicit a seroprevalence below 50% and it is also possible that using the additive during successive batches of pigs might reduce environmental *Salmonella* contamination and ultimately seroprevalence in pigs over time. This finding is similar to that obtained in a recent study from our group, which showed that dietary supplementation with sodium butyrate for 24–28 days prior to slaughter reduced seroprevalence but not to below the cut-off used for high-risk herds in Ireland (Walia et al., 2016). A treatment duration of at least 7 weeks may be necessary to reduce seroprevalence beyond that found in the present study (Creus et al., 2007; Visscher et al., 2009; Argüello et al., 2013a).

It is well documented that *Salmonella* infection can occur at any point during the growth of pigs and as such this presents a challenge as to where interventions should be focused for effective control. The main purpose in using pre-harvest *Salmonella* control strategies in pigs is to reduce the incidence of *Salmonella* carriers presented at slaughter (Argüello et al., 2013c). In this regard, much research has focused on the effect of dietary supplementation with organic acids at farm level to reduce the prevalence of *Salmonella* in the GIT of pigs at slaughter with conflicting results. Certain acid additives (sometimes used in combination with essential oils) have been successful in reducing *Salmonella* in the caecal digesta and/or lymph nodes (Creus et al., 2007; Boyen et al., 2008; Visscher et al., 2009; Willamil et al., 2011; Argüello et al., 2013a; Rasschaert et al., 2016), while others have not (De Busser et al., 2009; Willamil et al., 2011; Michiels et al., 2012; Argüello et al., 2013a; Walia et al., 2016; Rasschaert et al., 2016). The inability of the additive used in the present study to reduce *Salmonella* prevalence in the caecal digesta and ILN-MLN further illustrates the importance of additive selection and duration of feeding regarding control of *Salmonella* carriage in pigs. On the one hand, no significant differences were detected in *Salmonella* shedding at the end of the trial, which could explain the lack of differences observed in the caecal digesta and ILN-MLN. On the other hand, the fact that *Salmonella* was detected on the truck and in one lairage pen prior to introducing the animals, together with the fact that *S.* Derby and *S.* Typhimurium, two serotypes not present on the farm, were recovered from the caecum of two pigs, demonstrates that pigs could potentially have acquired a new infection during transport to the abattoir and/or in the lairage (Duggan et al., 2010; Argüello et al., 2014). It is also possible that multiple *Salmonella* serotypes were present, but undetectable, on the farm, although this is probably unlikely, as the finisher pens on this farm were artificially contaminated with *S.* 4,[5],12:i:- due to the fact that the pen and faecal samples collected prior to commencing the study were *Salmonella*-negative on multiple occasions. Nonetheless, factors such as the presence of multiple serotypes and/or acquisition of new infections immediately pre-slaughter may mask the success of control measures used at farm level. However, on-farm interventions are still considered a necessary first step in the overall hurdle approach to controlling *Salmonella* in pigs (Goldbach and Alban, 2006; Ojha and Kostrzynska, 2007). Moreover, it is possible that a longer treatment period with the formic-citric acid-essential oil additive used, i.e., >40 days, is needed in order to reduce intestinal *Salmonella* carriage. This demonstrates the importance of finding the correct balance between efficacy and cost-effectiveness. Additional feeding trials with a longer duration of treatment are therefore warranted.

Previous studies have demonstrated growth benefits as a result of dietary supplementation with organic acids, sometimes in combination with essential oils (Gálfi and Bokori, 1990; Partanen and Mroz, 1999; Øverland et al., 2000; Mroz et al., 2002; Partanen et al., 2002; Lawlor et al., 2005, 2006; Creus et al., 2007; Walsh et al., 2007; Øverland et al., 2009; Gebru et al., 2010; Htoo and Molares, 2012; Upadhaya et al., 2014; Zeng et al., 2015; Walia et al., 2016). Therefore, in addition to evaluating the efficacy of the feed additive as a *Salmonella* control measure, effects on growth performance and an associated cost-benefit analysis were investigated in the present study. Few studies have investigated the cost-benefit of dietary acidification in relation to *Salmonella* control (Creus et al., 2007; Walia et al., 2016) and none have evaluated it when essential oils are also present. Although pigs in the control group were heavier at the start of the study compared to those in the treatment group, the growth performance variables were adjusted for these weight differences and as such they do not impact the results and comparisons reported. Moreover, while treated pigs were leaner than pigs fed the control diet, they had numerically lower ADG and lighter carcasses, due to a significant reduction in feed intake. It therefore appears that the additive may have reduced feed acceptability. Consequently, sup-plementing the diet for 28 days with the formic-citric acid-essential oil blend increased the feed cost per kg of live-weight gain by €0.08. Therefore, for the present study, the organic acid-essential oil feed additive used was not cost beneficial, despite its efficacy in reducing *Salmonella* prevalence, albeit only at certain time points.

5. Conclusion

Overall, the results suggest that dietary supplementation with an encapsulated blend of formic acid, citric acid and essential oils, at 4 kg/tonne of feed, to finishing pigs for a strategic 28-day period prior to slaughter has potential to prevent increased *Salmonella* shedding at certain time points and seroprevalence. However, supplementation at this rate and for this duration did not influence intestinal carriage, nor did it reduce seroprevalence to below the cut-off used for the high *Salmonella* risk category in Ireland (50%). Furthermore, it did not improve growth performance and, in fact, increased the feed cost per kg live-weight gain during the trial. A longer duration of dietary supplementation is perhaps warranted, although the cost-benefit of this would have to be determined.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.prevetmed.2016.12.007.

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The efficacy of different cleaning and disinfection procedures to reduce *Salmonella* and *Enterobacteriaceae* in the lairage environment of a pig abattoir

Kavita Walia [a,b,d], Hector Argüello [a], Helen Lynch [a,c,d], Jim Grant [a], Finola C. Leonard [c], Peadar G. Lawlor [d], Gillian E. Gardiner [b], Geraldine Duffy [a,*]

[a] *Teagasc, Food Research Centre, Ashtown, Dublin 15, Ireland*
[b] *Department of Science, Waterford Institute of Technology, Waterford, Ireland*
[c] *School of Veterinary Medicine, University College Dublin, Dublin, Ireland*
[d] *Teagasc, Pig Development Department, Animal and Grassland Research and Innovation Centre, Moorepark, Fermoy, Co. Cork, Ireland*

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This study investigated several cleaning and disinfection protocols for their ability to eliminate *Salmonella* and to reduce levels of *Enterobacteriaceae,* within the lairage pens of a commercial pig abattoir.

Eight protocols were evaluated in each of 12 lairage pens at the end of the slaughtering day on 3 occasions (36 pens/protocol): (P1) high-pressure cold water wash (herein referred to as high-pressure wash); (P2) high-pressure wash followed by a quaternary ammonium compound (QAC)-based disinfectant without rinsing; (P3) high-pressure wash followed by a chlorocresol-based disinfectant without rinsing; (P4) high-pressure wash followed by a sodium hydroxide/sodium hypochlorite detergent with rinsing; (P5) P4 followed by P2; (P6) P4 followed by P3; (P7) P5 with drying for 24–48 h; and (P8) P6 with drying for 24–48 h. Two floor swabs and one wall swab were taken from each lairage pen before and after each protocol was applied, and examined for the presence of *Salmonella* and enumeration of *Enterobacteriaceae.*

High-pressure washing alone (P1) did not reduce the prevalence of *Salmonella* in the lairage pens. When high-pressure washing, the probability of detecting *Salmonella* following application of the chlorocresol-based disinfectant (P3) was lower than with the QAC-based disinfectant, P2 (14.2% versus 34.0%, respectively; $p < 0.05$). The probability of detecting *Salmonella* after the combined use of detergent and the chlorocresol-based disinfectant (P6) was also lower than application of detergent followed by the QAC-based disinfectant, P5 (2.2% versus 17.1%, respectively; $p < 0.05$). Drying of pens (P7 and P8) greatly reduced the probability of detecting *Salmonella.* Only 3.8% of swabs were *Salmonella*-positive 48 h after cleaning with detergent and the QAC-based disinfectant (P7); while an eradication of *Salmonella* was achieved 24 h after cleaning with detergent and the chlorocresol-based disinfectant, P8. A reduction in *Enterobacteriaceae* counts to below the limit of detection (LOD; 10 CFU/cm^2) was achieved following cleaning with detergent and disinfection with the chlorocresol-based disinfectant, regardless of drying ($p < 0.05$), whereas, applying detergent and the QAC-based disinfectant (P7) did not reduce *Enterobacteriaceae* counts to below the LOD.

Therefore ensuring that lairage pens are allowed to dry after intensive cleaning with detergent and a chlorocresol-based disinfectant is recommended as the most effective hygiene routine to eliminate *Salmonella* and reduce *Enterobacteriaceae* counts.

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1. Introduction

Following transport from the farm, the next stage in the pre-harvest control of *Salmonella* in pigs is within the lairage of the abattoir. Lairaging pigs in the abattoir, provides a buffer for the slaughter line, allowing pigs to recover from the stress of transport, and improves meat quality (Warriss, 2003). It is well documented that finishing pigs need as little as 2 h following exposure to a contaminated environment to acquire *Salmonella* (Boughton et al., 2007b; Hurd et al., 2001). As

Abbreviations: AMR, antimicrobial resistance; BG, brilliant green; BPW, buffered peptone water; CFU, colony forming unit; LB, luria-bertani; LOD, limit of detection; MIC, minimal inhibitory concentration; MRD, maximum recovery diluent; MSRV, modified semi-solid rapport-vassiliadis; NPSCP, Irish National Pig *Salmonella* Control Program; OD, optical density; PCA, plate count agar; PCR, polymerase chain reaction; QAC, quaternary ammonium compound; SAS, statistical analysis system; VRBGA, violet red bile glucose agar; XLD, xylose lysine deoxycholate.

* Corresponding author at: Food Safety and Microbiology Department, Teagasc Food Research Centre, Ashtown, Dublin 15, Ireland.
E-mail address: Geraldine.Duffy@teagasc.ie (G. Duffy).

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such, there is a clear risk of pigs acquiring *Salmonella* from the lairage, if the environment is contaminated. This fact has been highlighted in previous molecular typing studies that investigated *Salmonella* in abattoirs. Duggan et al. (2010) found that the lairage pens in all three Irish abattoirs investigated were highly contaminated with several serovars of *Salmonella enterica* (Derby, Typhimurium, and Manhattan). More importantly, strains isolated from pig carcasses and intestinal contents have been shown to be indistinguishable from those isolated from lairage pens (Argüello et al., 2013; Bolton et al., 2013; Duggan et al., 2010; Mannion et al., 2012; Rostagno et al., 2003).

One way to limit the occurrence and spread of *Salmonella* within the lairage environment is through appropriate cleaning and disinfection regimes. Several approaches have been investigated (Argüello et al., 2011; Boughton et al., 2007a; Swanenburg et al., 2001); however, difficulties in eliminating *Salmonella* remain. Reasons for this include production of biofilms, or developed resistance to the cleaning agents and/or disinfectants, or harboring sites (i.e., cracks and holes in the lairage pens, drains) that are not easily cleaned or disinfected, all of which allow *Salmonella* to survive (Boughton et al., 2007a; Corcoran et al., 2014; De Beer et al., 1994; De Busser et al., 2013; McLaren et al., 2011; Stewart et al., 2001).

While many studies highlight the usefulness of cleaning and disinfection in the lairage to reduce the level of *Salmonella* carriage in pigs before slaughter, very few have compared cleaning regimes (Boughton et al., 2007a; Schmidt et al., 2004; Swanenburg et al., 2001; van der Wolf et al., 2001). Moreover, no study to date has investigated the various combinations of power washing with detergent and disinfectants and a subsequent drying step as a means to eliminate *Salmonella* from lairage pens. Additionally, only a limited number of studies have examined the efficacy of specific detergent and/or disinfectant agents against *Salmonella* when used in the lairage area of pig abattoirs (Boughton et al., 2007a; Schmidt et al., 2004; Swanenburg et al., 2001). Quaternary ammonium compound (QAC) disinfectants are commonly used biocides, as their broad-spectrum of activity means that they are effective against a wide range of bacterial species (Hegstad et al., 2010; Holah et al., 2002; Sidhu et al., 2002). The main mode of action of QAC's against Gram-negative bacteria is disruption of the lipid bilayer of the cytoplasmic membrane and outer membrane leading to leakage of cytoplasmic components and eventually cell lysis (Quinn et al., 2011). Similarly, chlorocresol acts by causing a loss of cell membrane integrity and coagulation of cytoplasmic components, most likely due to protein denaturation (McLaren et al., 2011). Furthermore, chlorocresol was shown as the superior disinfectant as it consistently killed *Salmonella* in wet environments, albeit with poultry feces (McLaren et al., 2011), which is typical of lairage pens in pig abattoirs. For these reasons, and the fact that chlorocresol is not widely used in pig abattoirs as a disinfectant, its effect was compared to that of a QAC-based disinfectant in the present study.

Therefore, the objective of this study was to evaluate several cleaning and disinfection protocols, specifically with QAC-based or chlorocresol-based disinfectants, for their ability to eliminate *Salmonella* and to improve overall hygiene, as determined by measuring *Enterobacteriaceae* counts, within the lairage pen environment of a commercial pig abattoir.

2. Materials and methods

2.1. Abattoir and lairage area

One pig abattoir in the Republic of Ireland participated in this study. This abattoir routinely operates a Monday to Friday schedule, slaughtering approximately 2000 pigs per day from herds across the country. The lairage area consists of 12 main pens with solid concrete floors and walls. Each pen was 2.05 m × 14.95 m, with a capacity for holding 65 pigs during the day. During a slaughtering day, each pen was used multiple times and contained pigs from different herds.

Table 1
The eight different cleaning and disinfection protocols employed and sample collection conducted in each of 12 lairage pens in a commercial pig abattoir.

Cleaning and disinfection protocols[a]	Sampling day
(P1) High-Pressure Wash ('After Power Wash')	Mid-week (Tuesday/Wednesday)
(P2) High-Pressure Wash + QAC[b] Disinfectant ('After QAC Disinfectant')	
(P3) High-Pressure Wash + Chlorocresol[c] Disinfectant ('After Chlorocresol Disinfectant')	
(P4) High-Pressure Wash + Detergent[d] ('After Detergent')	End of week (Friday/Saturday)
(P5) High-Pressure Wash + Detergent + QAC Disinfectant ('After Detergent + QAC Disinfectant')	
(P6) High-Pressure Wash + Detergent + Chlorocresol Disinfectant ('After Detergent + Chlorocresol Disinfectant')	
(P7) Drying following cleaning with High-Pressure Wash + Detergent + QAC Disinfectant ('After QAC + Drying')	Sunday
(P8) Drying following cleaning with High-Pressure Wash + Detergent + Chlorocresol Disinfectant ('After Chlorocresol + Drying')	

[a] 2 floor and 1 wall swabs were taken after each cleaning and disinfection protocol was applied.
[b] QAC, Quaternary Ammonium Compound disinfectant was Holquat®, Holchem Laboratories Limited, UK.
[c] Chlorocresol disinfectant was Interkokask®, Hysolv, UK.
[d] Detergent was Rapier®, Holchem Laboratories Limited, UK.

2.2. Cleaning and disinfection protocols

Eight different cleaning protocols were evaluated in this study (Table 1). Each protocol consisted of the following: (P1) high-pressure cold (15–20 °C) water wash (herein referred to as high-pressure wash) to remove gross faecal matter; (P2) high-pressure wash followed by application of a QAC-based disinfectant (Holquat®, Holchem Laboratories Limited, UK) at a dilution rate of 2% without subsequent rinsing; (P3) high-pressure wash followed by application of a chlorocresol-based disinfectant (Interkokask®, Hysolv, UK) at a dilution rate of 2–3% without subsequent rinsing; (P4) high-pressure wash followed by an alkyl dimethyl amine oxide, sodium aryl sulphonate, sodium hydroxide and sodium hypochlorite detergent (Rapier®, Holchem Laboratories Limited, UK) at a dilution rate of 5%, with a contact time of 20 min followed by a high-pressure water rinse; (P5) protocol P4 followed by protocol P2; (P6) protocol P4 followed by protocol P3; (P7) combining protocol P5 with a drying step for 24–48 h; (P8) combining protocol P6 with a drying step for 24–48 h.

Each protocol was implemented in each of the 12 lairage pens at the end of the slaughtering day on three occasions (36 pens per protocol).

2.3. Sample collection

The 12 lairage pens were sampled, before and after implementation of the protocols listed above. In each pen, after it was emptied of pigs and before the protocol was implemented, two floor swabs (sterile sponges, 100 cm^2, pre-moistened with 10 mL Maximum Recovery Diluent (MRD), (Technical Services Consultants Ltd., Lancashire, UK) and one wall swab were collected ('Before Power Wash'). Each swab covered a 40 cm × 40 cm area. Immediately after applying P1 ('After Power Wash') or 25 min after applying P4 ('After Detergent') another 3 swabs per protocol were collected following the same procedure as above. Ten minutes after applying P2 ('After QAC Disinfectant') or after applying P3 ('After Chlorocresol Disinfectant'), 6 swabs per protocol containing a neutralizing buffer were used [four floor (2 per area) and two wall; sterile sponges, 50 cm^2, pre-moistened with 10 mL of neutralizing buffer; Technical Service Consultants Ltd., Lancashire, UK]. The neutralizing buffer consisted of the following compounds: Tween

(Polysorbate) 80, Saponin, Sodium chloride, Sodium thiosulphate, Lecithin, L-Histidine and Deionized water. Ten minutes after applying P5 ('After Detergent + QAC Disinfectant'), or 10 min after applying P6 ('After Detergent + Chlorocresol Disinfectant) only, P7 ('After QAC Drying'), and P8 ('After Chlorocresol Drying'), 3 swabs, as above, were taken after each protocol, using either the MRD swabs after application of the detergent or 6 swabs containing the neutralizing buffer after application of the disinfectants. All swabs were collected aseptically, kept at 4 °C and processed within 24 h.

2.4. Microbiological analysis

Each MRD sponge swab was suspended in 90 mL of buffered peptone water (BPW; Oxoid Limited, Hampshire, UK) and homogenized in a stomacher for 2 min. For the neutralizing buffer swabs, since 4 floor swabs were collected from 2 different areas in the pen, and 2 wall swabs were collected from the same area in the pen, the 2 swabs per floor or wall area were pooled and suspended in 90 mL of BPW and homogenized in a stomacher for 2 min, as above.

Isolation of *Salmonella* was carried out in accordance with International Organization for Standardization (ISO) 6579:2007 (Amendment 1: Annex D) method (International Organization for Standardization, 2007). All media and agar were obtained from Oxoid Limited (Hampshire, UK). Briefly, BPW swab suspensions were incubated at 37 °C for 19 h, after which 100 μL of each sample was pipetted onto modified semi-solid Rappaport-Vassiliadis (MSRV) agar plates and incubated at 42 °C for 24 h. If the MSRV plate was negative, it was incubated for a further 24 h. Presumptive *Salmonella* growth was streaked onto xylose lysine deoxycholate (XLD) and brilliant green (BG) agar and incubated at 37 °C for 24 h. Suspect colonies from XLD or BG agar plates were then streaked onto plate count agar (PCA) and incubated at 37 °C for 24 h. Afterwards, urea agar slants and *Salmonella* chromogenic agar plates were inoculated with colon(ies) from PCA and incubated at 37 °C for 24 h. Serological confirmation of colonies from PCA was performed using a *Salmonella* Latex Agglutination Kit (Oxoid).

In addition, *Enterobacteriaceae* counts from the floor swabs only were obtained before and after the implementation of each of the 8 protocols as follows. Ten-fold serial dilutions of the BPW swab suspensions were performed in MRD and appropriate dilutions pour-plated on violet red bile glucose agar (VRBGA; Oxoid). Plates were overlaid with VRBGA and incubated at 37 °C for 24 h. The limit of detection (LOD) was 10 CFU/cm^2.

2.5. Serotyping and antimicrobial resistance determination of Salmonella isolates

All presumptive *Salmonella* isolates recovered after the implementation of each of the 8 protocols was first tested using the real-time polymerase chain reaction (PCR) assay for the identification and differentiation of *Salmonella enterica* serotype Typhimurium and *S.* 4,[5],12:i:- as described by Prendergast et al. (2013). If isolates were not identified as *S.* Typhimurium or *S.* 4,[5],12:i:-, then serotyping was performed according to the White-Kauffmann-Le Minor scheme (Grimont and Weill, 2007) using commercial antisera (Pro-Lab Diagnostics, Cheshire, UK; SIFIN Institute, Berlin, Germany; and Statens Serum Institute, Copenhagen, Denmark).

The antimicrobial resistance (AMR) pattern of each isolate was determined using the Sensititre™ Gram Negative NARMS Plate (Thermo Scientific, Waltham, MA, USA). The following antimicrobials were tested: amoxicillin-clavulanic acid (AUG), ampicillin (AMP), azithromycin (AZI), cefoxitin (FOX), ceftiofur (XNL), ceftriaxone (AXO), chloramphenicol (CHL), ciprofloxacin (CIP), gentamicin (GEN), nalidixic acid (NAL), streptomycin (STR), sulfisoxazole (FIS), tetracycline (TET), and trimethoprim/sulfamethoxazole (SXT). Minimal Inhibitory Concentrations (MICs) were interpreted using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) epidemiological cut-off values.

2.6. Crystal violet biofilm assay and disinfectant resistance of Salmonella isolates

Analysis of biofilm formation was performed according to Chagnot et al. (2014) and an in-house methodology (Teagasc, Food Research Centre, Ashtown, Ireland) on selected isolates recovered after the implementation of all protocols except P8 ('After Chlorocresol Drying'), as no *Salmonella* was recovered following this protocol. Isolates were selected for analysis based on the uniqueness of their AMR profiles and serotypes, focusing on serotypes that are of concern in the Irish National Pig *Salmonella* Control Program (NPSCP) but also focusing on isolates that were obtained after application of the protocols that combined detergent and disinfectant with or without drying.

Isolates were grown on Luria-Bertani (LB; Oxoid) agar plates and incubated at 37 °C for 19 h. One colony from each LB agar plate was inoculated into 5 mL of LB without salt and incubated at 37 °C for 19 h. This overnight culture was diluted into 5 mL of fresh LB without salt so as to achieve an optical density ($OD_{600\ nm}$) of 0.02. Two hundred microliters of the OD-adjusted samples were then transferred into 4 wells of a 96-well microplate (for 4 technical replicates per isolate; F bottom microplate, NUNC™, ThermoFisher Scientific, Roskilde, Denmark) and incubated at 15 °C (to mimic the average temperature in the lairage of the abattoir) and at 37 °C (optimal temperature for *Salmonella* growth) for 48 h. After incubation, the liquid was removed and the wells washed with 200 μL of tryptone salt (Oxoid). Afterwards, 300 μL of pure ethanol (99.2%, Merck, Darmstadt, Germany) was added to each well and left for 20 min before being removed and allowing the well to air-dry for 1 h. Next, 200 μL of 0.1% crystal violet (Sigma-Aldrich Ireland Limited, Arklow, Ireland) was added to each well and left for 10 min at room temperature, after which it was removed and the wells washed twice with distilled water. Two hundred microliters of 33% acetic acid (Sigma-Aldrich Ireland Limited) was added to each well and the microplate agitated for 5 min on an orbital shaker (Stuart Scientific, Staffordshire, UK). Afterwards, 150 μL of this solution was transferred to a new 96-well microplate and the OD_{595nm} values were determined for each well using a plate reader (Multiskan™ FC Microplate Photometer, Thermo Scientific, Paisley, UK). For each isolate, this entire procedure was repeated for 4 biological replicates.

The biofilm formation ability of each isolate was determined according to Chelvam et al. (2014) as follows: no biofilm (OD isolate </= OD cut-off), weak biofilm producer (OD cut-off < OD isolate </= 2 × OD cut-off), moderate biofilm producer (2 × OD cut-off < OD isolate </= 4 × OD cut-off), and strong biofilm producer (4 × OD cut-off < OD isolate). The OD cut-off was defined as three standard deviations above the mean $OD_{595\ nm}$ of the negative control wells.

Resistance to the QAC-based or chlorocresol-based disinfectants was performed according to Andrews (2001) and Gantzhorn et al. (2014) on select isolates recovered from protocols P5 to P7. No isolates were recovered from P8. The concentrations that were tested to determine the minimum inhibitory concentration (MIC) of the QAC-based disinfectant were 2%, 1%, 0.5%, 0.25%, 0.125% and 0.0625%; while for the chlorocresol-based disinfectant the concentrations tested were 3%, 2%, 1%, 0.5%, 0.25%, 0.125% and 0.0625%.

2.7. Statistical analysis

Salmonella prevalence data and odds ratios were analyzed using the GLIMMIX procedure in Statistical Analysis System (SAS, V9.3, 2011), while *Enterobacteriaceae* data were log transformed and analyzed using the Mixed models procedure in SAS. In all cases the 'Before Power Wash' results were tested as a covariate in analysis and Tukey-Kramer least squares means adjustment for multiple comparisons was used to separate the treatment (protocol) means.

Residual checks were made to ensure that the assumptions of the analyses were met. For all analyses, statistical significance was established at $\alpha = 0.05$.

3. Results

3.1. Detection of Salmonella after application of the cleaning and disinfection protocols

If any one of the three swabs taken from each pen was found to contain *Salmonella*, then the pen was considered *Salmonella*-positive. Overall, all pens were *Salmonella*-positive before applying the cleaning protocols (Supplementary Table S-1), with just under 83% (450/543) of the swabs positive for *Salmonella* (Table 2). Likewise, all pens after power washing, after application of detergent, and after sole use of the QAC-based disinfectant were *Salmonella*-positive (Supplementary Table S-1). Power washing alone had no effect on *Salmonella* prevalence, with 87.2% (157/180) of swabs positive for *Salmonella*; while, using detergent alone after power washing resulted in a reduction in the number of *Salmonella*-positive swabs to 58/108 (54%, Table 2).

Ten pens were *Salmonella*-positive after sole use of the chlorocresol-based disinfectant (Supplementary Table S-1). Eighteen percent (13/72) of swabs taken after application of the chlorocresol-based disinfectant were *Salmonella*-positive as compared to 49/123 (40%) after application of the QAC-based disinfectant (Table 2). The probability of detecting *Salmonella* following power washing and application of the chlorocresol-based disinfectant was lower when compared with power washing and application of the QAC-based disinfectant ($p < 0.05$, Table 2).

Nine pens were *Salmonella*-positive after the combined use of the detergent and the QAC-based disinfectant (Supplementary Table S-1). In contrast, only one pen was *Salmonella*-positive following the combined use of the detergent and the chlorocresol-based disinfectant. The combined use of detergent and the QAC-based disinfectant resulted in a decline in the probability of detecting *Salmonella* ($p < 0.05$, Table 2) with 17/72 (24%) of swabs positive for *Salmonella*. A greater reduction in the number of *Salmonella*-positive swabs 1/72 (1%) was detected following the combined use of detergent and the chlorocresol-based disinfectant, resulting in a reduction in the probability of detecting *Salmonella* ($p < 0.05$, Table 2).

Only two pens were *Salmonella*-positive after drying following cleaning with detergent in combination with use of the QAC-based disinfectant, whereas all pens were *Salmonella*-negative after drying following cleaning with detergent and use of the chlorocresol-based disinfectant (Supplementary Table S-1). Allowing pens to dry greatly reduced the probability of detecting *Salmonella* ($p < 0.05$, Table 2). All swabs were *Salmonella*-negative 24 h after cleaning with detergent and use of the chlorocresol-based disinfectant followed by drying; whereas 2/72 (3%) of the swabs were *Salmonella*-positive 48 h after cleaning with detergent and use of the QAC-based disinfectant followed by drying.

3.2. Odds of Salmonella contamination after application of the cleaning and disinfection protocols

Odds ratios for comparing the likelihood of *Salmonella* contamination in the lairage pens following application of each cleaning/disinfection protocol were also determined (Table 3). Overall, power washing did not reduce *Salmonella* contamination in the lairage pens. Power washing alone was 8, 14, 44, 35, 333, 187 and 500 times more likely to result in *Salmonella* contamination than when sole use of detergent, QAC- or chlorocresol-based disinfectants, combined detergent and QAC- or chlorocresol-based disinfectants, and drying after combined use of detergent and the QAC- or chlorocresol-based disinfectants, respectively, were applied after power washing of the pens ($p < 0.05$, Table 3). Allowing the lairage pens to dry for 48 h following application of detergent and the QAC-based disinfectant was 13, 22, and 186.9 times less likely to result in *Salmonella* contamination than sole use of the QAC-based disinfectant, the detergent after power washing, or power washing alone, respectively ($p < 0.05$, Table 3). Allowing the pens to dry for 24 h after applying the detergent and the chlorocresol-based disinfectant was 41, 66, and 500 times less likely to result in *Salmonella* contamination than sole use of the chlorocresol-based disinfectant, the detergent after power washing, or power washing alone, respectively ($p < 0.05$, Table 3).

3.3. Enterobacteriaceae counts

The effect of the different cleaning and disinfection protocols on *Enterobacteriaceae* counts in the lairage pens is presented in Table 4. In line with the *Salmonella* results, a reduction in *Enterobacteriaceae* counts to below the LOD (10 CFU/cm^2) was achieved following application of the chlorocresol-based disinfectant after power washing as compared to a 0.86 log_{10} CFU/cm^2 reduction after application of the QAC-based disinfectant after power washing ($p < 0.05$). Sole use of detergent after power washing resulted in a 1.82 log_{10} CFU/cm^2 reduction in *Enterobacteriaceae* counts, as compared to a 0.59 log_{10} CFU/cm^2 reduction after the combined used of detergent and the QAC-based disinfectant ($p < 0.05$). On the other hand, a reduction in *Enterobacteriaceae* counts to below the LOD was also achieved following combined use of detergent and the chlorocresol-based disinfectant regardless of whether the pens were dry or wet ($p < 0.05$). A 2.9 log_{10} CFU/cm^2 reduction after pens were dried following application of the detergent and the QAC-based disinfectant was not enough to reduce *Enterobacteriaceae* counts to below the LOD.

Table 2
Number of *Salmonella*-positive samples and the probability of detecting *Salmonella* from 12 lairage pens in a commercial pig abattoir sampled before and after several cleaning and disinfection protocols were applied.

Cleaning and disinfection protocols	*Salmonella* prevalence [No. *Salmonella*-positive swabs/no. swabs taken (%)]			Probability of detecting *Salmonella* (%)[1]	sem (%)
	Floor	Wall	Total		
Before Power Wash	318/362 (87.8%)	132/181 (72.9%)	450/543 (82.9%)	N/A[e]	N/A[e]
(P1) After Power Wash	114/120 (95.0%)	43/60 (72%)	157/180 (87.2%)	87.9[a]	2.7
(P2) After QAC Disinfectant	39/82 (47%)	10/41 (24%)	49/123 (40%)	34.0[b]	5.0
(P3) After Chlorocresol Disinfectant	1/48 (2%)	12/24 (50%)	13/72 (18%)	14.2[c]	5.7
(P4) After Detergent	49/72 (68%)	9/36 (25%)	58/108 (54%)	45.8[b]	5.9
(P5) After Detergent + QAC Disinfectant	15/48 (31%)	2/24 (8%)	17/72 (24%)	17.1[c]	5.4
(P6) After Detergent + Chlorocresol Disinfectant	0/48 (0%)	1/24 (4%)	1/72 (1%)	2.2[d]	1.8
(P7) After QAC + Drying	1/48 (2%)	1/24 (4%)	2/72 (3%)	3.8[d]	2.2
(P8) After Chlorocresol + Drying	0/48 (0%)	0/24 (0%)	0/72 (0%)	1.2[d]	1.2

[a,b,c,d] Protocols sharing the same superscript are not significantly different ($p < 0.05$).
[e] N/A = not applicable, as data from 'Before Power Wash' were used as a covariate in the analysis.
[1] Values presented are probability of detecting *Salmonella* from total mean values of floor and wall swabs.

Table 3
Odds ratios for efficacy of the cleaning and disinfection protocols in removing *Salmonella* from 12 lairage pens in a commercial pig abattoir.

Cleaning and disinfection protocols	Odds ratios[a]							
	After Power Wash	After Detergent	After QAC Disinfectant	After Chlorocresol Disinfectant	After Detergent + QAC Disinfectant	After Detergent + Chlorocresol Disinfectant	After QAC Drying	After Chlorocresol Drying
After Power Wash	N/A[b]	8.62 ($p < 0.001$)	14.15 ($p < 0.001$)	44.00 ($p < 0.001$)	35.71 ($p < 0.001$)	333.3 ($p < 0.001$)	186.9 ($p < 0.001$)	500.0 ($p < 0.001$)
After QAC Disinfectant	0.07 ($p < 0.001$)	0.61 ($p = 0.80$)	N/A[b]	3.11 ($p = 0.35$)	2.49 ($p = 0.43$)	23.26 ($p = 0.006$)	13.21 ($p = 0.001$)	41.67 ($p = 0.007$)
After Chlorocresol Disinfectant	0.023 ($p < 0.001$)	0.196 ($p = 0.35$)	0.322 ($p = 0.35$)	N/A[b]	0.80 ($p = 1.0$)	7.46 ($p = 4.0$)	4.26 ($p = 0.54$)	13.33 ($p = 0.28$)
After Detergent	0.116 ($p < 0.001$)	N/A[b]	1.64 ($p = 0.80$)	5.09 ($p = 0.04$)	4.08 ($p = 0.04$)	37.93 ($p = 0.001$)	21.62 ($p < 0.001$)	66.67 ($p = 0.001$)
After Detergent + QAC Disinfectant	0.028 ($p < 0.001$)	0.245 ($p = 0.04$)	0.401 ($p = 0.43$)	1.25 ($p = 1.0$)	N/A[b]	9.26 ($p = 0.21$)	5.30 ($p = 0.26$)	16.67 ($p = 0.15$)
After Detergent + Chlorocresol Disinfectant	0.003 ($p < 0.001$)	0.026 ($p = 0.001$)	0.043 ($p = 0.006$)	0.13 ($p = 0.40$)	0.108 ($p = 0.21$)	N/A[b]	0.57 ($p = 1.0$)	1.78 ($p = 1.0$)
After QAC + Drying	0.005 ($p < 0.001$)	0.046 ($p < 0.001$)	0.076 ($p = 0.001$)	0.24 ($p = 0.54$)	0.19 ($p = 0.26$)	1.75 ($p = 1.0$)	N/A[b]	3.13 ($p = 0.98$)
After Chlorocresol + Drying	0.002 ($p < 0.001$)	0.015 ($p = 0.001$)	0.024 ($p = 0.007$)	0.08 ($p = 0.28$)	0.06 ($p = 0.15$)	0.56 ($p = 1.0$)	0.32 ($p = 0.98$)	N/A[b]

[a] Odds ratios < 1 indicate that the cleaning and disinfection protocols listed in the left column are more efficient in removing *Salmonella* than those given in the top row. Odds ratios > 1 indicate that the cleaning and disinfection protocols listed in the top row are more efficient in removing *Salmonella* than those given in the left column.
[b] N/A = not applicable, as the cleaning and disinfection protocols being compared are the same.

3.4. Serotyping and antimicrobial resistance of *Salmonella* isolates

Serotypes of the *Salmonella* isolates obtained from swabs taken after the use of detergent and/or disinfectants are listed in Table 5. Six serotypes were detected; *S.* 4,[5],12:i:-; *S.* Brandenburg, *S.* Bredeney; *S.* Derby; *S.* Typhimurium and *S.* Panama. Overall, as the protocol intensified, the number of different *Salmonella* serotypes recovered decreased; however, *S.* 4,[5],12:i- was recovered after implementation of all of the protocols listed in Table 5.

The AMR profiles of the same isolates recovered after cleaning and/or disinfection are also listed in Table 5. A total of 16 unique AMR profiles were detected among the various *Salmonella* serotypes, with most (i.e. 40 isolates) being multidrug resistant, showing resistance to ampicillin, streptomycin, trimethoprim/sulfamethoxazole and tetracycline; while fewer showed resistance towards cefoxitin (3 isolates), chloramphenicol (8 isolates), and gentamicin (3 isolates). None of the *S.* 4,[5],12:i:- or *S.* Typhimurium isolates showed the typical ASSUT (ampicillin, streptomycin, sulphonamide, tetracycline) resistance profile associated with Typhimurium.

Table 4
The effect of different cleaning and disinfection protocols on *Enterobacteriaceae* counts in 12 lairage pens in a commercial pig abattoir.

Cleaning and disinfection protocols	Mean *Enterobacteriaceae* count[1] (log_{10} CFU/cm^2)	sem
Before Power Wash	5.29	N/A[f]
(P1) After Power Wash	4.12[a]	0.10
(P2) After QAC Disinfectant	3.26[b]	0.13
(P3) After Chlorocresol Disinfectant	<LOD[e]	0.13
(P4) After Detergent	2.30[c]	0.08
(P5) After Detergent + QAC Disinfectant	3.53[b]	0.13
(P6) After Detergent + Chlorocresol Disinfectant	<LOD[e]	0.13
(P7) After QAC + Drying	1.23[d]	0.13
(P8) After Chlorocresol + Drying	<LOD[e]	0.13

[a,b,c,d] Protocols sharing the same superscript are not significantly different ($p < 0.05$).
[e] LOD – Limit of Detection.
[f] N/A - indicates not applicable, as data from 'Before Power Wash' were used as a co-variate in the analysis.
[1] Mean *Enterobacteriaceae* counts from floor swabs from all 12 pens sampled on 2–3 occasions.

3.5. Biofilm formation and disinfectant resistance of *Salmonella* isolates

The biofilm forming capability of selected isolates from within the six serotypes isolated after the various cleaning and/or disinfection protocols are detailed in Table 5.

At 15 °C (average lairage temperature), after sole use of the QAC-based disinfectant, a *S.* 4,[5],12:i:- was isolated and shown to be weak biofilm former. None of the *S.* 4,[5],12:i:- that were isolated after application of the chlorocresol-based disinfectant, either in combination with detergent or without, or after pens were allowed to dry following application of the detergent and the QAC-based disinfectant, were capable of forming biofilms. Likewise, none of the *S.* Derby or *S.* Panama isolates that were recovered after any of the protocols were shown to form biofilms at 15 °C. As the protocols intensified, the isolates recovered including *S.* Bredeney, showed moderate biofilm forming capability; while *S.* Typhimurium isolates recovered after application of the QAC-based disinfectant were better biofilm formers than isolates recovered after the detergent was applied in combination with the QAC-based disinfectant. At both 15 °C and 37 °C, *S.* Brandenburg isolates recovered after drying following cleaning with detergent and the QAC-based disinfectant showed moderate biofilm forming ability.

At 37 °C (optimum growth temperature for *Salmonella*), as the protocols intensified, *S.* 4,[5],12:i:-, *S.* Bredeney, *S.* Derby and *S.* Panama isolates showed weak to moderate biofilm forming capability. On the other hand, *S.* Typhimurium recovered after sole use of the QAC-based disinfectant, acted in a similar manner to the results at 15 °C. It possessed moderate biofilm forming ability, while those recovered after treatment with detergent and the QAC-based disinfectant showed weaker biofilm forming ability.

All 23 isolates recovered after the combined use of detergent and the QAC-based disinfectant or the chlorocresol-based disinfectant with or without a drying step were susceptible to the QAC-based and the chlorocresol-based disinfectants.

4. Discussion

This study evaluated eight cleaning and disinfection protocols for their ability to eliminate *Salmonella* and to reduce levels of *Enterobacteriaceae*, an indicator of overall hygiene, within the lairage pen environment of a pig abattoir in the Republic of Ireland.

Table 5
Serotypes, antimicrobial resistance (AMR) profiles and biofilm forming ability of *Salmonella* isolates recovered after different cleaning and disinfection protocols were applied in 12 lairage pens in a commercial pig abattoir.

Cleaning and disinfection protocols[a]	*Salmonella* serotype (no. per serotype)	AMR profiles (no. per serotype)[b]	Biofilm category [c] (no. per serotype)	
			15 °C	37 °C
After QAC Disinfectant	*S.* 4,[5],12:i:- (6)	AMP STR TET (3); STR TET (2)	None (1); Weak (2)	None (3)
	S. Brandenburg (2)	No Resistance (2)	–[d]	–
	S. Bredeney (1)	STR SXT (1)	–	–
	S. Derby (5)	No Resistance (4); STR (1)	–	–
	S. Panama (5)	AMP STR SXT (1); AMP STR SXT TET (4)	–	–
	S. Typhimurium (26)	No Resistance (2); AMP CHL STR (1); AMP CHL STR TET (2); AMP GEN STR TET (1); AMP STR TET (3); STR (16); STR TET (1)	None (2); Weak (2); Moderate (8); Strong (2)	None (2); Weak (2); Moderate (11)
After Chlorocresol Disinfectant	*S.* 4,[5],12:i:- (2)	No Resistance (2)	None (2)	None (2)
	S. Bredeney (1)	No Resistance (1)	Weak (1)	Weak (1)
	S. Derby (8)	No Resistance (5); AMP (1); AMP SXT (2)	None (6)	None (5); Weak (1)
	S. Panama (2)	No Resistance (2)	None (2)	None (1); Weak (1)
After Detergent	*S.* 4,[5],12:i:- (19)	No Resistance (2); AMP STR TET (12); STR TET (3); TET (1)	Moderate (1)	Weak (1)
	S. Brandenburg (7)	No Resistance (4); AMP STR SXT TET (1); STR (1); TET (1)	–	–
	S. Bredeney (1)	No Resistance (1)	–	–
	S. Derby (13)	No Resistance (6); AMP FOX STR TET (3); AMP STR (1); AMP STR SXT TET(1); STR (2)	–	–
	S. Panama (5)	AMP STR SXT (1); AMP STR SXT TET (4)	–	–
	S. Typhimurium (14)	AMP CHL STR (2); AMP CHL STR TET (3); AMP GEN STR TET (1); AMP STR SXT TET (1); AMP STR TET (4); STR (2); STR TET (1)	–	–
After Detergent + QAC Disinfectant	*S.* 4,[5],12:i:- (8)	AMP STR TET (4); STR (1); STR TET (3)	None (4); Weak (1)	None (2); Weak (2); Moderate (1)
	S. Bredeney (1)	No Resistance (1)	Moderate (1)	Moderate (1)
	S. Derby (1)	No Resistance (1)	–	–
	S. Typhimurium (10)	No Resistance (1); AMP GEN STR TET (1); AMP STR SXT (1); AMP STR SXT TET (1); AMP STR TET (3); STR (3)	None (3); Weak (5); Moderate (1)	None (1); Weak (5); Moderate (3)
After Detergent + Chlorocresol Disinfectant	*S.* 4,[5],12:i:- (1)	AMP STR SXT TET (1)	None (1)	None (1)
After QAC + Drying	*S.* 4,[5],12:i:- (1)	AMP STR TET (1)	None (1)	None (1)
	S. Brandenburg (1)	STR TET (1)	Moderate (1)	Moderate (1)

[a] *Salmonella* was not recovered from any pen After Chlorocresol + Drying protocol.
[b] Ampicillin (AMP), cefoxitin (FOX), chloramphenicol (CHL), gentamicin (GEN), streptomycin (STR), tetracycline (TET), and trimethoprim/sulfamethoxazole (SXT).
[c] Isolates were classified on the basis of biofilm formation as follows: None (OD isolate </= OD cut-off), Weak (OD cut-off < OD isolate </= 2 × OD cut-off), Moderate (2 × OD cut-off < OD isolate </= 4 × OD cut-off), and Strong (4 × OD cut-off < OD isolate). The OD cut-off was defined as three standard deviations above the mean OD_{595nm} of the negative control wells. Biofilm formation was only performed on selected *Salmonella* isolates, i.e. those with unique AMR profiles, selected serotypes, and/or those that were recovered after cleaning with the disinfectants alone, after cleaning with detergent plus the disinfectants, and after drying. This is the reason why numbers presented in the two columns do not add up to the total number of isolates found.
[d] '–' indicates that biofilm formation work was not carried out as per above.

The abattoir in the present study adhered to the recommendation that herds with a low *Salmonella* seroprevalence are slaughtered at the beginning of the day, whereas those with >50% seroprevalence are slaughtered at the end of the day in order to minimize the risk of cross contamination (Department of Agriculture Food and the Marine (DAFM), 2010). However, no pen(s) within the lairage of the study abattoir was specifically allocated to herds with *Salmonella* seroprevalence > 50%. All pens at one point in time, during the study, did, however, contain pig herds with at least 60% seroprevalence and more than half were allocated to herds with >80% *Salmonella* seroprevalence. This was most likely the reason that all pens were contaminated with *Salmonella*, as stress from transport may have initiated shedding even in pigs from low prevalence herds (Hurd et al., 2001, 2002; Williams and Newell, 1970).

Our results show that high-pressure washing alone was ineffective in reducing *Salmonella* in the lairage pens, and is in agreement with findings from previous research (Argüello et al., 2011; Boughton et al., 2007a; Schmidt et al., 2004; Swanenburg et al., 2001). Application of the chlorocresol-based disinfectant alone after power washing was better in terms of reducing *Salmonella* prevalence than the QAC-based disinfectant or sole use of detergent but results still showed the presence of *Salmonella* in the lairage pens. These results are also supported by the enumeration of *Enterobacteriaceae* and are also similar to those of previous work. For example, Boughton et al. (2007a) showed that reduction but not elimination of *Salmonella* was only achieved in lairage pens at weekends after intensive cleaning and disinfection was performed and pens were allowed to dry. Moreover, our finding that the chlorocresol-based disinfectant had better efficacy than the QAC-based disinfectant is supported by McLaren et al. (2011) and Gosling et al. (2016) who also found that chlorocresol was more effective than QAC's at reducing *Salmonella* in wet and dry environments. However, these studies were performed in the laboratory with inoculated poultry feces, and to our knowledge, the current study is the first to evaluate the use of a chlorocresol-based disinfectant in a commercial abattoir as a means of reducing *Salmonella* prevalence.

Our results are also in agreement with earlier research, which suggest that use of detergent and disinfectant is not fully effective in removing *Salmonella* from the lairage environment (Boughton et al., 2007a; Schmidt et al., 2004; Small et al., 2006; Swanenburg et al., 2001). The present study, however, illustrates that a 4-step protocol consisting of combined use of detergent and a chlorocresol-based disinfectant with subsequent drying for 24 h was the most successful in terms of removing *Salmonella* from the lairage pens. This is a novel finding that has not been shown to date. A previous study investigating the effect of different cleaning regimes on recovery of *Clostridium perfringens* from poultry crates, found that pressure washing with a QAC followed by drying for 48 h greatly reduced the amount of *C. perfringens* as compared to the cleaning regimes without a drying step (McCrea and Macklin, 2006). Although the study differed from the present study in that it focused on Gram-positive bacteria, poultry containers and a 3-step cleaning procedure, it nonetheless showed the effectiveness of drying in terms of reducing bacterial contamination. Further work in this area is needed, especially since our findings demonstrate that drying is a critical step in terms of elimination versus reduction of *Salmonella* from the lairage environment.

This finding, however, highlights the issue of the practicality of allowing lairage pens to dry for 24–48 h and highlights the risks associated with overnight accommodation of pigs in lairage pens when the environment is not dry. Considering the risk that the lairage represents in terms of acquisition of new *Salmonella* infections (Argüello et al., 2014; Duggan et al., 2010; Mannion et al., 2012), continual cleaning with detergent followed by efficient disinfection and drying for as long as possible is required to reduce levels of *Salmonella* in the lairage environment. While the pens in this study were naturally air dried, time is limited for this process and thus it may not be effective, particularly in cool weather with high humidity. As such, we recommend that heaters or other means of artificially drying the pens after cleaning and disinfection be used between batches of animals to shorten the drying time, although this would introduce a cost.

Despite the increasing amount of literature on bacterial resistance to QAC's (Hegstad et al., 2010), none of the isolates recovered in this study were resistant to the QAC-based disinfectant or the chlorocresol-based disinfectant in MIC tests. The fact that *Salmonella* was recovered after various cleaning and/or disinfection protocols were employed, in spite of showing no resistance to the disinfectants, highlights that more attention should perhaps be given to cleaning and disinfection procedures in the lairage, with appropriate use of the chemical agents (i.e., recommended concentrations and contact time) rather than focusing on disinfectant resistance. This finding is supported by several studies describing inadequate evidence of disinfectant resistant isolates from studies conducted in vitro, at farm level, or in abattoirs (Aarestrup and Hasman, 2004; Gantzhorn et al., 2014; Holah et al., 2002; Karatzas et al., 2007; McLaren et al., 2011). Emphasis of the cleaning and disinfection approach should therefore be directed towards problem areas in the pen including cracks and holes in the concrete flooring and walls, and cleaning/disinfecting the walls to the same standard as the floors.

In the transition to biofilm status, some characteristics of bacteria change, including their adherence, invasion, virulence, and resistance (Liu et al., 2014). Therefore, it is extremely difficult to eradicate biofilm-related contamination using routine cleaning methods such as disinfectants. The present study showed that a number of isolates, among the 6 serotypes of *Salmonella* recovered, were able to form biofilms at temperatures representative of those found in Irish abattoirs as well as at the optimal growth temperature for the organism (37 °C). Although the ability to form biofilms was variable, and depended not only on temperature but by the intensity of the cleaning protocol, combining detergents with disinfectants. Moreover, all isolates recovered after the combined use of detergent and the two disinfectants with or without a drying step were susceptible to the QAC-based and the chlorocresol-based disinfectants. As such, in this study, while it is possible that these two *Salmonella* isolates, *S.* Brandenburg and *S.* 4[5],12:i:-, were recovered as a result of their ability to form biofilms, it is more probable that they were recovered as a result of inadequate cleaning and disinfection (Brooks and Flint, 2008; Krysinski et al., 1992; Marin et al., 2009). These findings, suggest that a rigorous cleaning protocol, with for instance a chlorocresol-based disinfectant in combination with a drying step, can remove *Salmonella* from the lairage pen environment.

5. Conclusion

Overall, power washing alone was not successful in reducing the prevalence of *Salmonella* in the lairage pens of a commercial pig abattoir. The key recommendation from the present study is to ensure that lairage pens are allowed to dry after intensive cleaning and disinfection with a chlorocresol-based disinfectant in order to ensure that *Salmonella* is eliminated and *Enterobacteriaceae* counts reduced. Moreover, the ability of *Salmonella* isolates recovered from the lairage pens to form biofilms was variable, and was most common among *S.* Typhimurium, some of which were recovered after multiple step cleaning protocols.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijfoodmicro.2017.02.002.

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The efficacy of disinfectant misting in the lairage of a pig abattoir to reduce *Salmonella* and *Enterobacteriaceae* on pigs prior to slaughter

Kavita Walia [a, b, d], Helen Lynch [a, c, d], Jim Grant [a], Geraldine Duffy [a], Finola C. Leonard [c], Peadar G. Lawlor [d], Gillian E. Gardiner [b, *]

[a] *Teagasc, Food Research Centre, Ashtown, Dublin 15, Ireland*
[b] *Department of Science, Waterford Institute of Technology, Waterford, Ireland*
[c] *School of Veterinary Medicine, University College Dublin, Dublin, Ireland*
[d] *Teagasc, Pig Development Department, Animal and Grassland Research and Innovation Centre, Moorepark, Fermoy, Co. Cork, Ireland*

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Water misting/showers are used in abattoir lairages to improve meat quality, and to cool and calm pigs after transport and during hot weather. One novel approach, which has not been investigated to date, is to add a disinfectant to the misting water as a means of topically reducing *Salmonella* on pigs prior to slaughter, thereby potentially controlling this organism in the abattoir. The objective of this study was therefore to evaluate misting with water or with Virkon® S (an approved disinfectant for use in the presence of animals), for their ability to topically reduce *Salmonella* on high seroprevalence pig herds before stunning and to reduce *Enterobacteriaceae*.

Three experimental groups were investigated: control group (i.e., no misting); water group (misting with cold, 15–17 °C, water, herein referred to as water); and a disinfectant group (misting with 0.5% Virkon® S). As pigs entered the abattoir, each animal was swabbed along its back before being allocated to its experimental group. Each group was randomly assigned to one of 3 lairage pens that were separated by non-trial pens. After 30 min of misting with water or disinfectant, pigs were moved to the stunning area, where each pig was again swabbed, as above. Swabs were analyzed for the presence of *Salmonella* and enumeration of *Enterobacteriaceae*.

Before misting, *Salmonella* prevalence on the pigs was 79.0%, 72.1% and 83.6% for the control, water and disinfectant groups, respectively. After misting, *Salmonella* prevalence increased to 94.3% in the water group; whereas for the disinfectant group, the prevalence increased marginally to 85.9%. No change in *Salmonella* prevalence was detected for the control group. In line with the *Salmonella* results, no significant differences were observed in *Enterobacteriaceae* counts in the control group at either time point (4.37 and 5.01 $\log_{10}$ CFU/cm^2, respectively) or in the disinfectant group before and after misting (4.02 and 4.26 $\log_{10}$ CFU/cm^2, respectively). However, a 2.3 $\log_{10}$ CFU/cm^2 increase in *Enterobacteriaceae* was recorded for the water group after misting as compared to before misting ($p < 0.05$).

Since misting with water alone increased topical *Salmonella* contamination on pigs before slaughter, a risk assessment based on known *Salmonella* data, meat quality and welfare is recommended to determine whether its use is justifiable. On the other hand, the findings from this study suggest that misting with Virkon® S at 0.5% could have a role in topical antisepsis of pigs contaminated with *Salmonella* prior to slaughter and as such this warrants further investigation.

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Abbreviations: NPSCP, national pig *Salmonella* control program; psi, pounds per square inch; CFU, colony forming unit; APC, aerobic plate counts; BPW, buffered peptone water; PCA, plate count agar; TSB, tryptone soya broth; MRD, maximum recovery diluent; XLD, xylose lysine deoxycholate; MRSV, semi-solid rappaport vassiliadis; BGA, brilliant green agar; VRBGA, violet red bile glucose agar; PCR, polymerase chain reaction; AMR, antimicrobial resistance; MIC, minimal inhibitory concentration; EUCAST, European committee on antimicrobial susceptibility testing; SAS, statistical analyses system.

* Corresponding author.
E-mail address: ggardiner@wit.ie (G.E. Gardiner).

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1. Introduction

Holding pigs in lairage pens before slaughter presents challenges for *Salmonella* control. Numerous studies have shown that lairage pens are highly contaminated with *Salmonella,* which not only poses a risk to incoming naïve pigs but also for cross contamination along the slaughter line (Boughton, Egan, Kelly, Markey, & Leonard, 2007; Duggan et al., 2010; Mannion et al., 2012; Rostango et al., 2003; Swanenburg, Urlings, Keuzenkamp, & Snijiders, 2001; Walia et al., 2016). One approach to limit *Salmonella* contamination of pigs is to use misting devices or sprinklers already present in the lairage. During lairage holding, these devices mist or shower pigs with water, which aims to cool and calm the animals after transport, and especially during hot weather. Primarily, misting/showering is used as a means of reducing stress in order to improve meat quality with an added benefit of removing gross fecal matter on the animal before slaughter (Warriss, 2003). As a result, misting pigs may offer a means of topically reducing *Salmonella* on live pigs prior to slaughter and in the lairage environment, and as such, may be a potential strategy for *Salmonella* control in the abattoir, especially if a disinfectant can be added to the misting water. This is a novel approach which has not been investigated to date, although topical antisepsis, at weaning, of pigs previously exposed to shedding dams has proved successful in reducing subsequent *Salmonella* shedding (Patchanee, Crenshaw, & Bahnson, 2007). Previous research in cattle has shown that washing hides immediately before slaughter with water or bromide compounds can reduce carcass contamination with *Escherichia coli* O157:H7 (Byrne, Bolton, Sheridan, McDowell, & Blair, 2000; Schmidt, Wang, Kalchayanand, Wheeler, & Koohmaraie, 2012). Given that potable hot water is the only method currently approved for decontaminating pig carcasses in the EU (Regulation (EC) No 853/2004, 2004), and that carcass contamination rates in Ireland have still not declined below the 20% level found in the EU baseline survey (DAFM, personal communication; European Food Safety Authority, 2008), disinfectant misting is another possible approach to topically decontaminate infected pigs prior to slaughter. This novel misting strategy could complement the existing decontamination activities that occur after stunning (i.e., logistical slaughter, scalding, singeing), in the overall hurdle approach to control *Salmonella* in the abattoir.

Compounds such as chlorine and organic acids may be suitable for addition to the misting water, as they are effective in reducing *Salmonella* on pig skin, *in vitro,* when added to water (Kich et al., 2011). However, the challenge with topical antisepsis of pigs prior to slaughter is to ensure the use of compounds that are registered for use in the presence of live animals. Virkon® S is one such product (Chemours, 2015; Antec International Ltd, personal communication) and was chosen for the present study. It is a commercially available broad spectrum disinfectant that is widely used against microorganisms, including *Salmonella,* in the pig and poultry industry via boot dips, cold and thermal fogging, and misting or aerial spraying (Block, 2001). It is a stable oxidizing agent (a peroxymonosulphate) that generally denatures proteins, disrupts cell wall permeability, and oxidizes sulphydryl and sulphur bonds in proteins, enzymes, and other metabolites, ultimately leading to cell lysis and death (Block, 2001; Dunowska, Morley, & Hyatt, 2005).

Since the recommended minimum contact time for Virkon® S against *Salmonella* is 10 min, it was hypothesized that 30 min of constant misting with the disinfectant, at the recommended dilution rate for use in the presence of live animals, would reduce the prevalence of *Salmonella*, topically, in pig herds with high *Salmonella* seroprevalence (i.e., >80% *Salmonella* seroprevalence, as determined by the Irish National Pig *Salmonella* Control Programme, NPSCP). Therefore, the objectives of this study were to evaluate misting with water alone or with water containing Virkon® S disinfectant for their ability to: (1) topically reduce *Salmonella* on high seroprevalence pigs prior to slaughter, and (2) reduce *Enterobacteriaceae,* used as a measure of overall hygiene, both topically on the pigs as well as in the lairage environment.

2. Materials and methods

2.1. In vitro pig skin tests

Prior to conducting the trial on live pigs in the lairage, laboratory tests were conducted on pig skin, *in vitro*, to determine the efficacy of the Virkon® S disinfectant (a blend of potassium peroxymonosulfate, sulfamic acid, and sodium chloride; Antec International Limited, Sudbury, Suffolk, UK) in reducing *Salmonella* based on the method used by Kich et al. (2011) with modifications. Briefly, 18 pig skin samples, each taken from the neck of pig carcasses before chilling, were obtained from the study abattoir and each was cut uniformly to measure 18 cm × 10 cm. Skin samples were artificially inoculated with each of three suspensions of nalidixic acid resistant *Salmonella* Typhimurium containing 10^4, 10^5, or 10^6 CFU/mL, with 6 skin samples used for each inoculum. Briefly, the nalidixic acid resistant *S.* Typhimurium strain was grown overnight on plate count agar (PCA, Oxoid Limited, Hampshire, UK), and a single colony was inoculated into 90 mL of tryptone soya broth (TSB, Oxoid Limited), incubated overnight at 37 °C and then re-suspended in 10 mL of maximum recovery diluent (MRD, Oxoid Limited) to achieve suspensions containing ~10^4, ~10^5, and ~10^6 CFU/mL, respectively. Spread plate counts were performed on PCA to confirm the *Salmonella* concentration in each inoculum. A volume of 1 mL of *Salmonella* suspension was pipetted onto each skin sample and spread uniformly using a plate spreader. After 2 h at room temperature, each of the skin samples were swabbed with sterile 100 cm^2 sponges pre-moistened with MRD (Technical Service Consultants Ltd, Lancashire, UK) before being allocated to either a control group (no treatment), water group, or disinfectant group (i.e., 2 skin samples per group, per inoculum). A fine spray of either water or 0.5% Virkon® S disinfectant was applied to the surface of each skin sample in the water and disinfectant groups, respectively at 1 min intervals (i.e., 1 min spraying, 1 min not spraying) for 30 min, mimicking misting in the lairage in so far as possible. After spraying, each skin sample was again swabbed, as above, using the MRD sponges for the water and control groups or a 50 cm^2 sponge pre-moistened with neutralizing buffer (Technical Service Consultants Ltd) for the disinfectant group. Each swab was homogenized in 90 mL of buffered peptone water (BPW; Oxoid Limited), in a stomacher for 2 min. One hundred microliters of the homogenate was spread-plated on xylose lysine deoxycholate (XLD; Oxoid Limited) agar containing 30 μL/mL nalidixic acid (Sigma-Aldrich Ireland Limited) made up in 100% chloroform (Sigma-Aldrich Ireland Limited), in order to enumerate the nalidixic acid resistant *Salmonella*. The plates were incubated at 37 °C for 24 h.

2.2. Lairage trial

2.2.1. Experimental design

One commercial pig abattoir in the Republic of Ireland participated in this study. This abattoir routinely operates a Monday to Friday schedule, slaughtering approximately 2000 pigs per day from herds across the country. The lairage area (Fig. 1) consisted of 12 main pens with solid concrete floors and walls. Each pen is 2.05 m × 14.95 m, with a capacity for holding 65 pigs during the day and 45 pigs overnight. A 12.7 mm diameter pipe hangs above

the length of each pen and provides a mist of cold (15–17 °C) water (herein referred to as water) at a pressure of 2 bars (29 psi), delivering 1.4 L of water per minute over the entire lairage pen. One dosatron (0.2%–2% dose rate; Hingerose Limited, Northamptonshire, UK) was fixed to the water pipes in each of the lairage pens 3, 6 and 9 (Fig. 1) according to the manufacturer's instructions, in order to facilitate disinfectant addition to the misting water.

The experimental groups were as follows: (1) control group, in which no misting was performed; (2) water misting; and (3) disinfectant misting, in which misting with 0.5% Virkon® S was performed using a dosatron for Virkon® S addition. Pig herds with a *Salmonella* seroprevalence of greater than 80% based on data extracted from the Irish NPSCP were used in the study.

The 3 experimental groups were assigned to 3 lairage pens (either pen 3, 6 or 9; Fig. 1), ensuring that each pen was separated by non-trial pens. As the pigs entered the lairage area of the abattoir each animal was swabbed with one sterile MRD sponge, as used for the *in vitro* experiment outlined in Section 2.1, before being allocated to either the control, water or disinfectant group. The average length (from head to tail) of a finisher pig at market weight was determined to be ~160 cm. Therefore, each swab covered an area of ~10 cm × 160 cm along the length of each pig. In addition, 3 swabs from each of the trial lairage pens (2 floor swabs and 1 wall swab, each covering an area of 40 cm × 40 cm) were taken prior to entry of the pigs, also using the MRD sponges. Once pigs were in the lairage pens, the water or disinfectant misting was applied for 30 min continuously. Afterwards, the misting devices were switched off and as the pigs were moved into the stunning area, but prior to being stunned, each pig in the water and control groups was again swabbed with sterile MRD sponges, as outlined above, or with sponges containing neutralizing buffer for the disinfectant misting group, as outlined in the *in vitro* experiment in Section 2.1. Three additional swabs of each of the trial lairage pens (2 floor and 1 wall) were collected for each group after the pigs had exited, either using the sterile sponges with MRD for the control and water misting groups or the sponges with neutralizing buffer for the disinfectant misting group, as above. All swabs were collected aseptically and were kept at 4 °C and processed within 24 h.

The entire lairage experiment as outlined above was performed in triplicate, i.e., on 3 separate days with 3 different pig herds. Different pens were used for each experimental group each day to avoid any potential pen effect.

2.2.2. Microbiological analysis of lairage trial samples

Each sponge was homogenized in 90 mL of BPW in a stomacher for 2 min. This homogenate was then tested for the presence of *Salmonella* in accordance with International Organization for Standardization (ISO) 6579:2007 (Amendment 1: Annex D) method (International Organization for Standardization, 2007). All media were obtained from Oxoid Limited. Briefly, BPW homogenates were incubated at 37 °C for 19 h, after which 100 μL of each sample was inoculated onto modified semi-solid rappaport-vassiliadis (MSRV) agar plates and incubated at 42 °C for 24 h. If the MSRV plate was negative, it was incubated for a further 24 h. Presumptive *Salmonella* growth was then streaked onto XLD and brilliant green agar (BGA) plates and incubated at 37 °C for 24 h. Suspect colonies from XLD or BGA plates were then streaked onto PCA plates, and incubated at 37 °C for 24 h. Urea agar slants and *Salmonella* chromogenic agar plates were then inoculated with colonies from the PCA plates and incubated at 37 °C for 24 h. Serological confirmation of colonies from the PCA plates was performed using a *Salmonella* latex agglutination kit (Oxoid Limited).

In addition, *Enterobacteriaceae* counts were obtained from 5 pigs per group, both before and after treatment and from the 2 floor swabs from each trial lairage pen before and after treatment, as follows: 10-fold serial dilutions of the BPW homogenates were performed in MRD and appropriate dilutions pour-plated on violet red bile glucose agar (VRBGA, Oxoid Limited). Plates were overlaid with VRBGA and incubated at 37 °C for 24 h. The limit of detection was 10 CFU/cm^2.

2.2.3. Serotyping and antimicrobial resistance determination of Salmonella isolates

All presumptive *Salmonella* isolates recovered from both the pigs and the pens before and after misting were first screened using a real-time polymerase chain reaction (PCR) assay for the identification and differentiation of *Salmonella enterica* serotype Typhimurium and *S.* 4,[5],12:i:- as described by Prendergast et al. (2013). Isolates not identified as *S.* Typhimurium or its monophasic variant were then serotyped according to the White-Kauffmann-Le Minor scheme (Grimont & Weill, 2007) using commercial antisera (Pro-Lab Diagnostics, Cheshire, UK; SIFIN Institute, Berlin, Germany; Statens Serum Institute, Copenhagen, Denmark).

The antimicrobial resistance (AMR) profile of each *Salmonella* isolate was determined using the Sensititre™ Gram negative NARMS plate (Thermo Scientific, Serosep Ltd, Limerick, Ireland).

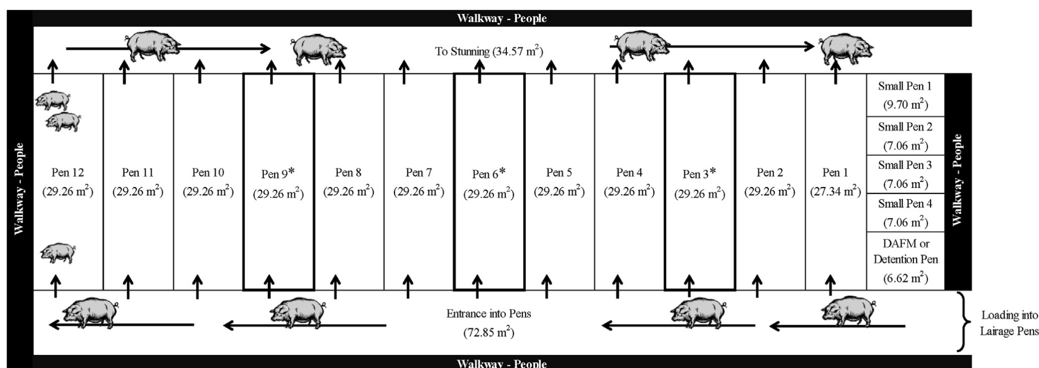

Fig. 1. Lairage pen set-up. *Lairage pens 3, 6 and 9 were used in the experiment. One dosatron was fixed to the water pipes in each of these three pens according to the manufacturer's instructions, in order to facilitate disinfectant (Virkon® S) addition to the misting water. Each pen was used on a different sampling day to avoid a potential pen effect.

The following antimicrobials were tested: amoxicillin-clavulanic acid (AUG), ampicillin (AMP), azithromycin (AZI), cefoxitin (FOX), ceftiofur (XNL), ceftriaxone (AXO), chloramphenicol (CHL), ciprofloxacin (CIP), gentamicin (GEN), nalidixic acid (NAL), streptomycin (STR), sulfisoxazole (FIS), tetracycline (TET), and trimethoprim/sulfamethoxazole (SXT). Minimal inhibitory concentrations (MICs) were interpreted using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) epidemiological cut-off values.

2.3. Statistical analysis

For *Salmonella* prevalence, the binary data were analyzed using the GLIMMIX procedure in Statistical Analyses System (SAS, V9.3, 2011). The differences between the groups were evaluated using the Tukey-Kramer least squares means adjustment for multiple comparisons, with the proportions of positive results in each of the groups before treatment (i.e., 'Control Before', 'Water Before Misting', 'Disinfectant Before Misting') used as a covariate in the model.

For *Enterobacteriaceae*, counts were log-transformed after which the data were analyzed as normally distributed data using the GLIMMIX procedure in SAS, with date as a blocking factor. Tukey-Kramer grouping for treatment least square means was performed for differences between group means.

Residual checks were made to ensure that the assumptions of the analyses were met. For all analyses, statistical significance was established at $\alpha = 0.05$.

3. Results

3.1. Enumeration of Salmonella from laboratory pig skin samples, in vitro

Mean counts of nalidixic acid resistant *S.* Typhimurium obtained from the pig skin samples before and after treatment are shown in Fig. 2. The *Salmonella* counts for the control (untreated) group remained the same for the three inocula used. After spraying with disinfectant, a 1.8, 1.3 and 1.7 $\log_{10}$ CFU/cm^2 reduction of the nalidixic acid resistant *S.* Typhimurium was achieved, at the three different inocula, respectively. This is compared to spraying with water where 1.9 and 1.0 $\log_{10}$ CFU/cm^2 reductions were achieved at the higher inocula, while a 0.9 $\log_{10}$ CFU/cm^2 increase was observed with the lower inoculum.

3.2. Salmonella prevalence in lairage trial samples

Mean prevalence of *Salmonella* before and after misting with either water or disinfectant or no treatment (control), i.e., the number of *Salmonella*-positive swabs as a percentage of the number taken, is shown in Fig. 3. In total 124, 122 and 128 pigs were swabbed for the control, water and disinfectant groups, respectively. Before pigs were allocated to the lairage pens (i.e. before misting) the *Salmonella* prevalence for each group, was 79.0% (98/124), 72.1% (88/122) and 83.6% (107/128), respectively (Fig. 3). After misting, the *Salmonella* prevalence increased by 30.7% in the water group to 94.3% (115/122); whereas for the disinfectant group, the prevalence increased by a marginal 2.7%–85.9% (110/128, Fig. 3). No change in *Salmonella* prevalence was detected for the control group (Fig. 3).

When the data were analyzed statistically, the disinfectant was better at preventing an increase in the probability of detecting *Salmonella* on the pig skin than misting with water alone (84.9% versus 96.3%, respectively, $p < 0.05$, Table 1). Likewise, not misting (i.e., control group) was also better at preventing an increase in the probability of detecting *Salmonella* than misting with water alone (80.5% versus 96.3%, respectively, $p < 0.05$, Table 1). On the other hand, no significant differences were observed between not misting versus misting with the disinfectant in terms of the probability of detecting *Salmonella* (80.5% versus 84.9%, respectively, $p > 0.05$, Table 1).

In terms of *Salmonella* prevalence within the trial lairage pens, before pigs were allocated to the 3 treatment pens, all pen swabs were *Salmonella*-positive for each experimental group. After water misting, all of the pen swabs (100%) were *Salmonella*-positive, while 89% (8/9) of swabs taken after disinfectant misting were *Salmonella*-positive and 7/9 (78%) of swabs taken from the control pen were *Salmonella*-positive.

3.3. Enumeration of Enterobacteriaceae in lairage trial samples

The overall effect of 'no misting', water misting or disinfectant

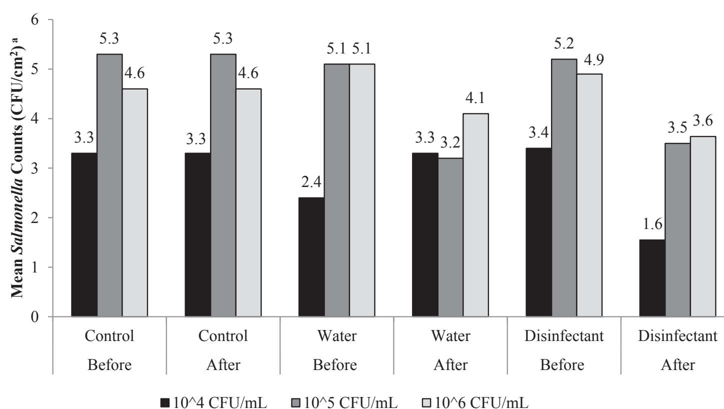

Fig. 2. Mean *Salmonella* counts ($\log_{10}$ CFU/cm^2) from samples of pig skin artificially inoculated with three different concentrations of *Salmonella* Typhimurium. Counts are shown before and after spraying with either water or disinfectant, or no treatment (i.e., control group). [a] Mean *Salmonella* counts from 2 skins samples per group, per inoculated concentration. Each skin sample was 18 cm × 10 cm.

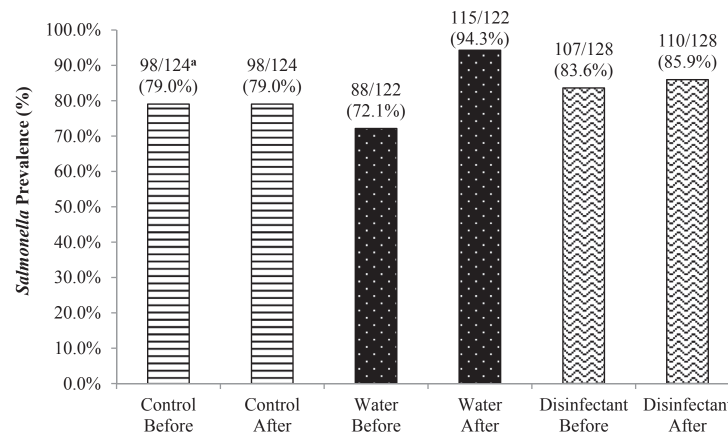

Fig. 3. Prevalence of *Salmonella* on live pigs swabbed before and after misting with either water or disinfectant, or no misting (i.e., control group) in a commercial pig abattoir. [a] *Salmonella* prevalence was calculated from the No. *Salmonella*-positive swabs/No. swabs taken.

Table 1
Effect of misting with water or disinfectant, or no misting on the probability of detecting *Salmonella* on the skin of pigs in a commercial pig abattoir.

Group	Probability (%)[a]	p-value
Control After[b] vs. Disinfectant After Misting[c]	80.5 vs. 84.9	0.65
Control After vs. Water After Misting[d]	80.5 vs. 96.3	0.001
Disinfectant After Misting vs. Water After Misting	84.9 vs. 96.3	0.01

[a] Values presented are the probability of detecting *Salmonella* as calculated from the mean *Salmonella* prevalence data. The values for the 'before' groups were used to construct a baseline for the 'after' groups, by using the proportions of positive results in each of the groups before treatment as covariates in the statistical model.
[b] Sem for 'control after' group was 0.037.
[c] Sem for 'disinfectant after misting' group was 0.033.
[d] Sem for 'water after misting' group was 0.016.

misting on topical *Enterobacteriaceae* counts on the live pigs is presented in Table 2. Similar to the *Salmonella* results, no significant differences were seen in *Enterobacteriaceae* counts for pigs in the control group before compared to after 'no misting' or in the disinfectant group before and after misting (Table 2). However, a 2.3 $\log_{10}$ CFU/cm^2 increase in *Enterobacteriaceae* was observed for the water group after misting as compared to before misting ($p < 0.05$, Table 2).

In terms of mean *Enterobacteriaceae* counts from the environmental swabs of the lairage pens, no differences were observed in the control and water misting groups after versus before treatment (6.04 versus 5.93 $\log_{10}$ CFU/cm^2 and 5.80 versus 5.67 $\log_{10}$ CFU/cm^2, respectively). On the other hand, a slight decline in *Enterobacteriaceae* counts was observed in the disinfectant group after misting compared to before misting (5.60 versus 5.95 $\log_{10}$ CFU/cm^2).

Table 2
The effect of no misting, water misting or disinfectant misting on topical *Enterobacteriaceae* counts from live pigs in a commercial pig abattoir.

Group	Mean *Enterobacteriaceae* counts[c] ($\log_{10}$ CFU/cm^2)
Control Before	4.37^{ab}
Control After	5.01^{ab}
Water Before Misting	3.31^{b}
Water After Misting	5.62^{a}
Disinfectant Before Misting	4.02^{ab}
Disinfectant After Misting	4.26^{ab}
Pooled sem	0.56

[a,b] Values within a group without a common superscript are significantly different ($p < 0.05$).
[c] Mean *Enterobacteriaceae* counts from 5 pigs per group, sampled on 3 occasions i.e. 15 pigs per group.

3.4. Serotyping and antimicrobial resistance profiling of Salmonella isolates from lairage trial samples

The serotypes and AMR profiles of the 90 *Salmonella* isolates recovered from the pigs before and after misting with water, disinfectant or no misting are detailed in Table 3. Overall, two serotypes, *S.* 4,[5],12:i:-, and *S.* Typhimurium, were detected on the pigs; and the same serotypes were also isolated from the trial lairage pens. Of the AMR profiles found for the pig isolates, 12 different profiles were detected for the *S.* 4,[5],12:i:- isolates, while 6 profiles were detected for the *S.* Typhimurium. Most of the isolates were multidrug resistant, demonstrating resistance to ampicillin, chloramphenicol, streptomycin, trimethoprim/sulfamethoxazole and tetracycline. However, a relatively small proportion of the 90 isolates showed additional resistance to: ceftiofur (1 isolate), ciprofloxacin (1 isolate), and gentamicin (15 isolates). Generally, after misting, more AMR profiles emerged on account of an increase in the recovery of *Salmonella* isolates. In addition, AMR profiles tended to include gentamicin as compared to before misting.

4. Discussion

In the present study, a disinfectant in the form of Virkon® S, added to the misting water at a commercial abattoir, was investigated as a novel means of reducing both environmental and skin contamination of *Salmonella* from high prevalence pig herds prior to slaughter.

Results from preliminary *in vitro* work demonstrated that application of Virkon® S, at 1 min intervals for 30 min, was successful in reducing the level of *Salmonella* contamination on pig skin, demonstrating the potential of topical antisepsis strategies. This finding is similar to that of Kich et al. (2011) who found that 10 s of disinfectant treatment was effective at reducing *Salmonella*

Table 3
Salmonella serotypes and antimicrobial resistance (AMR) profiles of isolates recovered from pigs, before and after water misting, disinfectant misting, or no misting (i.e., control group) were applied to live pigs in a commercial pig abattoir.

Group	*Salmonella* serotype (No. per serotype)	AMR profiles (No. per serotype)[a]
Control Before	*S.* 4,[5],12:i:- (5)	AMP CHL GEN STR SXT TET (1); AMP GEN STR TET (1); AMP GEN STR SXT TET (2)
	S. Typhimurium (9)	No Resistance (2); AMP CHL STR SXT TET (4); AMP STR TET (1); STR (1); SXT (1)
Control After	*S.* 4,[5],12:i:- (5)	No Resistance (1); AMP GEN STR SXT TET (1); AMP STR SXT TET (1); STR TET (2)
	S. Typhimurium (11)	AMP CHL STR SXT TET (4); AMP STR SXT TET (2); STR (5)
Water Before Misting	*S.* 4,[5],12:i:- (5)	AMP CIP STR TET (1); AMP STR TET (1); AMP STR SXT TET (3)
	S. Typhimurium (9)	AMP CHL STR SXT TET (4); AMP STR SXT TET (2); STR (2); STR SXT (1)
Water After Misting	*S.* 4,[5],12:i:- (5)	AMP CHL SXT TET (1); AMP GEN SXT TET (1); AMP GEN STR SXT TET (1); AMP; STR SXT TET (1); AMP SXT TET (1);
	S. Typhimurium (10)	No Resistance (1); AMP CHL STR SXT TET (2); AMP STR SXT TET (3); STR (4)
Disinfectant Before Misting	*S.* 4,[5],12:i:- (5)	AMP GEN STR TET (4); AMP GEN STR SXT TET (1)
	S. Typhimurium (10)	AMP CHL STR SXT TET (3); AMP STR SXT TET (2); STR (5)
Disinfectant After Misting	*S.* 4,[5],12:i:- (6)	AMP GEN STR TET (1); AMP GEN SXT TET (1) AMP GEN STR SXT TET (1); AMP STR SXT TET (2); AMP STR TET XNL (1)
	S. Typhimurium (11)	AMP CHL STR SXT TET (3); AM STR TET (2); AMP STR SXT TET (1); STR SXT (1); STR (4)

[a] Antimicrobials consisted of: ampicillin (AMP), ceftiofur (XNL), chloramphenicol (CHL), ciprofloxacin (CIP), gentamicin (GEN), streptomycin (STR), tetracycline (TET), and trimethoprim/sulfamethoxazole (SXT).

on artificially contaminated pig skin, albeit the agents used (chlorine and organic acids) differed from those employed in the present study.

On the other hand, when applied in the lairage of a commercial abattoir, our results showed that misting with Virkon® S for 30 min on high *Salmonella* prevalence pig herds had little effect in terms of reducing the organism, topically, prior to slaughter. However, *Salmonella* prevalence remained stable, as it did in the control group, which had no misting throughout the trial, while interestingly, misting with water alone increased the prevalence of *Salmonella*. This finding is in contrast to that of a study conducted in cattle where pressure washing with water for 3 min, although different from the low pressure longer duration misting used in the current study, significantly reduced *E. coli* O157:H7 from swabbed areas before animals were slaughtered (Byrne et al., 2000).

To our knowledge, this is the first study to investigate the effect of water misting in the lairage on *Salmonella* prevalence on live pigs. Interestingly, our results suggest that low pressure misting with water facilitates acquisition of *Salmonella* by pigs, probably from both the environment and from other pigs, during lairage holding. It is likely that the water droplets aid *Salmonella* dispersal or that the humid environment created by misting favours growth of the organism. Moreover, although no reductions in *Salmonella* prevalence were observed in the control or disinfectant groups, the prevalence remained constant, suggesting that misting with Virkon® S or not misting at all were better in terms of limiting *Salmonella* contamination than the current practice of misting animals with water. However, as the *Salmonella* prevalence on the animals in this study was extremely high, i.e., >80%, the biological significance of these results in relation to *Salmonella* control is questionable and necessitates additional research. For example, studies should be performed in herds with a lower *Salmonella* prevalence (but still high enough to warrant the use of control measures), as it is possible that the effects of disinfectant misting might be more pronounced in these, as the topical *Salmonella* load would be lower. In addition, had *Salmonella* been enumerated on the animals, we may have seen greater effects on *Salmonella* reduction. Future work should also investigate carcass contamination post-slaughter, as this was outside the scope of the present study. Furthermore, the fact that we observed a decrease in *Salmonella* contamination, *in vitro*, under controlled laboratory conditions with the disinfectant, but not on live pigs may be because *Salmonella* counts were performed *in vitro* but not *in vivo*. Nonetheless, our lack of correlation between *in vitro* and *in vivo* findings is similar to findings of an earlier study by Mies et al. (2004). The authors found that spray wash treatments of water, lactic acid, or chlorine on cattle pre-slaughter were unsuccessful in decreasing *Salmonella,* whereas when applied at higher concentrations to cattle hides, *in vitro*, a decrease in *Salmonella* counts was observed. This highlights the importance of field trials when evaluating any *Salmonella* control measure for use in the abattoir. Additionally, since all trial pens contained at least one *Salmonella*-positive sample before the trial commenced and after pigs were removed from the pens, this suggests that misting with or without disinfectant is not effective in reducing *Salmonella* in the lairage pens.

Although *Salmonella* counts were not performed on the animals, as outlined above, we performed *Enterobacteriaceae* counts as an indicator of the overall contamination of the pigs and lairage pens by enteric organisms. These data allowed for an indirect estimate of the effect of the treatments on *Salmonella* skin and pen contamination. In agreement with the *Salmonella* results, topical *Enterobacteriaceae* counts increased after water misting but not with disinfectant misting or when no treatment was applied. This, together with the fact that there was essentially no impact on *Enterobacteriaceae* counts in the lairage pens, suggests that water misting, as currently practiced at commercial abattoirs, will not reduce the level of *Enterobacteriaceae,* either in the lairage pens or on the animal, at least in high prevalence herds, and in fact has the opposite effect where the latter was concerned. This finding is supported by several studies, two of which were conducted by the authors, which found that power washing with water alone did not reduce *Enterobacteriaceae* counts either in lairage pens (Walia et al., 2016) or in transport trucks after unloading pigs in the abattoir (Mannion, Egan, Lynch, Fanning, & Leonard, 2008). Additionally, Mies et al. (2004) showed that spray washing water on cattle increased aerobic plate counts (APC), coliforms or *E. coli* before slaughter as compared to using a lactic acid solution, or chlorine. Likewise, Bell (1997) and Ellerboek, Wegener, and Arndt (1993) showed that spray washing water on cattle and sheep carcasses, respectively, was ineffective at reducing APC and *E. coli* contamination. While there were differences in these studies as compared to our study (i.e., high-pressure water, cattle and sheep, carcasses, lactic acid and chlorine), they nonetheless support our finding that washing with water alone does not decrease *Enterobacteriaceae*

counts.

In the abattoir used in this study, pigs are normally misted with water for between 30 min and 2 h, depending on the waiting time to stunning. Although 30 min of intermittently misting or showering pigs with water, is generally accepted as a means to cool, calm, and reduce aggression of pigs in lairage pens (Faucitano, 2010; Warriss, 2003; Weeding, Guise, & Penny, 1993), there is no agreement on the optimum duration of misting. The present study was conducted in a commercial abattoir where pigs are rested for a minimum of 30 min in the lairage pens prior to slaughter. Therefore, 30 min was chosen as the contact time for the water and Virkon® S misting groups and was standardised across replicates. Earlier research has also shown that removing organic matter, albeit on non-skin surfaces, prior to disinfection, increases the efficacy of various disinfectants, including oxidizing agents, against *Salmonella* (Gradel, Sayers, & Davies, 2004; Stringfellow et al., 2009). Therefore, removing visible organic matter on pigs prior to entry into the lairage pens, which is not current practice in the study abattoir, and increasing the disinfectant contact time, as well as examining different disinfection agents are possibilities for future research. It is also possible that the water pressure and flow rate exerted from the misting device used in the present study was inadequate for decontamination. While the animals looked visibly clean, they still had a high prevalence of *Salmonella*. As such, increasing the pressure and flow rate of the misting device to perhaps a minimum of 100 psi and 7.5 L per minute, respectively, as recommended by Pordesimo, Wilkerson, Womac, and Cutter (2002) for the reduction of microbial contamination on meat and carcasses may reduce *Salmonella* contamination. However, any adverse effects on animal welfare and/or meat quality would require prior investigation.

5. Conclusion

Results from the present study show, for the first time, that misting pigs in the lairage with water alone, as is the current practice in a number of commercial abattoirs, increases topical *Salmonella* contamination prior to slaughter. This suggests that a risk assessment should therefore be completed in abattoirs based on known *Salmonella* data, meat quality and welfare considerations as to whether its use should be avoided for high *Salmonella* prevalence herds. On the other hand, the findings from this study suggest that the addition of Virkon® S to the misting water can limit this contamination and may therefore have a role in topical antisepsis of pigs contaminated with *Salmonella* prior to slaughter in abattoirs that wish to use misting.

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