Contamination of Chicken Carcasses and the Abattoir Environment with *Salmonella* spp. and *Listeria monocytogenes* in Taiwan

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This thesis is presented for the degree of

Doctor of Philosophy

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Thesis Declaration

I, Chih-Hsien Lin, declare that:

This thesis is my account of my research and has been substantially accomplished during enrolment in this degree, except where other sources are fully acknowledged. All co-authors, where stated and certified by my Principal Supervisor or Executive Author, have agreed that the works presented in this thesis represent substantial contributions from myself. The thesis contains as its main content, work that has not previously been submitted for a degree at any other university. In the future, no part of this thesis will be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of Murdoch University and where applicable, any partner institution responsible for the joint-award of this degree.

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Abstract

Contaminated poultry and poultry products are the main food sources for human salmonellosis and listeriosis, with these contaminations predominantly occurring in abattoirs during the slaughtering and processing stages. Although poultry is the most commonly consumed meat in Taiwan, the epidemiological characteristics of contaminations during processing were not known prior to the studies described in this thesis.

A nationwide survey of 362 batches of broiler carcasses (1810 individual carcasses) processed at 45 abattoirs in Taiwan found that 56.4% (95% CI: 51.1-61.5) were positive for *Salmonella*. The results of a multivariable logistic regression analysis found that contamination was significantly associated with: season of sampling (warm season > cooler season, OR = 1.95; 95% CI: 1.2-3.2); location of the abattoir (southern region < northern region, OR = 0.45; 95% CI: 0.3-0.8); duration of scalding (scalding times longer than 90 seconds < shorter scalding times, OR = 0.2; 95% CI: 0.1-0.3); and bird type (commercial white broiler < Taiwan native chickens, OR = 0.21; 95% CI: 0.1-0.4).

Salmonella were detected in 156 of 622 samples (25.1%; 95% CI: 21.7-28.7) collected from a more intensive study undertaken at six abattoirs. The prevalence of *Salmonella* varied between sampling sites with 5.8, 17.6, 31.3 and 35.5% of cloacal swabs, environmental samples prior to processing, environmental samples during processing and carcass rinse samples, respectively, being positive. These 156 isolates represented 50 PFGE types. The presence of the same PFGE type at multiple stages during processing highlighted that the abattoir environment and intestinal contents are important sources of *Salmonella* in abattoirs. *Listeria monocytogenes* was not detected in any cloacal swabs (n = 120) or environmental (n = 256) samples collected before and

during processing, but 28 of 246 (11.4%; 95% CI: 7.7-16.0) rinse samples collected from carcasses post-evisceration were positive. These 28 isolates represented 5 PFGE types, confirming the presence of cross-contamination during processing.

An intensive study undertaken at one abattoir on 12 consecutive processing days involving repeated sampling of chickens from 12 farms detected *Salmonella* in 83.3, 22.9, 35.4, 34.4, 19.8 and 21.9% of carcass samples at post exsanguination, post plucking, post evisceration, post inside-outside bird washer, post wash tank, and post air-chilling, respectively. Fifty-seven PFGE types were characterized from the 223 isolates, and confirmed that *Salmonella*-infected flocks are important sources of contamination in the abattoir resulting in subsequent cross-contamination of carcasses. Distribution biomaps were developed in combination with *Salmonella* PFGE profiles to identify potential sources of cross-contamination in the abattoir.

Overall a total of 968 isolates belonging to 33 serotypes of *Salmonella* were detected in the three studies, with *S.* Albany (30.9%), *S.* Enteritidis (16.5%), *S.* Schwarzengrund (9.7%), and *S.* Typhimurium (6.7%) being most frequently isolated. These results demonstrate that the *Salmonella* serotypes commonly isolated from chicken carcasses and the abattoir environment were also those frequently affecting humans in Taiwan, supporting the belief that contaminated chicken meat is one source of human salmonellosis. The 28 isolates of *L. monocytogenes* were serotyped as either 1/2a (82.1%) or 1/2b (14.3%), with one isolate non-typeable (3.6%). These serotypes were the same as those causing the majority of human listeriosis cases in Taiwan and other countries of the world.

It is concluded that the information obtained from this research can be used to assess control measures to minimize the contamination of chickens processed at abattoirs in Taiwan with potentially pathogenic bacteria.

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This thesis contains work that has been published and prepared for publication.

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Abbreviations

WHO	World Health Organization
FAO	Food And Agriculture Organization Of The United Nations
OECD	Organization For Economic Cooperation And Development
CFU	Colony Forming Units
USA	The United States Of America
ROC	The Republic Of China (Taiwan)
DGBAS	Directorate-General Of Budget, Accounting And Statistics, Taiwan
COA	Council Of Agriculture, Executive Yuan, Taiwan
OIS	The Office Of Information Services, Executive Yuan, Taiwan
TNC	Taiwan Native Chickens
BAPHIQ	Bureau Of Animal And Plant Health Inspection And Quarantine, Taiwan
HPAI	Highly Pathogenic Avian Influenza
DOH	Department Of Health, Taiwan
AIA	Animal Industry Act
ESS	Establishment Standards For Slaughterhouse
RMI	Rules Of Meat Inspection
RSO	Requirements For Slaughter Operations
VMI	Veterinary Meat Inspectors
ATRI	Agricultural Technology Research Institute, Taiwan
MIA	Meat Inspection Assistant
NAIF	National Animal Industry Foundation, Taiwan
HACCP	Hazard Analysis And Critical Control Point
FSIS	Food Safety And Inspection Service, USDA
USDA	The United States Department Of Agriculture
TCDC	Taiwan Centers For Disease Control
EFSA	European Food Safety Authority
PFGE	Pulsed-Field Gel Electrophoresis
WGS	Whole Genome Sequencing
FDA	Food And Drug Administration, USA
ISO	International Organization For Standardization
SPI	*Salmonella* Pathogenicity Islands

CDC	Centers For Disease Control And Prevention, USA
ESR	Institute Of Environmental Science & Research Limited, New Zealand
ERS	Economic Research Service, USA
EU	European Union
MSs	Member States
NZFSA	New Zealand Food Safety Authority
GBD	Global Burden Of Diseases
DALY	Disability-Adjusted Life Year
TFDA	Taiwan Food And Drug Administration
UK	The United Kingdom
MOHW	Ministry Of Health And Welfare, Taiwan
BR	Commercial White Broilers
CI	Confidence Interval
OR	Odds Ratio
EC	European Commission
IOBW	Inside-Outside Bird Washer
MLVA	Multi-Locus Variable-Number Tandem-Repeat Analysis
MLST	Multi-Locus Sequence Typing

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Chapter 1: Introduction

1.1. Safety issues of meat and meat products

Food provides the essential energy requirements and nutrients to sustain human life (Fung et al., 2018). Wholesome food can, not only maintain the health and psycho-physical well-being of humans, but also prevent certain diseases (Gallo et al., 2020). Conversely, however, food can result in disease and have a negative effect on human health (Gallo et al., 2020). There are many aspects involved in the production of food for human consumption, and interference of one or more of these can result in food that is contaminated with harmful products/agents resulting in food unsafe for humans (Gallo et al., 2020). Food by nature is biological and hence can support the growth of microorganisms which are a potential source of foodborne diseases for humans and other animals (Fung et al., 2018). Contaminated meat and meat products are considered among the major sources of foodborne infections in humans (Das et al., 2019). Contamination by food poisoning agents, such as microorganisms or toxins, may occur at all stages of the food chain, including prior to harvesting, during slaughter or processing of the animals, or as a result of cross-contamination from food handlers (Singh & Mondal, 2019). The World Health Organization (WHO) estimated that approximately 600 million cases of diseases caused by contaminated food occurred in 2010, including about 350 million caused by pathogenic bacteria (Havelaar et al., 2015). In addition, it has been estimated that 3,895,914 foodborne cases occurred annually in Taiwan during the period 2012 to 2015 of which 1,445,384 required/sought medical care resulting in 50 deaths. The annual medical cost of these foodborne cases was estimated at NT$1.3 billion resulting in a total loss of 4,974 disability-adjusted life years (DALYs) (Lai et al., 2020).

Meat, being a rich nutrient matrix with proteins, vitamins, minerals, micronutrients, and fat, has been a first-choice food for people all over the world, and is believed to have a higher biological value than proteins from other sources, including plants (Das et al., 2019; Gul et al., 2016). In the past few decades, global meat production has tripled, with a 20% increase in the last ten years. Pork is the most widely eaten meat in the world, accounting for more than 36% of the world's meat intake, followed by poultry and beef, accounting for about 35 and 22%, respectively (FAO, 2014). Although production of all major meat types have been increasing in absolute terms, low production costs, a short production cycle, high feed conversion ratios, and low product prices make poultry the meat of choice for producers and consumers (OECD/FAO, 2021), and the consumption of poultry is increasing more quickly than any other major meat (Chai et al., 2016).

Meat may be contaminated at the beginning of the food production chain on farm, such as with *Salmonella* infections of farm animals. In poultry flocks, contamination is favored by various factors, including raising in confined spaces, which is conducive to the rapid spread of infections (Gallo et al., 2020). Poultry can act as reservoirs for many types of foodborne pathogenic bacteria, including *Salmonella* serotypes, *Campylobacter jejuni*, *Listeria monocytogenes*, *Clostridium perfringens*, and *Staphylococcus aureus.* However, most of the bacteria present in live poultry are not pathogenic, but can be associated with meat spoilage (Davis et al., 2010). These spoilage microorganisms include *Enterobacteriaceae* spp., *Acinetobacter* spp., *Aeromonas* spp., *Alcaligenes* spp., *Moraxella* spp., *Flavobacterium* spp., *Staphylococcus* spp., *Micrococcus* spp., and *Pseudomonas* spp. (Das et al., 2019). When the total number of such microorganisms reaches 10^7 colony forming units (CFU) per gram, spoilage of meat will occur, thereby reducing its quality, sensory attributes and shelf-life (Das et al., 2019). More than 8 billion chickens or more than 30 billion pounds of meat are processed in the United

States of America (USA) each year, 80% of which is sold as fresh product. It is estimated that 2 to 4% of this meat is lost due to spoilage, equivalent to an annual loss of $300 to $600 million USD (Russell, 2010). In addition, during 2009–2015, a total of 5,760 foodborne outbreaks were reported in the USA, resulting in 100,939 illnesses, 5,699 hospitalizations and 145 deaths, and chicken was the meat responsible for the most illnesses (12% of all foodborne cases). In addition, 36.6% (34/93) of raw chicken meat samples from traditional markets and supermarkets in northern Taiwan in 2017–2019 were positive for *Salmonella* and the evidence from epidemiological, laboratory, and supply-chain investigations identified raw poultry as the vehicle for the widespread nature of *Salmonella* infections (Feng et al., 2020). Outbreaks caused by *Salmonella* and *Listeria* are important targets for public health intervention efforts, and improving the safety of chicken meat is considered a top priority by producers and processors (Dewey-Mattia et al., 2018). In addition, contamination of fresh chicken meat with *Salmonella* has been demonstrated to mainly occur at abattoirs during the slaughtering and processing stages (Cardinale, Tall, et al., 2005; Goksoy et al., 2004; Rasschaert et al., 2007). It has also been reported that *L. monocytogenes* in poultry meat originates from infected flocks (Ryser & Buchanan, 2012) or from environmental contamination during processing (Franco et al., 1995; Lawrence & Gilmour, 1994; Loura et al., 2005; Sakaridis et al., 2011). The processing plant must provide hygienic environmental and operating conditions so that products can be produced in a safe, sanitary, and wholesome manner to ensure the microbiological safety of chicken meat (Davis et al., 2010).

1.2. Overview of the chicken industry in Taiwan

Taiwan, officially named the Republic of China (ROC), is located in East Asia. It faces the Pacific Ocean to the east, People's Republic of China to the west, Japan to the

northeast, and the Philippines to the south. Taiwan, with associated islands, covers a total area of 36,197 km^2. The country is divided into six special municipalities, 13 counties and three provincial municipalities (Figure 1.1) (OIS, 2013). These are further subdivided into smaller entities, including county municipalities, towns and villages. In 2019, Taiwan had a human population of 23.6 million with a population density of approximately 652 people per km^2 making it one of the most densely populated countries in the world (MOI, 2020). The contribution from the agricultural sector to the Gross Domestic Product of Taiwan in 2019 was 1.77% (DGBAS, 2020). Livestock and poultry production made up 31.6% (US $5.8 billion) of all agricultural production in 2019, and chicken meat production accounted for 24.7% of all livestock and poultry production (COA, 2020a). These values highlight the importance of the poultry industry to the country.

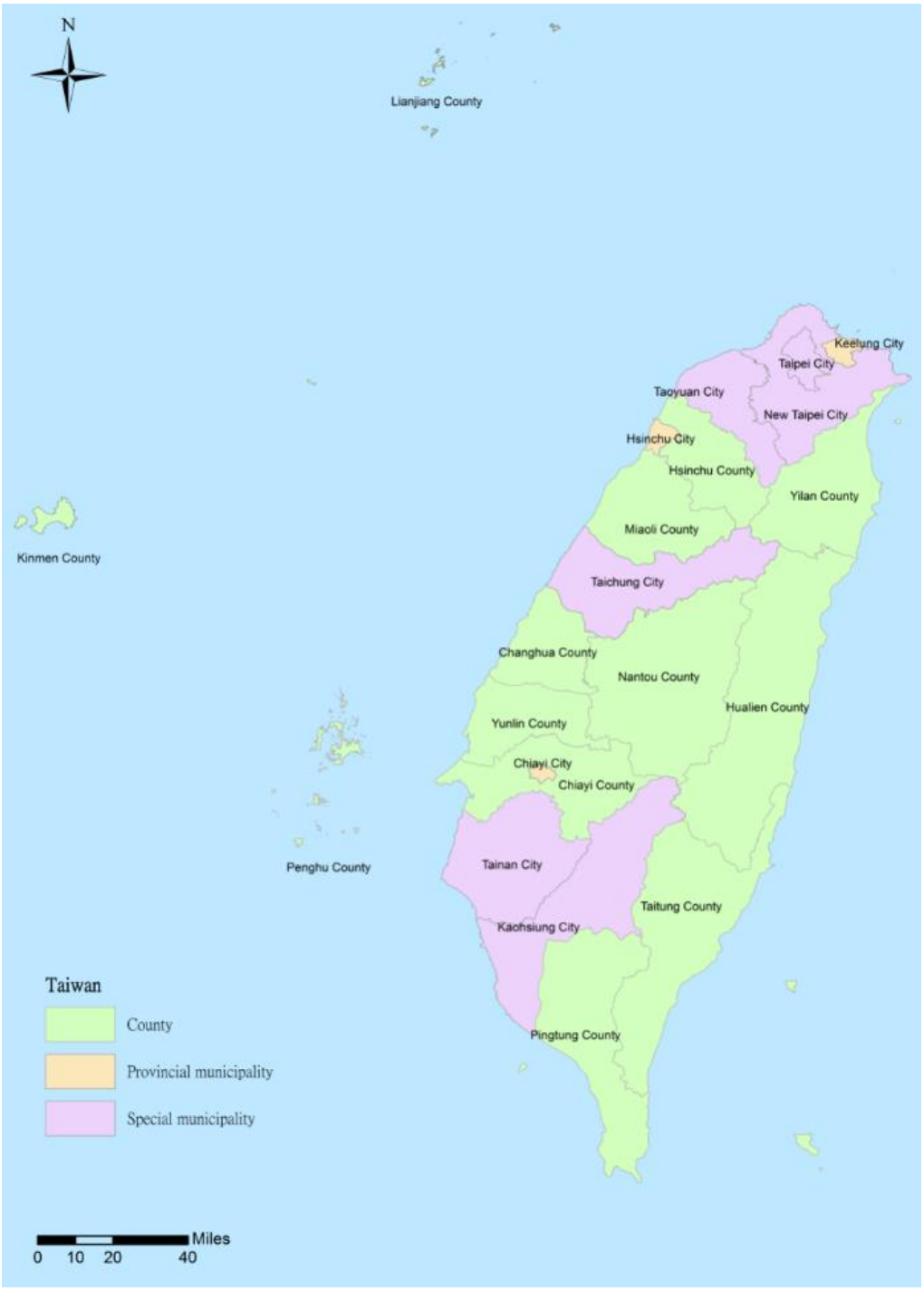

Figure 1.1 Taiwan administrative map (OIS, 2013).

Chicken meat in Taiwan is produced by three major chicken types: white broilers; Taiwan native chickens (TNC); and culled layers (COA, 2020a). The TNC represents a number of locally developed slow-growing breeds favored by Taiwanese consumers (Cheng et al., 2008). Genetically they are not native at all but have been crossed with

foreign breeds, notably French chickens around 1980 (Lee, 2006). The TNC have the characteristics of a single comb, red to black feathers, and black shanks (Chao & Lee, 2001). Layers are culled and processed for their meat when they are approximately 2 years old or when their rate of egg production drops (personal communication with some interviewed chicken farmers). Over the past decade both the number of chickens kept on farms and the number slaughtered annually has changed only slightly from 99 and 335 million, respectively in 2010, to 98.7 and 374 million in 2019, respectively (Table 1.1) (COA, 2020a). In 2019, the white broilers, TNC, and culled layers accounted for 64.3, 30.6, and 5.1% of all chickens slaughtered, respectively (Table 1.2) (COA, 2020a). Production of live chickens in Taiwan is mainly undertaken in the central and southern regions of the country (Figure 1.2) (COA, 2020a). The production cost (per 100 chickens) of white broilers and TNC was US $322 and 595, respectively in 2019 (COA, 2020a). The main reason for this large difference is the age and live weight difference at marketing, with the TNC taking approximately 80-110 days to achieve a market live weight of 2–2.5 kg. In contrast, the white broiler requires only 35-40 days to achieve a live weight of approximately 1.5 kg (Cheng et al., 2008; Chumngoen et al., 2016; Huang et al., 2007).

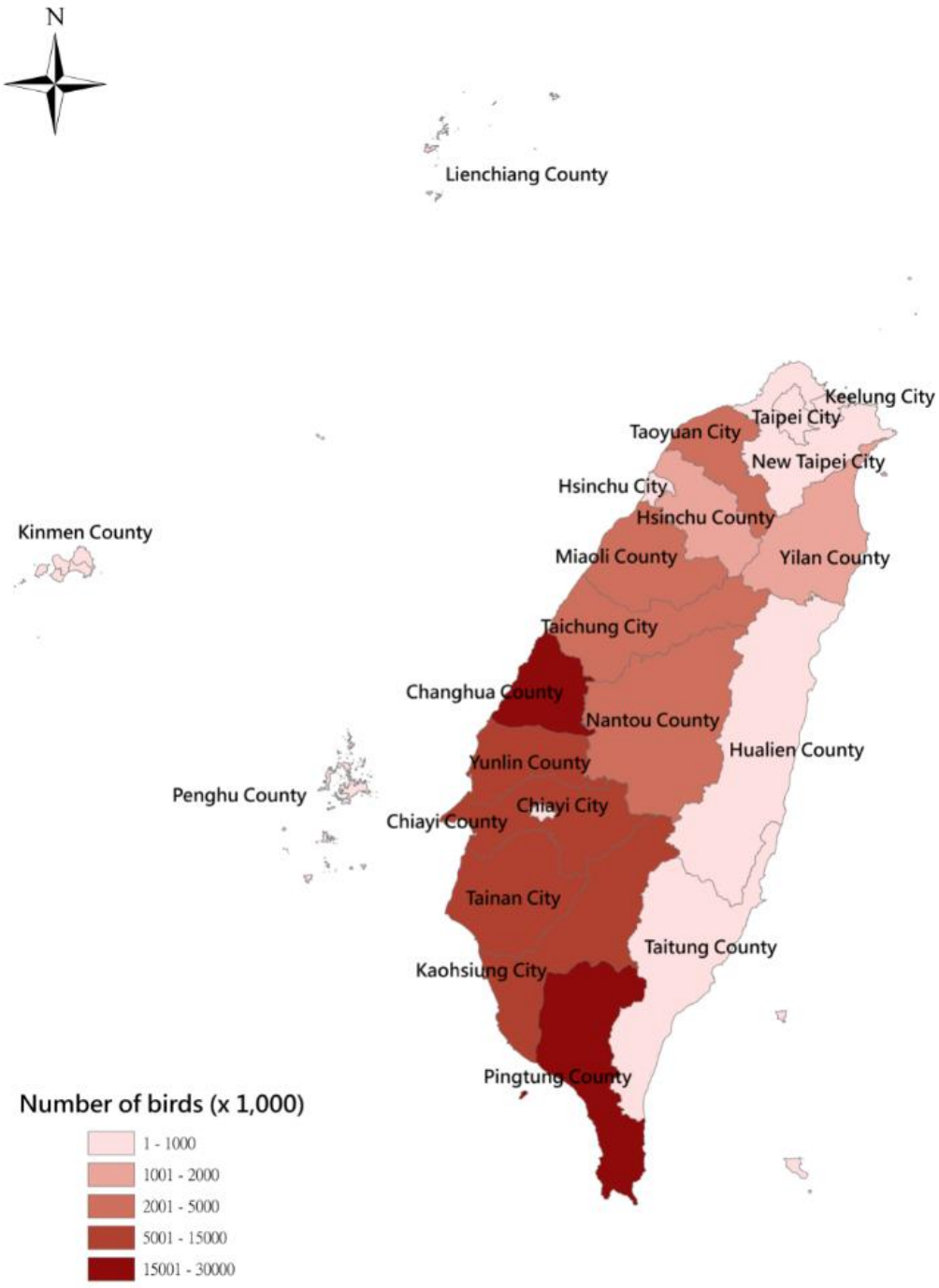

Figure 1.2 The distribution of chickens in Taiwan districts in 2019 (COA, 2020a)

Table 1.1 Number of chickens in Taiwan (2010 – 2019)

Year	**Number of birds** (x 10^3)					
	Total	Breeders of layers	Layers	Breeders of broilers	White broilers	Taiwan native chickens
2010	**98,989**	350	36,125	3,794	24,198	34,522
2011	**96,850**	320	36,235	3,816	20,977	35,502
2012	**91,597**	299	36,666	3,487	21,746	29,399
2013	**91,070**	277	36,716	3,944	21,486	28,647
2014	**94,523**	390	37,602	4,086	22,757	29,688
2015	**90,975**	414	38,205	3,928	19,804	28,624
2016	**94,646**	342	39,270	4,354	21,445	29,235
2017	**96,002**	371	39,646	4,709	21,313	29,963
2018	**100,995**	370	40,979	4,586	23,122	31,938
2019	**98,676**	425	42,838	4,427	23,205	27,781

Source: COA (2020a)

Table 1.2 Numbers and total weight of chickens slaughtered in Taiwan (2010 – 2019)

Year	**Total**		Culled layers		White broilers		Taiwan native chickens	
	Head ($\times 10^3$)	**Carcass weight m.t.**	Head ($\times 10^3$)	Carcass weight m.t.	Head ($\times 10^3$)	Carcass weight m.t.	Head ($\times 10^3$)	Carcass weight m.t.
2010	**334,760**	**558,197**	18,918	19,297	191,993	284,738	123,849	254,162
2011	**350,121**	**589,462**	18,576	18,947	200,707	304,354	130,838	266,161
2012	**324,520**	**544,326**	18,767	19,143	186,994	283,776	118,759	241,407
2013	**307,487**	**510,167**	18,863	19,240	185,650	281,571	102,974	209,356
2014	**326,298**	**543,160**	18,839	19,215	198,449	301,151	109,010	222,794
2015	**321,139**	**533,073**	18,904	19,282	196,539	298,085	105,696	215,706
2016	**340,754**	**565,485**	19,518	19,898	209,170	317,374	112,066	228,213
2017	**335,214**	**555,322**	17,598	17,939	211,111	320,278	106,505	217,105
2018	**353,047**	**597,316**	17,568	17,934	226,540	343,796	108,939	235,586
2019	**373,771**	**631,770**	19,248	19,647	240,167	364,164	114,356	247,959

Source: COA (2020a)

The number of domestic chickens slaughtered only increased slightly between 2010 and 2019 in Taiwan (Table 1.2), in contrast the amount of chicken meat and related products imported increased by 92% during this period, with an average of 152,100 tonnes of chicken meat and related products imported annually (Table 1.3) (COA, 2020a). The average *per capita* consumption of all meat types was 77.63 kg between 2010 and 2019 in Taiwan (COA, 2020b) of which 36.56 kg (50.4%) was pork and 34.53 kg (42.2%) poultry meat (Table 1.4) (COA, 2020b). The consumption of all kinds of meat increased from 75.91 kg in 2010 to 84.84 kg in 2019 (Table 1.4). During this decade the amount of pork and mutton consumed decreased, in contrast to poultry meat and beef which increased. Poultry has become the most commonly consumed meat in Taiwan since 2018 (COA, 2020b).

Table 1.3 Quantity and value of chicken meat and related products imported into Taiwan (2010 – 2019)

Year	**Total**		Fresh or chilled chicken meat		Frozen chicken meat		Preserved chicken meat		Canned chicken meat		Offal	
	Quantity (m.t.)	**Value (US$1,000)**	Quantity (m.t.)	Value (US$1,000)	Quantity (m.t.)	Value (US$1,000)	Quantity (m.t.)	Value (US$1,000)	Quantity (m.t.)	Value (US$1,000)	Quantity (m.t.)	Value (US$1,000)
2010	**110,567**	**121,054**	-	-	109,586	119,515	237	845	16	74	728	620
2011	**108,240**	**132,947**	26	35	107,305	131,453	210	748	17	63	708	683
2012	**123,127**	**174,725**	-	-	121,695	172,732	198	749	12	58	1,222	1,186
2013	**111,446**	**154,466**	-	-	110,179	152,750	216	805	28	65	1,023	845
2014	**140,977**	**180,622**	26	33	140,137	178,850	274	1,079	55	145	511	548
2015	**180,500**	**176,504**	1	8	179,286	173,863	549	1,774	106	296	559	571
2016	**160,813**	**135,171**	-	-	160,052	133,419	308	1,116	135	307	318	329
2017	**157,955**	**156,408**	-	-	157,053	154,461	304	1,152	152	389	446	406
2018	**214,685**	**217,004**	-	-	213,528	214,600	365	1,467	141	358	651	579
2019	**212,671**	**199,640**	-	-	211,235	196,862	378	1,432	141	396	917	950

Source: COA (2020a)

Table 1.4 *Per capita* consumption of meat in Taiwan (2010 – 2019)

Units：Kg.

Year	**All meat**	Pork	Poultry	Beef	Mutton	Others
2010	**75.91**	36.98	32.70	4.90	1.29	0.04
2011	**77.17**	37.30	33.84	4.85	1.14	0.04
2012	**75.17**	37.18	32.54	4.39	1.03	0.03
2013	**71.49**	34.94	30.63	4.84	1.04	0.04
2014	**75.56**	35.44	33.70	5.16	1.21	0.05
2015	**78.06**	37.56	34.26	5.07	1.12	0.05
2016	**76.98**	35.66	34.63	5.69	0.96	0.04
2017	**77.66**	36.50	34.26	5.88	0.97	0.05
2018	**83.40**	37.25	38.57	6.41	1.11	0.06
2019	**84.84**	36.84	40.16	6.83	0.94	0.07

Source: COA (2020b)

Since the 1980's the Taiwanese government has provided assistance to the white broiler industry to improve the genetics of the birds to improve their feed conversion rate and growth rate. In conjunction, funds have also been provided for upgrading the processing of poultry through improved slaughter plants, cold storage and packing facilities (Lin, personal observation). White broilers are raised by farmers to market weight (5-6 weeks old), and then sold as a batch directly to the meat processors for slaughtering and processing in Taiwan (personal communication with some interviewed chicken farmers). The supply chain for white broilers and cull layers is strictly controlled, with the birds slaughtered and processed only in officially approved abattoirs under the Taiwanese government's meat inspection system. Most of these carcasses are chilled or frozen and are primarily used by the fast-food and meat processing industry. In contrast to the white broiler industry, the TNC industry is poorly organized with different marketing channels than for the former larger industry. Small chicken dealers usually purchase chickens directly from farmers, and later sell them to local consumers or to large dealers in urban areas for on-sale to retail stores. Prior to 2013, most TNC were slaughtered in

traditional markets without official meat inspection, and were then sold to consumers as fresh carcasses (Lin, personal observation). However, due to the threat of zoonotic highly pathogenic avian influenza (HPAI), the Taiwan government declared a ban on the slaughter of birds in traditional (live bird) markets from May 2013 to prevent the dissemination of disease from live birds to humans (Juan, 2013). To facilitate this, the government has assisted the industry to set up small to medium sized abattoirs specifically for TNC (Yu, 2012).

Overall there were only 19 poultry abattoirs in Taiwan in 2002 but this number increased to 115 in 2019 (BAPHIQ, 2020). The location of poultry abattoirs in Taiwan in 2019 is displayed in Figure 1.3. In 2019, the annual number of TNC processed in approved abattoirs was 87 million, more than twice the number slaughtered in 2002 (BAPHIQ, 2020).

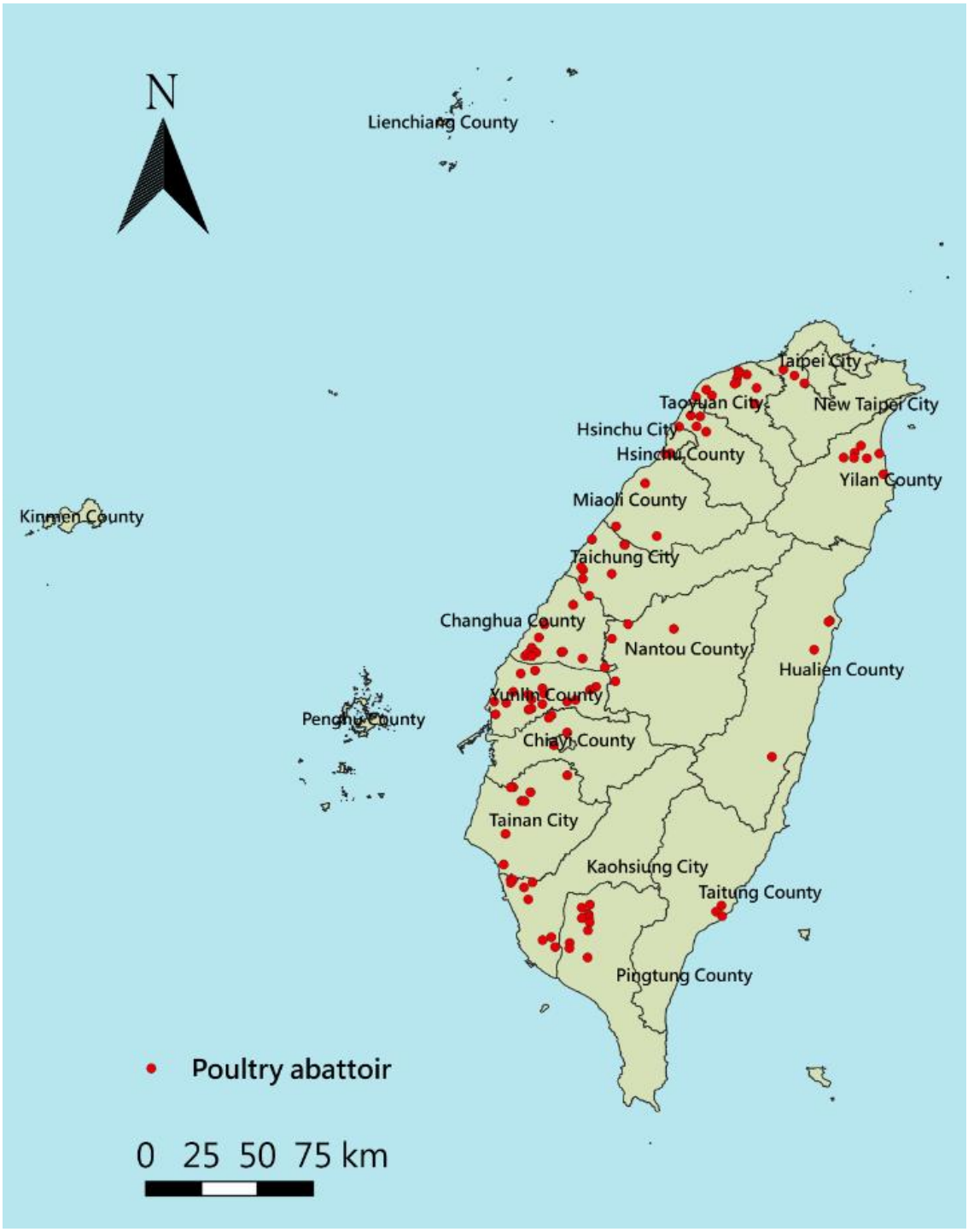

Figure 1.3 The location of poultry abattoirs in Taiwan, 2019 (BAPHIQ, 2020).

1.3 The meat inspection system in Taiwan

Prior to 1998, meat inspection in Taiwan was conducted by the Central Food Safety Authority, Department of Health (DOH, restructured to Ministry of Health and Welfare in 2013), in accordance with the Act Governing Food Safety and Sanitation. Following

the implementation of the Animal Industry Act (AIA) in June 1998, the responsibility for meat inspection was transferred to the Bureau of Animal and Plant Health Inspection and Quarantine (BAPHIQ) under the Council of Agriculture (COA). The BAPHIQ was established in 1998 in order to consolidate the administration and execution of policies and regulations relating to animal and plant health inspection, quarantine and meat inspection (BAPHIQ, 2015a). Between 1998 and 2000 BAPHIQ developed many regulations, including the Establishment Standards for Slaughterhouse (ESS), Rules of Meat Inspection (RMI), and Requirements for Slaughter Operations (RSO) to guide the sanitary management of meat and poultry establishments. After a two-year period of transition, in January 2001 BAPHIQ replaced the DOH as the body responsible for the meat inspection service in Taiwan (Lin, personal observation).

Since the AIA was implemented in 1998, all pigs, cattle and goats have been required to be slaughtered, processed and inspected at approved abattoirs. The development of poultry inspection was undertaken into two stages. Firstly, all commercial poultry (including chickens, ducks and geese) processed for human consumption from July 2004 to May 2013 were required to be slaughtered in approved abattoirs. Two exceptions were granted to these regulations for official inspection: firstly was for poultry which were slaughtered in traditional markets; and secondly was for the slaughter of poultry for non-commercial use (poultry slaughtered by the owner for their own or their family's/friend's consumption and which were not offered for sale). However after May 2013 the slaughter of poultry in traditional markets was banned, and only poultry for non-commercial use were allowed to be slaughtered without official inspection (Juan, 2013).

The purpose of meat inspection is to ensure that meat and meat products are free of diseases and suitable for human consumption (Huneau-Salaün et al., 2015). Meat

inspection is composed of two main components: ante-mortem inspection involving the examination of the live animals prior to slaughter; and post-mortem inspection of the animals' carcass and viscera after slaughter (Blagojevic & Antic, 2014). Carcasses and product which pass the meat inspection process are stamped "BAPHIQ inspected and passed" on the carcasses of large carcasses/ruminants/pigs, or on the packages of poultry in Taiwan. This measure confirms that the meat has been examined and is considered wholesome and safe for human consumption. Condemned parts, viscera and animals dead on arrival are marked with dye prior to rendering or incineration to prevent these condemned materials from entering the human food chain (Chiang & Liu, 2002).

In Taiwan, meat inspection is conducted by Veterinary Meat Inspectors (VMI). These inspectors are qualified veterinarians who have completed additional training courses run by the Agricultural Technology Research Institute (ATRI, formerly the Animal Technology Institute Taiwan). These courses include information on the relevant regulations, methods of enforcement, meat safety and sanitation, ante-mortem and post-mortem inspection procedures, and control of restricted, condemned and inedible products. Monitoring of carcass microbiology and off-line inspection procedures are also covered in the advanced course (Du, 2002). A qualified VMI undergoes a two-week training course and in-plant experience under a senior inspector before they are able to inspect animals/carcasses for human consumption. Also under the AIA, a person who has a senior high school or higher educational background and who has passed the necessary training (five weeks of coursework and 14 weeks of on-plant training) can act as a Meat Inspection Assistant (MIA). The MIA can assist in the ante-mortem, post-mortem and other related inspection work under the direction of the VMI (Lin, personal observation). BAPHIQ established the meat inspection program in 1989, with

the National Animal Industry Foundation (NAIF) being responsible for implementing the program. When VMIs and MIAs are qualified and certified, they may be employed by the NAIF, although their wages are provided from the BAPHIQ budget. Once VMIs and MIAs are employed by the NAIF, they report to the BAPHIQ Branch Office and are allocated to an abattoir to practice meat inspection (Figure 1.4) (Lin, personal observation).

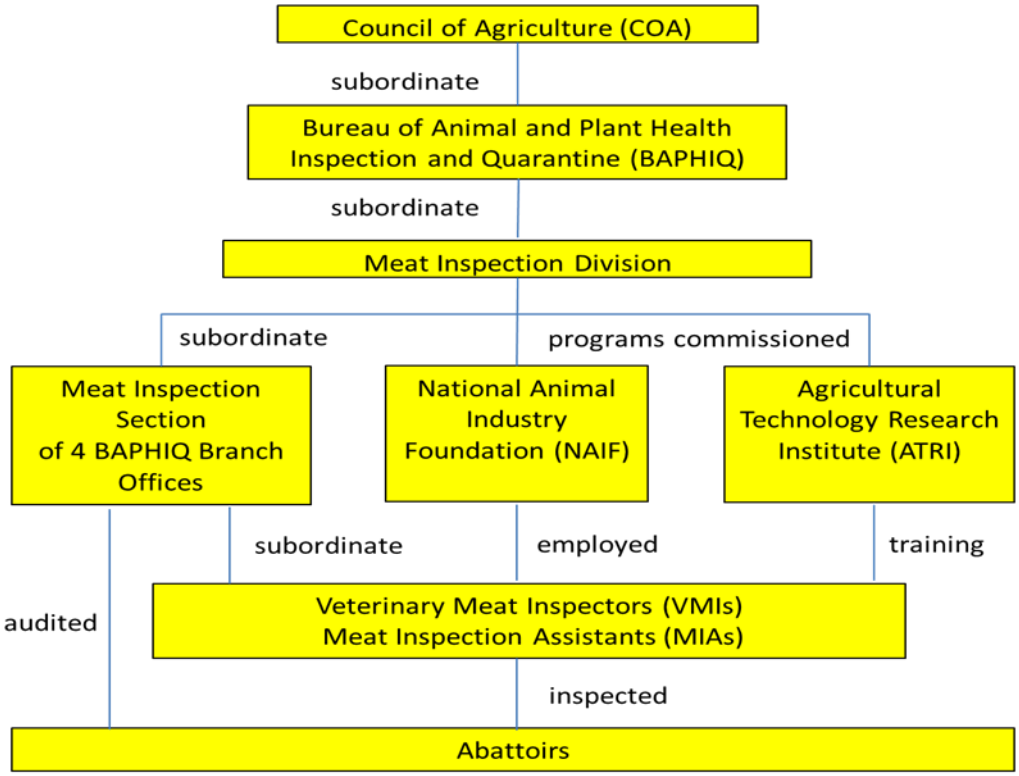

Figure 1.4 The framework of the meat inspection system in Taiwan.

Although the muscle tissues of healthy livestock and poultry is generally sterile (Das et al., 2019), carcasses can potentially be contaminated with pathogenic bacteria and spoilage microorganisms during slaughtering and processing, particularly if slaughter facilities are badly constructed and/or sanitation is poorly maintained, potentially leading to foodborne diseases in humans and spoilage of the meat (Arguello et al., 2013; Das et al., 2019; Davis et al., 2010; Gallo et al., 2020). To ensure both the regulations of ESS and RSO have been properly observed, the VMIs also have to supervise the

sanitary management of the abattoirs in Taiwan. If there is any violation of the regulations of the ESS and RSO, the VMI shall write a cautionary advice to the abattoir, and then the abattoir is required to take corrective action within a prescribed period of time. Meanwhile, a copy of the advice is also sent to the BAPHIQ Branch Office and the staff of BAPHIQ will audit the improvement of the offending abattoir at the expiration date of the prescribed period of time. Where the offender fails to comply with the direction, a fine (NT$ 30,000-60,000) is imposed until the violation is corrected. If the violation is not corrected the slaughter operation will be suspended in part or in whole. Abattoirs that have been ordered for suspension but continue their slaughter operations shall have their registration certificates revoked and rendered null and void (Lin, personal observation).

1.4 Microbiological screening program in abattoirs

The inspection of food animals at the time of slaughter has historically focused on identifying signs of disease conditions that make the carcass or parts of the carcass unfit for human food by visual inspection, palpation and incision of relevant lymph nodes and organs (Alvseike et al., 2018). However, bacteria are not detectable by visual inspection and bacterial contamination of carcasses has become an important food safety issue (Alvseike et al., 2018). To keep the microbial load of raw meat under control, different food safety measures/guidelines should be followed strictly (Das et al., 2019). The Pathogen Reduction, Hazard Analysis and Critical Control Point (PR/HACCP) Systems is an established food safety system designed by the Food Safety and Inspection Service (FSIS) of the United States Department of Agriculture (USDA). The HACCP system is used to help meat and poultry processing establishments identify and evaluate hazards that can impact the safety of their products, set up controls necessary to prevent these hazards from occurring or keeping them within acceptable

limits, monitor the effect of control measures, and maintain records of these practices. Microbiological sampling is a critical component of HACCP which can be used to verify that preventive efforts undertaken by meat and poultry establishments are successfully controlling pathogens and ensuring products are safe and wholesome (FSIS, 2011).

Effective HACCP systems have to be based on accurate baseline data on the types and levels of contamination at each stage of meat processing (Gill et al., 1996). They also need to employ accurate baseline data in the ongoing establishment of appropriate critical limits, as a means of monitoring the effectiveness of developed/implemented control measures (Pearce et al., 2004). Therefore, it is necessary to investigate the nationwide microorganism profile of chicken carcasses to establish the baseline standard of specific pathogens and to assess the effectiveness of the processing operations. Although BAPHIQ did not require abattoirs to implement the HACCP system before 2019, a microbiological screening program for chicken carcasses was launched in 2006 in Taiwan, which was based on the framework of the Nationwide Microbiological Baseline Data Collection Program designed by the FSIS (Lin, personal observation). Routine baseline surveying of poultry carcasses to determine the prevalence of selected pathogens of concern for food safety can provide valuable information regarding the effectiveness of the processing steps/stages or identify the need for interventions.

1.5 Aims and Objectives of the research described in this thesis

Salmonella are a common cause of foodborne and infectious disease throughout the world, resulting in more than 100,000 human deaths globally each year (Dougan et al., 2011; Majowicz et al., 2010; WHO, 2013) and are a leading foodborne pathogen in Taiwan (Lai et al., 2020). Although listeriosis caused by *L. monocytogenes* in humans is

a relatively rare disease compared with salmonellosis in Taiwan, it is important because it has the highest case fatality rate of all foodborne infections (Huang et al., 2015). The Taiwan Centers for Disease Control (TCDC) listed listeriosis as a notifiable disease on January 1, 2018 in an effort to improve its control (TCDC, 2018). In addition, contaminated poultry and poultry products have been identified as a major food source responsible for human salmonellosis and listeriosis (Finstad et al., 2012; Mead et al., 2010; Morar et al., 2014; Olsen et al., 2005) with contamination commonly occurring at abattoirs during the slaughtering and processing stages (Cardinale, Tall, et al., 2005; Cox et al., 1997; Rørvik et al., 2003; Rasschaert et al., 2007). Therefore, the studies reported in this thesis were designed to focus on investigating the contamination of chickens slaughtered in Taiwan with *Salmonella* and/or *L. monocytogenes*.

Prior to the study outlined in this thesis, the microbiological screening program used in Taiwan for chicken carcasses was not used to identify factors responsible for the contamination of carcasses with specific pathogens of concern for food safety. In addition, there was limited information available about when and where any cross-contamination occurred, and what the potential sources of this contamination were during the processing of white broilers and TNC in Taiwan. Therefore, the main aim of this thesis was to gather data about *Salmonella* and *L. monocytogenes* contamination of chickens slaughtered in Taiwan to support and strengthen the existing processing procedures and to identify areas that required improvement. The main objectives of the studies outlined in this thesis are:

- To estimate the prevalence of *Salmonella* in Taiwanese broilers at slaughter and to identify risk factors associated with the presence of *Salmonella* in batches of broilers at processing.
- To investigate the potential sources of *Salmonella* and *L. monocytogenes*

contamination, and pathways for cross-contamination within abattoirs processing broiler chickens in Taiwan.

- To describe where in an abattoir processing line *Salmonella* contamination of broiler carcasses occurred, and to investigate the potential sources of *Salmonella* contamination, so that possible interventions could be applied.

Chapter 2: Literature Review

2.1 Important bacterial foodborne pathogens in poultry meat

Poultry meat is one of the most commonly consumed and globally traded meat products, and understanding factors which contribute to poultry associated outbreaks of foodborne disease has important implications for food safety (Antunes et al., 2016; Chai et al., 2016). Chai et al. (2016) analyzed 149 poultry-associated foodborne disease outbreaks reported to the United States Foodborne Disease Outbreak Surveillance System from 1998 to 2012, and found that *Salmonella* caused the largest number of outbreaks (64, 43%), followed by *Clostridium perfringens* (39, 26%), *Campylobacter* spp. (10, 7%), norovirus (10, 7%), *Staphylococcus aureus* enterotoxin (8, 5%), *Listeria monocytogenes* (5, 3%), *Bacillus cereus* (4, 3%), and other agents (9, 6%). Of these pathogens in poultry meat, *Salmonella* is the most frequent cause of foodborne disease both internationally (Antunes et al., 2016) and in Taiwan (Lai et al., 2020). However, *L. monocytogenes* is considered to be the most frequent cause of death of all foodborne acquired infections internationally (EFSA, 2014; Thomas et al., 2020) as well as in Taiwan (Huang et al., 2015).

2.1.1 *Salmonella* spp.

Salmonella are enteric bacteria that infect humans and other animals, and are a common cause of zoonotic disease throughout the world (Duggan et al., 2010). Georg Gaffky first successfully cultured the typhoid bacillus, *Salmonella* Typhi, from patients in Germany in 1884 (Barnett, 2016). Later, Theobald Smith isolated what became known as *Salmonella* Choleraesuis from the intestines of pigs in 1885 (Schultz, 2008). The genus *Salmonella* was named after the American veterinarian Daniel Elmer Salmon, who was Smith's director and the administrator of the United States Department of

Agriculture (USDA) at that time (Ellermeier & Slauch, 2006).

2.1.1.1 Taxonomy and typing methodology of *Salmonella* spp.

The genus *Salmonella* is within the family *Enterobacteriaceae*, and is comprised of two species: *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* is further divided into six subspecies: *S. enterica* subsp. *enterica*, *S. enterica* subsp. *salamae*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp. *houtenae* and *S. enterica* subsp. *indica* (Porwollik et al., 2004).

The most common way to classify *Salmonella* is by serotyping, in which the bacteria are distinguished based on differences in their: somatic (O) lipopolysaccharides (LPS) on the external surface of the bacteria's outer membrane; flagellin (H) antigens associated with the peritrichous flagella; and capsular (K) antigens which occur only in *S.* Typhi, *S.* Paratyphi C, and *S.* Dublin (Li et al., 2012). *Salmonella* are serotyped based on surface antigens according to the Kauffman-White Scheme, and the WHO Collaborating Centre for Reference and Research on *Salmonella* is responsible for updating this scheme (Ellermeier & Slauch, 2006). Based on this classification system, more than 2,500 *Salmonella* serotypes have been identified and more than 99% of isolated *Salmonella* strains are serovars/serotypes in the species *enterica* (Grimont & Weill, 2007). Additionally, further subtyping may be performed using phage typing or molecular subtyping methods. Phage typing depends on the ability of specific bacteriophages to infect bacteria with complementary surface receptors. Phages can combine with the receptors, infect and lyse the bacterium with the lysis zone easily detectable as a zone of clearing (plaque) within a lawn of bacteria on a media plate. These types are denoted by phage type (PT) or definitive phage type (DT) numbers. Phage typing can be used to identify possible links between sporadic cases, which helps trace the main source of an outbreak and has been used to further differentiate *S.*

Enteritidis isolates from different geographical locations. For example, phage type *S.* Enteritidis PT 4 is most commonly detected in Europe, while PT 8 is more prevalent in the USA (Ricke et al., 2013).

Although serotyping or phage typing are useful as preliminary tools for *Salmonella* classification, Pulsed-Field Gel Electrophoresis (PFGE) is one of the most important molecular typing methods used to further classify and differentiate strains of *Salmonella* (Arguello et al., 2013; Ricke et al., 2013). PFGE profiling is a DNA fingerprinting method which utilizes restriction enzyme digestion of purified genomic DNA. The selective ability of rare-cutting restriction enzymes, primarily *Xbal* or *AvrII* (*BlnI*), are used in PFGE for *Salmonella* to digest the bacterial genome into a limited number of restriction fragments. These fragments are then separated using specialized electrophoretic conditions in which the field polarity is varied during the run to facilitate the two dimensional separation of large DNA fragments (10 to 800 kb in length) to produce specific banding patterns or DNA fingerprints (Whittam & Bergholz, 2007). In general, the gels are scanned and converted to digital images, and then analyzed using specialized computer software. PFGE is often considered the 'gold-standard' method for *Salmonella* typing (Arguello et al., 2013; Ricke et al., 2013; Whittam & Bergholz, 2007). During outbreak investigations, PFGE can detect differences between very closely related strains and PFGE profiles can be classified as epidemiologically linked with a high degree of confidence to trace the main source of an outbreak or contaminated food (Whittam & Bergholz, 2007). For instance, in some studies, transmission of *Salmonella* from contaminated broiler meat to humans has been confirmed by PFGE (Cardinale, Perrier Gros-Claude, et al., 2005; Liebana et al., 2001). Moreover, several authors have used PFGE to investigate the relationships between *Salmonella* isolates obtained from abattoirs to identify potential sources of

contamination and the contamination cycles of the slaughter process (Arguello et al., 2013; Botteldoorn et al., 2004; De Busser et al., 2011; Duggan et al., 2010; van Hoek et al., 2012). In recent years, new molecular methods based on whole genome sequencing (WGS) have been adopted by many researchers as they have higher accuracy and superior discriminatory power than PFGE (Pietzka et al., 2019; Singh et al., 2021; Yin et al., 2020). Nevertheless, PFGE remains an affordable and relevant technique for small laboratories and hospitals, especially given the current high costs associated with the purchase of next generation sequencing equipment and the computational analyses required for WGS (Neoh et al., 2019).

2.1.1.2 Characteristics of *Salmonella* spp.

Salmonellae are gram-negative, oxidase negative, rod-shaped, facultative anaerobic bacteria. The cells are typically 0.7-1.5 μm by 2-5 μm in size. *Salmonella* strains ferment glucose with the production of acid and gas, and most of them are motile due to the presence of flagella (Ellermeier & Slauch, 2006; FAO/WHO, 2007). Although salmonellae are generally considered mesophilic in nature, with an optimum growth temperature between 35℃ and 43℃, some strains can grow at elevated temperatures of up to 54℃, whilst others are able to grow in food stored at 2℃ to 4℃. The pH for optimal growth of *Salmonella* is between 6.5 and 8.2, with pH values greater than 9 and lower than 4 inhibiting growth. *Salmonella* grows optimally at a water activity of 0.995, and the minimum water activity for growth is 0.94 in media with a neutral pH or as low as 0.93 in rehydrated dry soups after 3 days of incubation at 30℃ (Ellermeier & Slauch, 2006; FAO/WHO, 2007; Li et al., 2012). *Salmonella* can grow in the presence or absence of air. Normally, high concentrations of CO_2 (60-80%) inhibit the growth of *Salmonella* at a temperature of 10 to 11°C in meat, but at 20°C there is little inhibition (FAO/WHO, 2007; Li et al., 2012).

Salmonella can survive for long periods under refrigeration, and lower storage temperatures generally extend survivability (FAO/WHO, 2007; Li et al., 2012). Different serotypes of *Salmonella* have been shown to survive storage at 25 and 35℃ on dried paper disks for 35 to 70 days, whereas storage at 4℃ can increase survival to 22 to 24 months (Hiramatsu et al., 2005). In minced chicken breast (pH 5.8), 60-83% of *Salmonella* cells were found to survive for 126 days at -20℃, in contrast at -2 and -5℃, only 1.3 to 5.8% were viable after 5 days (FAO/WHO, 2007). Most importantly, temperature is only one factor, and the other factors of the food such as pH, water activity, product, and serotype of isolates must be considered when evaluating survivability (Farakos et al., 2014; FDA, 2012).

Salmonella spp. have numerous biochemical characteristics useful for identification and differentiation from other *Enterobacteriaceae* genera. A typical *Salmonella* isolate produces acid and gas from glucose in triple sugar iron (TSI) agar and does not utilize lactose or sucrose in TSI or in differential plating media i.e. xylose lysine desoxycholate (XLD), and Hektoen enteric (HE) agars (Li et al., 2012). Furthermore, salmonellae produce an alkaline reaction from the decarboxylation of lysine to cadaverine in lysine iron agar (LIA), generate hydrogen sulfide (H_2S) gas in both LIA and TSI, and do not hydrolyze urea. Due to the production of H_2S, *Salmonella* colonies appear pink on XLD agar plates and blue-green to blue with or without black centers on HE agar plates. Many *Salmonella* colonies have large, glossy black centers or may be completely black on HE or XLD agars (Andrews et al., 2014; Li et al., 2012). These characteristics of *Salmonella* have been used to create protocols for isolation of the organism from food, tissue, and environmental samples and include the Bacteriological Analytical Manual of the U.S. Food and Drug Administration (FDA), the Microbiology Laboratory Guidebook of the U.S. Department of Agriculture (USDA), and the *Salmonella* method

ISO 6579: 2002 (E) of the International Organization for Standardization (ISO). Although there are a diverse range of media available, all of these three protocols include the following steps: a non-selective enrichment step to ensure recovery and growth of injured bacteria cells; selective enrichment to allow for propagation of *Salmonella*; selective plating to isolate and presumptively identify *Salmonella* colonies; and confirmation of colony identity with various biochemical/serological tests (Li et al., 2012; Ricke et al., 2013).

2.1.1.3 Virulence of *Salmonella* spp.

Following ingestion *Salmonella* are successively exposed to the acidic environment of the stomach, to the detergent-like activity of bile, to decreasing oxygen supply, to the normal gut-flora and their associated metabolites, and finally to the cationic antimicrobial peptides present on the surface of epithelial cells (Rychlik & Barrow, 2005). Colonization and invasion of intestinal epithelial cells is enhanced by the ability of *Salmonella* to express virulence associated genes that enable survival in macrophages and dendritic cells (Ricke et al., 2013). Many of these virulence genes are clustered together at focal points or loci within the bacterial chromosome into "islands" referred to as *Salmonella* Pathogenicity Islands (SPI). These gene-clusters are thought to have been acquired by *Salmonella* from other species of bacteria through horizontal gene transfer (Groisman & Ochman, 1996). Many of these genomic islands in *Salmonella* encode important functions that are essential to the pathogen's virulence, and these loci define this genus and its species. The distinct genomic clusters, classified as SPI-1 through to SPI-5, are conserved in *S. enterica*; however, there are genetic variabilities within several of these loci in different serovars (Li et al., 2012). Some *Salmonella*, such as *S.* Typhi, may possess additional pathogenicity islands, SPI-6 through to SPI-10, which appear to be unique to *S.* Typhi and other serovars associated with enteric fever

in humans (Li et al., 2012). Although the role of some SPI in the pathogenesis of disease is well described, the function of many SPI associated genes on virulence is currently unknown (FAO/WHO, 2007).

Certain *Salmonella* serovars, such as *S.* Abortusovis, *S.* Choleraesuis, *S.* Dublin, *S.* Enteritidis, *S.* Gallinarum-Pullorum and *S.* Typhimurium, carry a large, low-copy-number plasmid that contains virulence genes and is required to trigger systemic disease. All plasmids contain the 7.8 kb plasmid virulence (*spv*) locus which encodes products for the prolific growth of salmonellae in the host's reticuloendothelial tissues (Rotger & Casadesús, 1999). This locus harbors five genes designated *spv* RABCD. The product of the *spv* R is a positive regulatory protein essential for the expression of the other *spv* genes. Expression of the *spv* genes might play a role in the multiplication of intracellular salmonellae, but the exact function of the encoded proteins is not fully known. Other virulence factors of *Salmonella* include production of toxins, and presence of fimbriae and flagella. However, the role of these factors in the pathogenesis of *Salmonella* spp. is also not fully established (van Asten & van Dijk, 2005).

2.1.2 *Listeria monocytogenes*

In 1924 Murray et al. (1926) first isolated a small, Gram-positive rod bacterium and named the organism *Bacterium monocytogenes*. They suspected an oral route resulted in bacterial infection in rabbits and guinea pigs. At approximately the same time, Harvey Pirie isolated and described the same organism from veldt rodents (*Tatera lobengulae*) in South Africa and named the bacterium *Listerella hepatolytica* (FAO/WHO, 2004; Gant & McKenzie, 1956; Wiener, 1957). In 1940, Pirie recommended the bacteria be renamed to *Listeria monocytogenes*, which was retained in the Approved Lists of Bacterial Names (Dortet et al., 2009; Pirie, 1940). *Listeria monocytogenes* is a member

of the *Listeria* genus, which comprises 10 species (EFSA, 2014). Of this genus, only *L. monocytogenes* and *L. ivanovii* can cause disease in animals, and only *L. monocytogenes* appears to cause disease in humans, although there are sporadic reports of *L. innocua* and *L. seeligeri* also causing disease in humans (Batt, 2014; EFSA, 2014). Pathogenic infection by *L. monocytogenes* results in listeriosis and the disease was considered the most frequent cause of death among all foodborne infections in 2012 (EFSA, 2014).

2.1.2.1 Taxonomy and typing methodology of *L. monocytogenes*

For the purposes of public health surveillance and to assist in outbreak investigation, *L. monocytogenes* isolates are often further subdivided by serotyping (Ryser & Buchanan, 2012). Serological typing of *L. monocytogenes* is based on antibodies that specifically react with somatic (O) lipopolysaccharides (LPS) on the external surface of the bacteria's outer membrane and flagellin (H) antigens (Gasanov et al., 2005).

A robust serotyping scheme allows for subspecies designation of isolates, and the flagellar H antigen is determined using cultures grown at 25℃, while the O antigen is determined on cultures grown at 35℃. Thirteen different serotypes/serovars have been identified - 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7 (Batt, 2014; Pizarro-Cerdá & Cossart, 2019). Serotypes 1/2a, 1/2b, and 4b are responsible for 98% of cases of human listeriosis (Zeinali et al., 2016), with most outbreak strains in humans belonging to serovar 4b (Batt, 2014; Ryser & Buchanan, 2012). In contrast serotypes 1/2a, 1/2b, 1/2c, 3b and 4b are commonly found in chickens and their products (Jamshidi & Zeinali, 2019; Maung et al., 2019; Oliveira et al., 2018; Zeinali et al., 2016; Zhang et al., 2007). Although serotyping is still useful for first-level discrimination between isolates before application of more-sensitive subtyping methods (Ryser & Buchanan, 2012), it is not sufficient to determine the origin of the causative strains

(Todd & Notermans, 2011). In order to be able to confirm the source(s) of outbreaks, establish patterns of transmission, and determine and monitor reservoirs of epidemic strains, investigative techniques, such as molecular typing methods, have been developed (Gasanov et al., 2005). Pulsed-Field Gel Electrophoresis is one of the most discriminating of the molecular typing methods that is primarily used in conjunction with restriction enzyme (endonuclease) digests of DNA (Miettinen et al., 2001). Similar to the molecular subtyping of *Salmonella*, PFGE is considered the ‘‘gold standard’’ technique for molecular subtyping of *L. monocytogenes* because it has high accuracy and discriminatory power and reproducible results (Bouayad et al., 2015; Foerster et al., 2013). The operating method of *L. monocytogenes* PFGE is similar to that for *Salmonella* (described in Section 2.1.1.1); however, the selective ability of rare-cutting restriction enzymes, primarily *Xbal*, *AscI*, or *ApaI*, are used in PFGE for *L. monocytogenes* to digest the bacterial genome into a limited number of restriction fragments (CDC, 2013b). As outbreaks are linked with a food product, it is important to understand the epidemiology of *L. monocytogenes* in food industry plants. Epidemiological studies and techniques such as PFGE are able to indicate the potential sources of contamination, trace the contamination in the plant and enhance knowledge about the environmental locations where *L. monocytogenes* can survive and develop in a plant (Chasseignaux et al., 2001).

2.1.2.2 Characteristics of *L. monocytogenes*

Listeria monocytogenes is a Gram-positive rod, aerobic and facultatively anaerobic, non-spore forming, catalase-positive, oxidase-negative bacterium which can ferment sugars to produce acid without producing gas (Gasanov et al., 2005). It displays a typical tumbling motility at 20-25℃, but not at 35℃ (FAO/WHO, 2004). The cells are usually 0.5-2 μm in length. It produces a β-hemolysin on blood agar plates, which is

part of the CAMP (so named for Christie, Atkins, and Munch-Petersen) diagnostic test (Batt, 2014). The organism is psychrotrophic and grows over a temperature range of 0 to approximately 50℃, with an optimum temperature of 30-37℃ (Batt, 2014). When temperatures are below 0℃, the bacterium is preserved or moderately inactivated (Ryser & Buchanan, 2012). The pH range for the growth of *L. monocytogenes* is between 4.4 and 9.4 at water activities ≥ 0.92 with sodium chloride (NaCl) as the solute (FAO/WHO, 2004), although optimal growth occurs at neutral pH and 0.5% NaCl (Dortet et al., 2009). *Listeria monocytogenes* is inactivated by heating at 60℃ for 30 min, making pasteurization a useful technique for deactivating the bacteria in dairy products (Dortet et al., 2009).

Listeria monocytogenes has numerous biochemical characteristics useful for differentiation from other *Listeria* spp. and other bacterial genera. Currently, cultural methods for the detection of *L. monocytogenes* are based on a two-stage enrichment procedure: isolation using selective agars; and then confirmation with biochemical assays (Hegde et al., 2007). The protocols created by the USDA-FSIS for the detection of *Listeria* in meat and poultry products are the most widely used internationally. These involve incubation in an enrichment broth for 24-48 h (Donnelly & Diez-Gonzalez, 2013). Two selective enrichment media are recommended in the USDA protocols: University of Vermont (UVM) broth; and Fraser broth. These usually contain antimicrobial agents to which *L. monocytogenes* is resistant, and allow resuscitation of sublethally injured cells (Beumer & Curtis, 2003). Following incubation, a portion of the enrichment mixture is plated onto the final selective agar, modified Oxford agar (MOX), and incubated for 24 h. After that, colonies with typical characteristics (surrounded by a small zone of β-hemolysis) are processed to confirm the final identification using the commercial identification kit, API *Listeria* test system

(bio-Merieux, Marcy-Etoile, France) (FSIS, 2013).

Numerous selective agars have been developed for the isolation of *L. monocytogenes*. Enhanced hemolysis agar (EHA) can be used to detect a few *L. monocytogenes* colonies amongst colonies of other listeriae on the basis of hemolysis, the CAMP reaction and xylose fermentation. These three features are used to differentiate different *Listeria* spp. commonly found in foods and food environments (Beumer & Curtis, 2003; Beumer et al., 1997). In addition, selective chromogenic culture media have also been developed for the detection of pathogenic *L. monocytogenes*. The media can detect the virulence factors phosphatidylinositol-phospholipase C (PI-PLC) and phosphatidylcholin-phospholipase C (PC-PLC) produced by pathogenic *L. monocytogenes* (Hegde et al., 2007). BCM chromogenic agar (Biosynth International, Naperville, USA) is one type of media which results in the formation of blue colonies by *L. monocytogenes* and *L. ivanovii,* whilst other *Listeria* spp., lacking the specific enzyme, produce white colonies (Beumer & Curtis, 2003; Restaino et al., 1999). BBL CHROMagar *Listeria* agar is another chromogenic media with a high degree of specificity for the confirmation of suspect *L. monocytogenes* colonies. On BBL CHROMagar *Listeria* agar, *L. monocytogenes* forms a translucent white precipitation zone (halo) surrounding blue-pigmented colonies of 2-3 mm in diameter, with an entire border which can be used to visually differentiate *L. monocytogenes* colonies from all other *Listeria* spp., including *L. innocua* (Hegde et al., 2007).

2.1.2.3 Virulence of *L. monocytogenes*

Infection of humans with *L. monocytogenes* occurs via ingestion of contaminated food. After ingestion *L. monocytogenes* can cross the intestinal barrier and is thought to spread from the mesenteric lymph nodes to the spleen and liver. It may then reach the brain or the placenta, resulting in septicemia, meningitis, encephalitis, and/or

intrauterine infections (Dortet et al., 2009).

During invasion of the intestinal epithelial cells, *L. monocytogenes* may gain entry via nonphagocytic cells using two of its cell-surface proteins, internalin (InlA) and InlB, that interact with E-cadherin and Met (or the hepatocyte growth factor receptor), respectively (Dortet et al., 2009). Once internalized, the organisms must escape from the vacuole before fusion with the lysosome, which would result in the death of the bacterium. The pore-forming toxin listeriolysin O (LLO) and two secreted phospholipases PlcA and PlcB act to disrupt the vacuole and allow the bacteria to enter the cell's cytoplasm, where they multiply and polymerize actin to form actin polymers at one of the poles through the presence of the characteristic actin tails. The effect of the actin tail is to move the bacterium around the cytoplasm of the host cell. Actin polymerization allows bacteria to pass into neighboring cells by forming protrusions in the plasma membrane, resulting in a second infection cycle. It is through this mechanism that *L. monocytogenes* can move through its host, in the most extreme cases from the intestinal epithelium to the brain (Batt, 2014; Dortet et al., 2009).

2.2 Characteristics of important bacterial foodborne diseases in humans arising from consumption of poultry meat

2.2.1 Salmonellosis

Salmonellosis is one of the most common and widely distributed foodborne diseases which is caused by eating food contaminated with non-typhoid *Salmonella* (WHO, 2013). Non-typhoidal salmonellae are a leading cause of bacterial diarrhea worldwide and it is estimated that 93.8 million cases of gastroenteritis and 155,000 deaths occur globally each year (Majowicz et al., 2010).

2.2.1.1 Overview of salmonellosis

The clinical manifestations of salmonellosis can be grouped into two distinct disease

syndromes: gastroenteritis; and systemic disease (Cox & Pavic, 2014). Most people infected with *Salmonella* develop diarrhea, fever, abdominal pain, gastroenteritis, nausea and sometimes vomiting within 6 to 72 hours (usually 12-36 hours) of ingestion of the contaminated food. The illness is usually self-limiting, lasts 2 to 7 days and, in most cases, does not require specific treatment (CDC, 2015b; EFSA, 2014; WHO, 2013). However, certain individuals are particularly susceptible to disease, including those younger than 1 year of age, the elderly, and immunocompromised patients, especially HIV infected patients, as the associated dehydration can be severe and life-threatening (Cox & Pavic, 2014; EFSA, 2014; Ellermeier & Slauch, 2006; WHO, 2013). These susceptible groups may account for up to 60% of all notified cases and, although the overall mortality of cases is low (0.1-0.2%), these susceptible groups are overrepresented (Cox & Pavic, 2014). When *Salmonella* results in systemic infections, such as septicemia, antibiotic treatment is necessary (Cox & Pavic, 2014; EFSA, 2014) and fluid replacement may be required, especially in the elderly or in young children (Gilbert et al., 2010; MPI, 2013). In a small proportion of cases long term sequelae may occur, including appendicitis, endocarditis, pericarditis, meningitis, peritonitis and urinary tract infections (Cox & Pavic, 2014). Three to four weeks following the presence of gastrointestinal symptoms, reactive arthritis or Reiter's syndrome may occur, and this may last for up to a year or longer (MPI, 2013). The infectious dose required to cause disease varies from 1-100 CFU ingested, depending on the type of contaminated food ingested and the serovar (Cox & Pavic, 2014). Food high in fat or protein has been shown to offer significant protection for the organism; for example, chocolate or peanut butter may protect cells from gastric juices resulting in infection after consuming a lower dose than usual (Gilbert et al., 2010). In addition, host factors can also influence the infectious dose. A relative lower dose is required in the very young with a poorly

developed immune system and low gastric acidity, and the elderly and immunocompromised with a weakened immune response against infection (Cox & Pavic, 2014).

In the USA, the number of laboratory-diagnosed non-typhoidal salmonellae infections in 2019 was 8,556 cases with 2,430 (28%) of these requiring hospitalization, and the average annual incidence of salmonellosis was 17.1 cases per 100,000 individuals with the most common serotypes being *Salmonella* Enteritidis and *Salmonella* Typhimurium (Tack et al., 2020). The CDC estimated that approximately 1.35 million individual cases and 420 deaths occurred annually in that country, resulting in an estimated US$ 400 million in direct medical costs (CDC, 2019). The total annual cost of these infections has been estimated at US$ 4.1 billion in the USA alone (ERS, 2021).

In the European Union (EU), 28 Member States (MSs) provided data on salmonellosis and reported an average incidence of 19.7 per 100,000 humans in 2017, with the highest rates in the Czech Republic and Slovakia (≥106 cases per 100,000). A total of 91,662 human cases were confirmed by the 28 MSs in 2017, with 42.5% of the confirmed cases requiring hospitalization. The two most commonly reported *Salmonella* serovars in 2017 were also *S.* Enteritidis and *S.* Typhimurium, representing 49.1% and 13.4%, respectively of all cases of salmonellosis (EFSA, 2018). The overall economic burden of human salmonellosis in the EU has been estimated to be as high as EUR€ 3 billion (US$ 3.6 billion) a year (EFSA, 2011).

In New Zealand, the average annual incidence for notifiable salmonellosis was 42.8 cases per 100,000 population and 3.6 cases per 100,000 population for hospitalizations over a 12-year period (1997-2008), with the most common serotypes again *Salmonella* Typhimurium and *Salmonella* Enteritidis (Lal et al., 2012). The annual cost of salmonellosis was estimated to be NZ$ 4.8 million (US$3.4 million), with foodborne

infections costing NZ$ 2.8 million (US$ 2 million) (Lake et al., 2010). In Australia, 11,265 cases of salmonellosis were notified in 2012 (49.6 cases per 100,000 population) (Corvisy et al., 2015).

In Taiwan, non-typhoidal salmonellosis was responsible for 5.6% of all foodborne outbreaks for the period 1986-1995 (Pan et al., 1997). One laboratory estimated that at least 10,000 isolates of *Salmonella* could be recovered each year from hospitals across Taiwan and, using figures from the USA, they suggested that there were approximately 200,000 episodes of salmonellosis in Taiwan each year (Kuo et al., 2014). In addition, a study over a 6-year period (1991-1996) reported a case fatality rate of 8% (20/249) for patients who underwent clinical and microbiological analyses after presenting with salmonellosis to a hospital (Chen et al., 1999). The annual cost for salmonellosis-related hospitalizations was estimated at US$ 3.2 million for the period 2006-2008 in Taiwan; however, this value did not include the costs associated with outpatients, post-hospital care and associated productivity losses (Chen et al., 2012). A recent study that adopted the WHO Foodborne Disease Burden Epidemiology Reference Group (FERG) methodology framework based on the Taiwan National Health Insurance research database during 2012-2015, estimated that 185,977 cases of foodborne salmonellosis (with 6 deaths) occurred annually in Taiwan. This was estimated to result in a total loss of 510 disability-adjusted life years (DALYs) with an annual medical cost of NT$ 268 million (US$ 9.6 million) (Lai et al., 2020). According to the Taiwan Centers for Disease Control (TCDC), *Salmonella* Enteritidis and *Salmonella* Typhimurium were the two most prevalent serovars isolated from humans from 2003 to 2017 (Chiou et al., 2019; Chu et al., 2009).

2.2.1.2 Implicated foods and factors that influence transmission of *Salmonella* spp.

Although salmonellae may be spread to humans via person-to-person dissemination,

contaminated food or water, contact with infected animals, or from a contaminated environment, non-typhoidal salmonellosis is primarily a foodborne disease (FAO/WHO, 2007) and the fecal-oral route is the most frequent method of transmission (Gilbert et al., 2010). It has been shown that 95% of non-typhoidal salmonellosis cases in the USA (Mead et al., 1999) and 63% of human salmonellosis cases in New Zealand (NZFSA, 2007) each year were the result of foodborne exposure. Non-typhoid *Salmonella* was also the leading foodborne pathogen in Taiwan for the period 2012 to 2015 (Lai et al., 2020).

In recent years, large outbreaks of salmonellosis have been reported throughout the world due to a range of contaminated foods. Specifically in Taiwan in July 2010, a nationwide outbreak of salmonellosis associated with sandwiches contaminated with *Salmonella* Enteritidis and other bacterial pathogens purchased via an online shopping service in Taiwan was investigated. In that outbreak, a total of 886 consumers were contacted of which 36.6% had become ill with major signs and symptoms of diarrhea, abdominal pain, fever, headache and vomiting (Wei et al., 2014). In April 2018, another salmonellosis outbreak linked to French toast sandwiches in Taiwan resulted in 19 persons becoming sick, with 12 requiring hospitalization and 1 dying (Chueh et al., 2020).

Contamination of meat and meat products with zoonotic bacteria are considered one of the most important public health risks (Morar et al., 2014). Red and white meats are considered the main food sources of human salmonellosis, although a wide variety of other foods have also been implicated in some outbreaks (Gilbert et al., 2010). As the intestinal tract of a large range of food animals can act as a source of *Salmonella*, direct or indirect contamination of food can arise from fecal contamination (EFSA, 2014). Poultry, particularly chicken, has been identified as a major food source responsible for

human salmonellosis (FAO/WHO, 2007; Finstad et al., 2012; Gilbert et al., 2010; Mead et al., 2010; Morar et al., 2014; Tack et al., 2020). In the EU, fresh broiler meat has been identified as the most frequently contaminated meat product with *Salmonella* (4.9% of broiler meat tested), followed by fresh turkey meat (4.2%) and fresh poultry meat other than broiler meat (2.7%). Furthermore in the EU, 2.2% of *Salmonella* foodborne disease outbreaks were linked to the consumption of broiler meat in 2017, which was far less than those linked to contaminated eggs and egg products (36.8%) and bakery products (16.7%) (EFSA, 2018). The Danish Zoonoses Centre (DZC) modelled the estimated contribution of major animal and food sources to human infections with *Salmonella* in 2005, and estimated that the mean number of human cases (per 100,000 inhabitants) that could be attributed to broilers and imported poultry products were 1.3 and 4.0, respectively (FAO/WHO, 2007). In the USA, there are more than 40,000 human salmonellosis cases reported each year, and *Salmonella* from animal origin, in particular chickens, is considered the most likely source of infection (Finstad et al., 2012). This is highlighted by the finding that *Salmonella* have been recovered from 10–50% of chicken meat samples collected from retail outlets in the USA (Ellermeier & Slauch, 2006). In addition, a report summarizing the results of food samples from traditional markets and supermarkets in northern Taiwan in 2017–2019 found that 36.6% (34/93) of raw chicken meat samples were positive for *Salmonella* and the evidence from epidemiological, laboratory, and supply-chain investigations identified raw poultry as the vehicle for the widespread nature of *Salmonella* infections (Feng et al., 2020).

In general, the muscle tissue of healthy animals is sterile (Das et al., 2019). However, meat products can easily be contaminated with *Salmonella* spp. during animal slaughter, carcass dressing and cutting, meat processing, storage, merchandizing, preparation, and serving as a result of careless processing or improper hygiene (Chlebicz & Śliżewska,

2018; Sofos, 2014). In addition, different studies on the prevalence of *Salmonella* found that contamination of poultry meat occurred by contact with contaminated hands of workers or equipment, or by direct contact with contaminated carcasses mainly during the slaughtering and processing stages (Chotinun et al., 2014; Das et al., 2019; Goksoy et al., 2004; Rasschaert et al., 2007; Rasschaert et al., 2008; Zutter et al., 2005), with the most critical processing steps being plucking, evisceration and chilling (Keener et al., 2004).

The proportion of chicken carcasses contaminated with *Salmonella* has been investigated at abattoirs in many countries. In the USA, 3.9% of 8,861 carcass rinse samples from 178 establishments that killed young chickens (broilers) in 2014 were positive for *Salmonella* (FSIS, 2015). In the EU countries, of 51,093 fresh broiler meat sample units (single or batch) collected from 24 MSs and 1 non-MS in 2012, 4.1% were positive for *Salmonella* (range 0 to 22.7%) (EFSA, 2014). In New Zealand, 2.0% of rinse samples from whole poultry carcasses were positive for *Salmonella* in 2003 (Lake et al., 2004). In contrast, in Thailand 9% of samples of chicken carcasses swabbed under the wing and around the cloaca during 2000 to 2003 were shown to be positive for *Salmonella* (Padungtod & Kaneene, 2006). In Taiwan, a study was conducted by Chen et al. (2004) involving rinse samples from chicken carcasses collected from 14 Taiwanese abattoirs to investigate the prevalence of pathogenic microorganisms from 2000 to 2002. The prevalence for *Salmonella* species was 4.5, 0.7 and 1.7% for 2000, 2001 and 2002, respectively. In contrast, a high prevalence was reported in a study from the Republic of Korea with 42.7% of the carcasses sampled from 15 poultry abattoirs being contaminated with *Salmonella* (Bae et al., 2013).

It is apparent that salmonellosis is an important public health concern associated with the consumption of food of animal origin, especially fresh broiler meat (EFSA, 2014).

Contamination of chicken carcasses mainly occurs during slaughtering and processing and there is the potential for cross-contamination between carcasses, as well as from the environment to the carcasses (Keener et al., 2004). Consequently the monitoring of both carcasses and the abattoir environment for *Salmonella* is important in controlling this bacterium (Lin et al., 2009) and this forms the basis of the research reported in this thesis.

2.2.2 Listeriosis

Listeriosis, resulting from infection with *L. monocytogenes*, is important because it is the most frequent cause of death of all foodborne acquired infections (EFSA, 2014), although listeriosis in humans is a relatively rare disease compared with salmonellosis (CDC, 2020a, 2020b). In Taiwan, listeriosis was listed as a notifiable disease in January 2018 in an effort to improve its control (TCDC, 2018) and whole-genome sequencing (WGS) of all case-reported isolates is now performed to further investigate and track related cases (Huang et al., 2021).

2.2.2.1 Overview of listeriosis

The clinical manifestations of listeriosis can be grouped into two categories: invasive; and non-invasive (FAO/WHO, 2004). Invasive listeriosis are cases where *L. monocytogenes* crosses the intestinal barrier and leads to invasion of otherwise sterile body sites, such as the pregnant uterus, the central nervous system (CNS) or the blood (Dortet et al., 2009; FAO/WHO, 2004). The mortality rate from invasive listeriosis in healthy adults is generally low but can be as high as 30% in high risk groups, including pregnant women, neonates, adults with underlying disease (cancer, AIDS, diabetes, chronic hepatic disorder, transplant recipients), the elderly, and other persons with weakened immune systems (Batt, 2014; EFSA, 2014; Ryser & Buchanan, 2012; Turner, 2010). For example, a nationwide epidemiological outbreak investigation conducted in

Switzerland demonstrated that 12 of 13 infected people were immunosuppressed, of which 9 had comorbidities with tumors, diabetes, and other diseases, 8 were older than 60 years, and 1 was pregnant (Stephan et al., 2015). Of 123 human listeriosis cases at a medical center in northern Taiwan from 2000 to 2015 almost all had underlying conditions including malignancies (61.7%), steroid usage (39.1%), diabetes mellitus (31.3%), renal insufficiency (27.8%), and liver cirrhosis (17.4%) and overall the affected group had an average age of 63.9 years old (Huang et al., 2021).

Listeriosis in pregnant women is typically a mild influenza-like illness (fever and myalgia with or without diarrhea) or an asymptomatic infection, but can result in fetal loss, premature labor, invasive neonatal infection or death of the fetus (Cartwright et al., 2013; CDC, 2011; Craig et al., 2019). In newborns, the most common features of the infection are meningitis and/or septicemia (Craig et al., 2019; Jackson et al., 2010). The main presentations in immunosuppressed adults are as a result of central nervous system infection and/or septicemia leading to meningitis and primary bacteremia (McLauchlin et al., 2004). Other infrequent manifestations of listeriosis in persons with underlying cardiac lesions and various types of focal infections are endo-ophthalmitis, septic arthritis, osteomyelitis, pleural infection and peritonitis (Ryser & Buchanan, 2012). A systematic review of human listeriosis in China highlighted that of 147 clinical cases reported between 1964 and 2010, 31% involved infection of the central nervous system, 46% had septicemia and 23% were focal infections or gastroenteritis, with an overall case-fatality rate of 26% (Feng et al., 2013). The clinical presentations of 123 human listeriosis cases from 2000 to 2015 in northern Taiwan included bacteremia (74.8%), neurolisteriosis (20.0%), and bacterial peritonitis (5.2%) (Huang et al., 2021).

The clinical manifestations of non-invasive listeriosis (referred to as febrile listerial gastroenteritis) develop after a short incubation period and include diarrhea, fever,

headache and myalgia (FAO/WHO, 2004). These digestive manifestations, with a short-lived gastroenteritis, occur in healthy, as well as in immunocompromised, individuals who have ingested highly contaminated food products; however these presentations are usually self-limiting and quickly resolve (Dortet et al., 2009; Foerster et al., 2013).

The incubation period for listeriosis ranges from 1 to more than 90 days (Turner, 2010), although it is typically 2 to 3 weeks (FAO/WHO, 2004). A study conducted by Goulet et al. (2013) reported that the incubation period for listeriosis varied according to the clinical presentation of the disease, being 14 days for cases involving the central nervous system or bacteremia, and 6 weeks in pregnancy-associated cases. The infective dose is not known and is likely to differ between strains, although it is assumed to be approximately 1000 cells (Batt, 2014). Apparently healthy people who develop epidemic or sporadic foodborne listeriosis have been shown to ingest food contaminated with more than 100 cfu/gm of food (Gudbjörnsdóttir et al., 2004). As with other foodborne infectious bacteria, listeriosis is treated with antibiotics; however, it is important to start treatment as early as possible in cases with bacteremia due to the severity of the disease and the high associated mortality (Hernandez-Milian & Payeras-Cifre, 2014). Most experts suggest adding gentamicin to ampicillin for the treatment of listeriosis, but a study conducted by Fernández Guerrero et al. (2012) considered that combined ampicillin-gentamicin therapy did not improve survival, whereas trimethoprim sulfamethozaxole may be an effective alternative therapy for infections. The duration of treatment of bacteremia is usually about two weeks, but in immunocompromised patients there have been reported recurrences after two weeks of treatment. Therefore, it is recommended to prolong the duration of therapy in such cases depending on the clinical manifestations of the patients (Hernandez-Milian &

Payeras-Cifre, 2014).

The annual incidence of listeriosis has been investigated in many countries. In the EU, the annual incidence of listeriosis was reported as 0.48 per 100,000 population in 2017 with a total of 2,480 confirmed human cases and 227 deaths (EFSA, 2018). In the USA, the annual incidence of listeriosis in 2016-2019 was 0.3 cases per 100,000 individuals (Tack et al., 2020) with an estimated 1,591 illnesses and 306 deaths annually, and the annual cost of these infections was estimated to be US$ 3.2 billion (ERS, 2021). In Australia, 93 cases of invasive *L. monocytogenes* infection were officially notified (0.4 per 100,000) in 2012 (Corvisy et al., 2015). In Taiwan, a recent study using the well-established Global Burden of Diseases (GBD) methodology to estimate disease burden from foodborne illnesses, reported that, on average, 17 listeriosis cases (with 0.5 deaths) occurred annually in Taiwan, resulting in a total loss of 10 DALYs with associated annual medical costs of NT$ 2.8 million (Lai et al., 2020).

In some countries, data on the annual incidence of listeriosis are not available due to this disease being either rare or not a notifiable disease, although some epidemiological studies have been undertaken. For example, listeriosis is not a notifiable disease in China, and there is no national monitoring system for this disease; however, in a study conducted in China from 1964 to 2010, a total of 147 clinical cases in 28 (90%) provinces were reported. The overall case-fatality rate was 26% (34/130) among clinical cases with known outcomes and 46% (21/46) among neonatal cases (Feng et al., 2013). Similarly, before the Taiwan CDC listed listeriosis as a notifiable disease on January 1, 2018, listeriosis had been rarely reported in Taiwan (Tai et al., 2020; TCDC, 2018). A study undertaken from 1996 to 2008 identified 48 patients with listeriosis at a Taiwan hospital, and detected an increase in the average annual incidence from 0.029 cases per 1,000 hospital admissions for the period 1996-2004 to 0.118 cases per 1,000 hospital

admissions for the period 2005-2008, with 28% of admissions dying by day 14 of hospitalization (Huang et al., 2011). Another study reported that the annual incidence of invasive listeriosis at four medical centers in Taiwan from 2010-2012 was > 0.125 cases per 1,000 hospital admissions (Huang et al., 2015). A recent study examined the medical records of neonatal and maternal patients with pregnancy-associated listeriosis at two hospitals in Taiwan for the period from January 2000 to December 2018. The study reported that the annual incidence of perinatal listeriosis increased significantly from 0.94/10,000 cases in 2000–2011 to 5.45/10,000 cases in 2012–2018 (Tai et al., 2020). These studies highlight that further disease monitoring and surveillance are crucial to determine the trend of incidence in Taiwan (Huang et al., 2021).

2.2.2.2 Implicated foods and factors that influence transmission of *L. monocytogenes*

Listeria monocytogenes has become of major concern to public health authorities and the food industry since the organism was responsible for a disease outbreak in Canada in 1981 associated with the consumption of contaminated coleslaw (Capita et al., 2005; Cartwright et al., 2013). Most cases of human listeriosis occur through ingestion of contaminated food, and both outbreaks and sporadic cases are predominately associated with ready-to-eat (RTE) foods (CDC, 2011; EFSA, 2014; FAO/WHO, 2004; McLauchlin et al., 2004). Common-source outbreaks have been associated or linked epidemiologically with the consumption of milk, cheeses, ice cream, vegetables, fruits, seafood, and meat and meat products (Batt, 2014; Cartwright et al., 2013; EFSA, 2014; FAO/WHO, 2004).

Since the Canadian outbreak some large outbreaks of listeriosis have been reported. Between 2006 and 2007, an outbreak linked to cheese made from pasteurized milk was reported in Germany. The outbreak was widespread with cases being reported in 13 of the 16 German federal states affecting a total of 189 people with 81% hospitalized and

14% of cases dying (Koch et al., 2010). Between June and November 2008, a multi-province outbreak caused by contaminated delicatessen meat occurred in Canada. Fifty-seven people were reported to have been infected resulting in 41 hospitalizations and 24 deaths (Currie et al., 2015). In 2009 in Denmark an outbreak, involving 8 people, was traced back to infected beef from a meals-on-wheels delivery service. All patients developed septicemia and one developed meningitis with two of the eight cases dying (Smith et al., 2011). In 2011, a multistate outbreak associated with cantaloupe melons was reported in the USA. This outbreak was the largest in that country's history with 147 individuals affected from 28 states. Of these 143 were hospitalized and 33 died (McCollum et al., 2013). From 2017 to 2018 the largest outbreak ever reported occurred in the Republic of South Africa. This outbreak contained 937 cases of which 193 died and was caused by a contaminated RTE processed meat (polony) (Thomas et al., 2020).

Although there is no record of a large-scale outbreak of listeriosis in Taiwan, *L. monocytogenes* has been isolated from various local food products in Taiwan for several decades (Huang et al., 2015). In 1990, Wong et al. (1990) found that 58.8% of pork samples, 50% of chicken carcasses, 12.2% of vegetables and 10.5% of seafood sampled at food markets were contaminated with *L. monocytogenes*. The Taiwan Food and Drug Administration (TFDA) performed a large food surveillance study in 2014 and demonstrated that 1.0% of fruit/vegetables and 1.4% of RTE foods were contaminated with *L. monocytogenes*. These findings highlight that disease monitoring and surveillance are crucial to determine the current level of contamination of foods in Taiwan with *L. monocytogenes* and to determine if this value is reducing through the adoption of appropriate intervention methods (Huang et al., 2021).

Although chicken meat has never been identified as the source of an outbreak of listeriosis internationally (Aury et al., 2011; Bouayad et al., 2015; Rørvik et al., 2003;

Rothrock et al., 2017), ready-to-eat and undercooked chicken have been linked to sporadic cases of human listeriosis worldwide (Kerr et al., 1988; Rothrock et al., 2017; Schwartz et al., 1988), with a single outbreak linked to turkey meat in the USA (Olsen et al., 2005). Internationally, the prevalence of *L. monocytogenes* in chicken carcasses or fresh chicken meat is reported to range from 25.7 to 70% (Cox et al., 1997; Praakle-Amin et al., 2006; Rørvik et al., 2003; Uyttendaele et al., 1997). Due to the ubiquitous nature of *L. monocytogenes*, the organism is widely distributed in the processing environments; particularly in processing areas that are cool and wet (Aury et al., 2011; Batt, 2014; EFSA, 2014; FAO/WHO, 2004). It has also been reported that *L. monocytogenes* in poultry meat can originate from infected flocks (Ryser & Buchanan, 2012), as well as from environmental cross-contamination during processing (Franco et al., 1995; Lawrence & Gilmour, 1994; Loura et al., 2005; Sakaridis et al., 2011). A study in the United Kingdom (UK) conducted by Lawrence and Gilmour (1994) found that 26% (21/79) of environmental samples and 59% (34/58) of raw poultry products collected from poultry processing plant were positive for *L. monocytogenes.* Ojeniyi et al. (1996) investigated the prevalence of *L. monocytogenes* on neck skin of processed chickens before and after the spin chiller in seven Danish abattoirs. They found a prevalence of 22.5% for samples collected before the spin chiller and 45% in samples collected after the carcasses had been in the spin chiller, highlighting the important role of cross-contamination between carcasses during processing. A study in the USA of processed broiler carcasses after the final chiller found that approximately one quarter (27 of 105) of the carcasses were contaminated with *L. monocytogenes* (Cox et al., 1997). Another study of 150 broiler carcasses from seven poultry abattoirs in Norway detected *L. monocytogenes* in 50% of the carcasses, with one abattoir having all carcasses positive (n = 6) (Rørvik et al., 2003). However, in a study of freshly dressed

(processed) broiler chickens in Jordan a much lower prevalence of 9.4% was found (Osaili et al., 2011). Another study in Algeria evaluated the changes in the prevalence of *L. monocytogenes* in broilers during processing at three abattoirs. Overall 8.9% of samples were positive, with a higher prevalence at the end of processing (17%) than at the evisceration stage (1%) (Bouayad et al., 2015). In contrast, a lower prevalence was reported by Chen et al. (2004), who collected rinse fluid samples from chicken carcasses at the end of processing in 14 Taiwanese abattoirs and investigated the prevalence of pathogenic microorganisms from 2000 to 2002. The prevalence of *L. monocytogenes* was 2.8, 4.4 and 0.6% in 2000, 2001 and 2002, respectively. These findings highlight the need to understand the survival of *L. monocytogenes* to minimize or prevent contamination of the environment in poultry processing plants (Chasseignaux et al., 2002).

2.3 Risk factors associated with important foodborne pathogens in poultry processed at commercial abattoirs

The main processing steps of the abattoirs in Taiwan include holding, stunning and exsanguination, scalding, plucking, evisceration, post-mortem inspection, carcass washing and chilling, followed by packing and labeling with an inspection certificate (Figure 2.1) (Lin, personal observation). During the slaughtering and processing of poultry, there is the potential for contamination of carcasses with fecal/gastro-intestinal material and further cross-contamination between birds (FSANZ, 2005). The proportion of poultry carcasses contaminated and the level of foodborne pathogens on carcasses at abattoirs are dependent upon several factors associated with the status of the birds prior to slaughter, and contamination at different steps/stages of processing. The management conditions on the source poultry farms, the hygienic practices implemented during the slaughtering operations and the subsequent handling of carcasses are all potentially

associated with contamination (Brizio & Prentice, 2015; FSANZ, 2005).

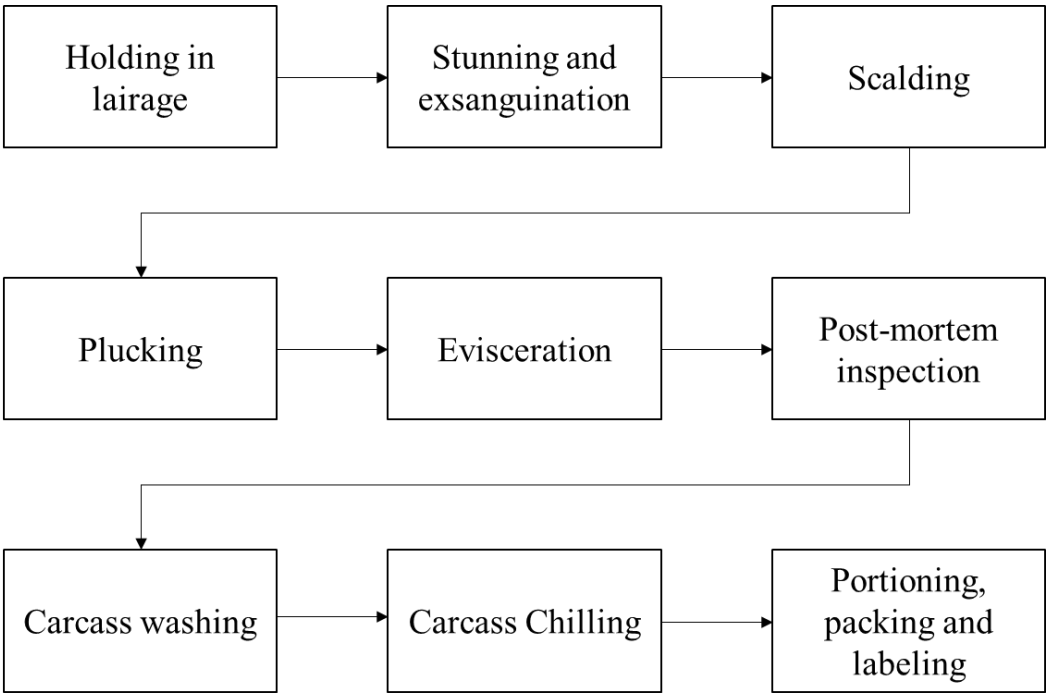

Figure 2.1 The main processing steps of the poultry abattoirs in Taiwan

To ensure consumers are provided with high-quality, safe and wholesome products, the processing of poultry is designed to minimize the numbers of bacteria on the outer and inner surfaces of the carcass (FAO/WHO, 2009a). However, improper slaughtering operations or ineffective cleaning and disinfection of machines/equipment in poultry abattoirs may lead to the contamination of poultry carcasses (Miettinen et al., 2001). Many publications have reported cross-contamination occurring during carcass processing, especially during scalding, de-feathering, evisceration and chilling (FAO/WHO, 2007; Fries, 2002; FSANZ, 2005; Hue et al., 2010; King et al., 2011; Morar et al., 2014; Russell, 2012b). Furthermore, several studies have indicated that cross-contamination might originate from the slaughter environment which becomes contaminated before birds are processed; or the processing of birds from infected flocks

(FAO/WHO, 2009a; Henry et al., 2012; King et al., 2011; Nógrády et al., 2008). In order to reduce cross-contamination, several interventions have been developed that include: withholding feed from broilers for several hours on-farm prior to transporting and slaughtering; establishing different time/temperature combinations for inactivating bacteria in the scalding tank; and using antimicrobial substances in the processing line (Fries, 2002).

In this section, the major stages of poultry slaughtering are considered with regard to the potential spread of foodborne pathogens with the majority of the data referred to in this review applying to broiler chickens.

2.3.1 Transportation from farms to abattoirs and in lairage

Several studies have demonstrated that transportation of poultry from the source farms to the processing plants increases the prevalence of *Salmonella* and that transport cages or crates are an important source of cross-contamination between birds and batches (Byrd & McKee, 2005; King et al., 2011). Poultry can shed salmonellae in their feces, and the concentration of these bacteria can be up to 10^4 cfu/g of gut content or feces (King et al., 2011). During transportation the crates/cages holding birds are usually stacked on top of each other, and this, along with the fact that the stress of transportation leads to increased fecal excretion, results in a greater opportunity for contamination of birds with feces and subsequent cross-contamination of potential pathogens between birds (FAO/WHO, 2002; FSANZ, 2005).

Rigby et al. (1980) demonstrated that before birds were loaded into crates, 14.0% of the crates were already contaminated with *Salmonella* which highlights the potential for contamination of birds prior to their entry to the abattoir, resulting in contamination of the abattoir and its environs and potentially the processed carcasses. Therefore after unloading, cages/crates need to be thoroughly cleaned and disinfected to avoid

cross-contamination to the next flock/batch of birds transported (Van Immerseel et al., 2009). However, in a study by Rigby et al. (1982), although 86.6% of plastic transport crates were positive for *Salmonella* prior to loading with birds, 73.5% of them were still positive for this bacterium after passing through the crate washer. In a Danish study, the percentage of *Salmonella* contaminated crates after cleaning and disinfection was still 34.3% (Olsen et al., 2003). These studies highlight that good cleaning and disinfection protocols and monitoring the efficacy of the disinfection process is essential (Van Immerseel et al., 2009). Washing the live bird transport crates with water and disinfectant, and then leaving them to dry for 48 hours has been recommended to reduce the levels of residual *Salmonella* in and on the transport cages (FAO/WHO, 2011; King et al., 2011).

A study conducted by Mainali et al. (2009) found that a longer transport time was also associated with an increased prevalence of *Salmonella* in neck skin samples of broiler chickens. These authors concluded that, as birds were fasted for several hours on-farm prior to transportation, if the transport time was longer there would be increased likelihood of birds eating litter, and hence an increased risk of infection with *Salmonella* from bacteria shed onto the litter by infected birds. These authors also observed that a longer time in lairage was associated with an increased prevalence of *Salmonella* in the crops and ceca. In contrast a study by Lawes et al. (2012) found that transit time to the abattoir of greater than 2.5 h was protective for *Campylobacter* in the ceca of broilers. These authors believed that this finding was likely to be an indicator of farm size rather than a causal relationship between transit time and *Campylobacter* infection. They reported that large poultry farms were more likely to be located close to the company abattoir and concurrently larger farms were more likely to be infected with *Campylobacter*, whereas flocks from smaller farms might have to be transported further

to be slaughtered and initially had a lower prevalence of *Campylobacter*.

In addition, fans are commonly used in lairage to prevent birds overheating. However, Harbaugh et al. (2006) demonstrated experimentally that *Salmonella* could be transmitted within two hours from caged contaminated turkeys to previously non-contaminated turkeys when a fan was used, and the authors hypothesized that aerosolized dust was an important route for this transmission. When a broiler chicken arrives at a processing plant, it can be heavily contaminated with feces and pathogenic bacteria, such as *Salmonella* and *Campylobacter*. These microorganisms are found on the skin, feet and feathers or in the crop, colon, ceca and cloaca of the birds (FAO/WHO, 2009a; Finstad et al., 2012; Russell, 2012b). In contrast, *Listeria* infrequently enters the processing plant on the live broilers (Cox et al., 1997; FSANZ, 2005). However, Liang et al. (2013) identified bioaerosols containing *L. monocytogenes* could be distributed throughout a chicken abattoir by air movement, such as from the live-bird holding area to the slaughtering, evisceration, cutting, and packing areas. *Listeria* appear to take up residence in the abattoir, a situation which ultimately leads to cross-contamination to carcasses during processing (Cox et al., 1997; Nógrády et al., 2008; Olsen et al., 2003; Van Immerseel et al., 2009).

To reduce cross-contamination during processing, slaughter of pathogen-free flocks has been implemented in some European countries. However, the studies by Olsen et al. (2003) and Zutter et al. (2005) indicated it could not always avoid cross-contamination between birds from different flocks. This is likely due to the presence of viable organisms remaining on processing equipment as a result of inefficient cleaning and disinfection (Corry et al., 2002; Lillard, 1990; Olsen et al., 2003; Rasschaert et al., 2007; Rasschaert et al., 2008; Rigby et al., 1980; Rigby et al., 1982; Zutter et al., 2005). Additionally, minimizing the stress on birds through the use of low intensity lighting,

minimizing handling and avoiding delays in processing, as well as withholding feed (but not water) for 4-12 hours before slaughter (including catching and transportation time), have been recommended since they can reduce the likelihood of contamination of carcasses by fecal material and ingesta without significantly affecting carcass weight (FAO/WHO, 2011; King et al., 2011).

2.3.2 Stunning and exsanguination

As mentioned previously, birds can be contaminated with large numbers of pathogenic bacteria through cross-contamination during transportation and holding, prior to stunning. Both electrical and gaseous stunning are unlikely to change the levels of contamination or cross-contamination and these practices have not been identified as factors for the spread of *Salmonella* on poultry carcasses (FSANZ, 2005). It is highly likely that the microbial results during stunning and exsanguination reflect the status of microorganisms in the chickens when they arrive at the abattoir, and positive carcasses indicate that these birds were already contaminated with a substantial number of bacteria on arrival (Finstad et al., 2012).

During exsanguination, the neck of the bird is cut and the bird "bled-out" over a period of one to three minutes. This step is unlikely to influence the level of contamination (King et al., 2011), even though birds can carry large numbers of pathogenic bacteria. Kotula and Pandya (1995) reported that the percentage of birds positive for *Salmonella* spp. was between 27.5 and 75%, 42.5 to 100% for *Escherichia coli*, and 45 to 82.5% for *Campylobacter jejuni/coli*, with the number of *Salmonella* spp., *E. coli*, and *C. jejuni*/*coli* ranging between 5.8 and 8 $\log_{10}$ cfu/g after the chickens were exsanguinated but before the carcasses were scalded. The blade of the knife used to cut the neck might be a potential source of cross-contamination at slaughter (FAO/WHO, 2009a). Mead et al. (1994), using an antimicrobial-resistant strain of *E. coli* as a marker, inoculated the

knives in an automated “throat-cutting” machine. In that study the marker organism was spread to the 500th bird passing through the machine; however, using chlorinated spray-wash water directed at the knife reduced the spread of the marker organism by 50%, and a higher concentration of chlorine may have provided even better control.

2.3.3 Scalding

During scalding, poultry carcasses are immersed in a scald tank (temperature range: 50 - 65℃) to loosen the feathers to facilitate plucking (FSANZ, 2005). This is the first place in the poultry abattoir where fecal material containing potentially pathogenic bacteria is able to come off the birds and enter the surrounding water (FSANZ, 2005; King et al., 2011; Russell, 2012b). Pathogenic bacteria potentially could survive in scalding water because of the protective effect of fecal material, feathers or even the carcass temperature which would not reach the temperature of the scald water (Henry et al., 2012). If the scalding water is not stirred or the temperature is not high enough to kill bacteria, viable microorganisms could be transmitted between carcasses during scalding. As such, this stage may be a significant site of cross-contamination (Finstad et al., 2012; FSANZ, 2005; King et al., 2011; Russell, 2012b).

In general, when scalding is operated properly, this procedure can reduce the levels of pathogenic bacteria on carcasses (FAO/WHO, 2007). For instance, the numbers of *Campylobacter* on carcasses have been shown to be reduced by 2-3 $\log_{10}$ (Byrd & McKee, 2005) and the number of *Salmonella* positive poultry carcasses decreased by 38% post scalding (Georgnaras et al., 1997). A study by Dan et al. (2013) demonstrated that after scalding at a temperature of 56-60℃ for 3 minutes, a reduction of 0.75 $\log_{10}$ cfu/g for the total bacterial count, 1.05 $\log_{10}$ cfu/g for *E. coli*, and 0.67 $\log_{10}$ cfu/g for *Listeria innocua*, was achieved. The temperature of the scalding tank is critical for bacterial survival; however, it does vary depending upon the poultry species being

processed. A high temperature scalding process has a beneficial effect in reducing the number of bacteria (FSANZ, 2005). Yang et al. (2001) inoculated *Salmonella* Typhimurium and *C. jejuni* into scald water and onto chicken skins to determine the effects of scalding temperature (50℃ and 60℃) on bacterial survival. After scalding at 50℃ and 60℃, the reductions of *C. jejuni* were 1.5 and 6.2 $\log_{10}$ cfu/mL in water and < 1 and > 2 $\log_{10}$ cfu/cm^2 on chicken skins, respectively; and the reductions of *Salmonella* Typhimurium were < 0.5 and > 5.5 $\log_{10}$ cfu/mL in water and < 0.5 and > 2 $\log_{10}$ cfu/cm^2 on skins, respectively.

To inhibit the growth of microorganisms, scald water should be kept at a temperature at least 5℃ above the microorganism's maximum growth temperature. Owing to the maximum growth temperature for *Salmonella* being 45℃, the temperature of scald water is recommended not to be lower than 50.5℃ to prevent any multiplication of *Salmonella* present in the scalding tank (Russell, 2012b). Additionally, Byrd and McKee (2005) suggested that the greatest reductions in both *Salmonella* and *Campylobacter* counts were at scald temperatures of 58-60℃, compared to 52℃. Although the higher scalding temperatures may be favorable in reducing contamination, they do contribute to tearing of carcass skin and blemishes of the epidermis, potentially resulting in carcasses with undesirable appearances (FSANZ, 2005; King et al., 2011; Löhren, 2012; Zweifel et al., 2015). The microtopography of chicken skin was investigated by Kim et al. (1993) at varying scalding temperatures to determine the least favorable skin surface for the attachment of salmonellae. The study found that skins scalded at 52 and 56℃ retained most of the epidermis, whereas skins scalded at 60℃ began to lose most of the epidermal layers during scalding and exposed the dermal surface after plucking. The number of salmonellae attached to 60℃ processed skins was 1.1-1.3 logs higher than to the skins processed at 52 and 56℃, as measured by scanning electron microscopy (Kim

et al., 1993). Furthermore, chicken fat is predominantly unsaturated and the fat under the skin becomes liquefied after only 2 minutes when carcasses are placed in a scalding tank at a temperature of 54.5℃. As carcasses are suspended upside down, liquefied fat will drain out from under the skin as the carcasses move along the line, and also when the carcasses are in the chilling tank. This can result in the chilling water containing high levels of fat due to the higher temperature scalding, reducing the effect of disinfection by chlorination in the chilling tank (Russell, 2012b).

Contamination during scalding can be minimized by the use of countercurrent flow and multi-staged tanks (FAO/WHO, 2011; Russell, 2012b). This countercurrent flow of water can wash the birds and remove contamination from the birds as they travel through the scalding tank, with more than 1 L per bird recommended (Russell, 2012b). A study by Waldroup et al. (1993) demonstrated that a properly run countercurrent scalding tank could reduce the percentage of carcasses with *Salmonella* by 88.5% without the use of chemicals. Another study by Cason et al. (2000), examined scald water samples from a broiler abattoir to evaluate the numbers of coliforms, *E. coli*, and salmonellae with a multiple-tank, counter-flow scalding procedure. The results demonstrated the mean coliform concentrations in tanks 1, 2, and 3 (where tank 3 is the last tank that carcasses passed through before being de-feathered) were 3.4, 2.0, and 1.2 $\log_{10}$ cfu/ml, respectively. Burdens of *E. coli* followed the same pattern with means of 3.2, 1.5, and 0.8 $\log_{10}$ cfu/ml in tanks 1, 2 and 3, respectively, with significant differences in the concentrations of both coliforms and *E. coli* between the tanks, whereas the *Salmonella* contamination was only reduced after the third tank. This study highlighted that most bacteria removed from carcasses during scalding were washed off during the early part of scalding; however, the numbers of aerobic bacteria in carcass rinses were not affected by the scalding tank design (Cason et al., 2000).

Using pre-scald wash systems and approved chemicals in scalding tanks are also recommended to reduce contamination during scalding (FAO/WHO, 2011). Some abattoirs have installed a bird brush and washer before scalding. Brushes and chlorinated water can physically remove feces from the feathers and skin of the birds and reduce the amount of fecal material in the scalding tank by approximately 90% (Russell, 2012b). In the USA chemicals can be added to the scalding tank to reduce the number of pathogenic bacteria. Russell (2008) evaluated the effect of an acidic, copper sulfate-based commercial sanitizer on the prevalence of *Salmonella* and on the total aerobic bacteria (APC) and *E. coli* counts on broiler carcasses during scalding. The results showed that the prevalence of *Salmonella* contaminated carcasses was reduced by an average of 30%, and the average $\log_{10}$ reduction overall was 3.80 and 3.05 for APC and *E. coli*, respectively.

2.3.4 Plucking

When a broiler chicken arrives at the abattoir, it is already contaminated with a substantial number of bacteria (FAO/WHO, 2009a; Finstad et al., 2012). Rigby et al. (1980) found that contamination of the feathers of birds with *Salmonella* was more common than intestinal carriage. Moreover some bacteria can survive on the feathers even after scalding, and these contaminated feathers are likely to be an important means of introducing bacteria into the abattoir environment during de-feathering (Rigby et al., 1980). Rasschaert et al. (2007) found that 50% of samples of feathers from the breast and the wings of broilers collected after scalding were positive for *Salmonella*. In addition, many reports and studies have considered that de-feathering is a processing stage resulting in significant cross-contamination (Byrd & McKee, 2005; FAO/WHO, 2009a; FSANZ, 2005; Hänninen, 2010; King et al., 2011; Morar et al., 2014). A study by Gruntar et al. (2015) examined the presence and quantity of contaminating *C. jejuni*

during various stages of processing, and found the highest skin contamination was detected after de-feathering, suggesting that the majority of *Campylobacter* contamination actually occurred prior to evisceration, probably during the preceding plucking stage. Several other studies have also observed that the average *Campylobacter* counts on broiler carcasses tended to increase after de-feathering (Berrang et al., 2001; Duffy et al., 2014; Zweifel et al., 2015). Moreover, the studies of both Nde et al. (2007) and Rasschaert et al. (2007) reported significant increases in the prevalence of *Salmonella* after de-feathering than before de-feathering. Also Mead et al. (1994), using a marker strain of *E. coli* inoculated onto several carcasses prior to entering the de-feathering machines¸ demonstrated that the marker bacterium was detected on the 200th carcass following de-feathering of the inoculated birds.

Nde et al. (2007) used molecular subtyping by PFGE of *Salmonella* serotypes isolated from the fingers of the plucking machines and the carcasses before and after de-feathering to trace cross-contamination. Their study found that the PFGE subtypes of *Salmonella* isolated from the fingers of the plucking machines were similar to the subtypes isolated from the carcasses before and after de-feathering, indicating that the fingers facilitated carcass cross-contamination during de-feathering. Other studies have shown that the surface of the rubber fingers becomes roughened with increasing use, allowing bacteria to transfer from the fingers to the carcasses during the next de-feathering process (Fries, 2002; FSANZ, 2005; King et al., 2011). Moreover, the feather follicles in the skin at this stage are open and the rubber fingers used in de-feathering can drive microorganisms into the skin tissue and feather follicles, which may decrease the effect of subsequent surface carcass washing (Byrd & McKee, 2005; Hänninen, 2010; Sofos et al., 2013).

The other possible route of microbial cross-contamination of broiler carcasses during

de-feathering is via aerosols (FSANZ, 2005; King et al., 2011; Russell, 2012b; Sofos et al., 2013). Allen et al. (2003) used an identifiable strain of *E. coli* as a marker microorganism to determine the sources, routes and patterns of microbial cross-contamination during mechanical de-feathering of broiler carcasses. The results showed that the marker bacterium could be transferred to other carcasses and equipment by aerosol or by the large airborne droplets created during de-feathering. From these studies Russell (2012b) concluded that there was significant potential for cross-contamination of bacteria between carcasses during de-feathering arising from direct contact between carcasses, the action of the fingers of the plucking machines and through aerosols.

To minimize cross-contamination at de-feathering, a range of interventions are recommended. For instance, as de-feathering is considered a "dirty" activity, it should be physically separated from later primary processing activities to prevent exposure to aerosols or the large airborne droplets generated during de-feathering (King et al., 2011). Furthermore, continuous spraying of equipment and carcasses with water during de-feathering, and dismantling of the equipment for a complete clean and disinfection after processing can prevent the build-up of feathers and bacteria on the equipment. Regular inspection and replacement of worn fingers of the plucking machines also will reduce cross-contamination (FAO/WHO, 2011; FSANZ, 2005).

2.3.5 Evisceration

In addition to de-feathering, evisceration is also considered an important processing stage which can result in bacterial cross-contamination (Byrd & McKee, 2005). During evisceration, the crop, gut and other internal organs are removed, but some of these organs can be heavily contaminated with pathogenic bacteria (FSANZ, 2005; King et al., 2011). When these organs are damaged, the leakage of crop and intestinal contents

during the evisceration process may not only cause contamination of the carcasses of this flock, but can also contaminate the slaughter equipment leading to extensive cross-contamination of the carcasses of subsequently slaughtered flocks (Russell, 2012b; Sofos et al., 2013; Van Immerseel et al., 2009). Brizio and Prentice (2015) reported that one of the biggest problems in poultry processing was carcass contamination by fecal leakage during evisceration.

A study by Smith et al. (2007) identified that 5.5 to 25.2% of processed broilers received damage to their digestive tract during evisceration. Similarly Russell (2003) reported that 2 to 34% of broilers at one processing plant had damaged intestines during the evisceration process. Moreover, Rivera-Perez et al. (2014) reported an increase in the percentage of contamination with *Salmonella* (10 to 40%) during evisceration, and tearing of the digestive tract was considered responsible for this. Similarly, most studies summarized by the FAO/WHO (2002) showed a two to five fold increase in the prevalence of *Salmonella* species after evisceration, although one study in the USA showed little effect of evisceration (Morris & Wells, 1970). In addition, Dan et al. (2013) collected samples from poultry carcasses at the various steps of the slaughtering process and found that the load of *E. coli* and *L. innocua* during evisceration increased significantly by 1.55 $\log_{10}$ cfu/g and 0.44 $\log_{10}$ cfu/g, respectively, compared with the results obtained in the scalding step. Furthermore a study by Bouayad et al. (2015) also suggested that contamination of broiler carcasses with *Listeria* spp. at abattoirs increased during and after the evisceration stage.

Contamination of carcasses arising from the rupture of viscera might be caused by poorly controlled abattoir processes (FSANZ, 2005), particularly if mechanical eviscerating equipment is not designed or adjusted properly (King et al., 2011; Russell, 2012b; Russell & Walker, 1997). For example, unless equipment is correctly maintained

and calibrated, at a higher processing speed there is a greater likelihood of rupture of the viscera resulting in fecal contamination of the carcass (FSANZ, 2005). Furthermore, if the machine(s) used for evisceration cannot adapt to the natural variation in the carcass sizes within a batch of processed birds, it also can increase the likelihood of rupture (Cossi et al., 2010; Hue et al., 2010). To reduce the potential for rupture of the viscera, careful setting of the evisceration machines and adjustment when birds of a different weight/size are being processed are necessary (FAO/WHO, 2011; Hue et al., 2010; Mead, 2000). Brizio et al. (2015) highlighted the need to limit the size variation of birds within batches to ensure processing of birds of similar sizes. They also reported the benefit in adjusting the period of pre-slaughter fasting (they recommended 8-12 hours) to ensure that the digestive tract was empty at processing, and therefore the potential impact of a ruptured digestive tract during mechanical processing was reduced. Pre-slaughter fasting is considered critical in controlling the amount of intestinal spillage that occurs during evisceration (FSANZ, 2005).

In general, high-capacity abattoirs adopt either a semi-automated or fully automated evisceration process; however, manual evisceration is used in small abattoirs. Chiarini et al. (2009) compared the level of *L. monocytogenes* in two poultry facilities following the same standards but only differing in the form of evisceration (manual vs automated). The results of that study showed the products from the plant adopting manual evisceration were more contaminated than those from the plant with highly automated evisceration; however, the pathogen was more frequently detected in environmental samples from the abattoir with automated evisceration. Similarly, Cossi et al. (2010) investigated the presence of *Salmonella* spp. and microbiological indicators at different stages of processing at two abattoirs (a high-capacity plant with automated evisceration and a plant with a lower capacity and adopting manual evisceration). They similarly

observed more microbial contamination of carcasses after evisceration in the abattoir with manual evisceration. In contrast Nunes (2013) reported that manually-eviscerated carcasses were less likely to be contaminated than carcasses eviscerated automatically. He concluded that flocks containing birds of different sizes contributed to increasing the risk of fecal and bile contamination during automated evisceration operations.

In addition to the interventions mentioned to reduce the likelihood of rupture of the viscera, other measures are also recommended to reduce cross-contamination during the evisceration process. Use of continuous sprays to rinse equipment and birds during evisceration is recommended (King et al., 2011). This helps wash the surface of the processing equipment reducing potential for cross-contamination by pathogens (Russell, 2012b). Also, equipment should be thoroughly cleaned and sanitized between shifts in a day to minimize the buildup of contamination (FSANZ, 2005). Furthermore, spraying water containing 20-50 ppm of chlorine subsequent to de-feathering and carcass evisceration has been shown to reduce the prevalence of *Salmonella*-positive broiler carcasses from 34% to 26% and from 45% to 36%, respectively (FAO/WHO, 2011). However some countries, such as those in the EU, have banned the addition of antibacterial agents to water during processing to appease the concerns of consumers about the use of chemicals during processing of poultry (Russell, 2012d).

2.3.6 Post-mortem inspection

During post-mortem inspection, carcasses are visually and manually inspected for defects by meat inspectors. However, a pathogen-contaminated carcasses cannot be identified by the naked eye (Byrd & McKee, 2005). Furthermore, when inspectors manually examine carcasses with their hands, they may inadvertently increase cross-contamination between carcasses (Oosterom et al., 1983).

In the USA, the FSIS allows a carcass with visually detectable fecal contamination

during post-mortem inspection to be passed after appropriate treatment(s) which may include trimming, vacuuming and washing, or a combination of these (Russell, 2012b). If internal contamination is present, in addition to reprocessing treatments, the entire carcass must be washed with water containing 20 ppm chlorine (Russell, 2012b). However, if fecal contamination is still evident after reprocessing, the carcass is then condemned (Finstad et al., 2012). Similar regulations are also adopted in Canada (Powell et al., 1995). Powell et al. (1995) investigated the number and presence of bacteria in inspection-passed and re-processed (with 5-sec inside/outside spray wash followed by vacuuming) broiler carcasses. They found that in initially inspection-passed carcasses the average CFU (log_{10}/ml) for a standard plate count (SPC) was 3.99; coliforms was 3.49; *E. coli* was 3.34, and the detection frequencies for *Salmonella* and *Campylobacter* were 4% and 84%, respectively. However, in carcasses after reprocessing the average CFU (log_{10}/ml) for a SPC was 3.66; coliforms was 3.21; *E. coli* was 3.06, and the detection frequencies of carcasses with *Salmonella* and *Campylobacter* were 0% and 77%, respectively. The lower SPC, coliform and *E. coli* levels after reprocessing, when compared to inspection-passed carcasses, highlights the advantages of these additional processes.

During post-mortem inspection, the eviscerated carcasses and their viscera should be operating synchronously to enable the inspector to conduct an inspection of the carcass and its associated viscera. Two different types of viscera presentation are currently used in abattoirs: one where the viscera hangs outside of, but remains attached to, the carcass; and the second that uses either shackles or trays to present the viscera separately from the carcass (Fries, 2002). Russell and Walker (1997) determined the effect of the two evisceration systems (Nu-Tech method with the viscera separated from the carcass; conventional streamlined inspection system (SIS) with the viscera remaining attached to

the carcass) on visible contamination and the microbiological profile of fresh broiler chicken carcasses. Their results clearly demonstrated that evisceration using the Nu-Tech method resulted in fewer visibly contaminated carcasses and lower aerobic plate counts, total coliform counts, and total *E. coli* counts than the traditional SIS evisceration system.

To minimize cross-contamination at the meat inspection stage, adequate light and the ability to adjust the line speed during inspection are required to allow detection of visibly contaminated carcasses and carcasses displaying gross pathology (FAO/WHO, 2011).

2.3.7 Carcass washing

Eviscerated carcasses may be washed internally and externally by using high pressure sprayers to remove any visible fecal contamination before they are chilled. Generally, carcass washing is able to reduce visible contamination on carcasses, but reports on the effectiveness of washing on carcass microbiology have been conflicting (Smith et al., 2007).

A study to investigate the microbiological impact of spray washing broiler carcasses with different concentrations of chlorine and water temperatures before chilling was undertaken by Northcutt et al. (2005). They found that spray washing could reduce *Salmonella* on carcasses from 4.0 $\log_{10}$ to 3.2 $\log_{10,}$ *Campylobacter* from 6.3 $\log_{10}$ to 3.5 $\log_{10}$, total aerobic counts from 6.7 $\log_{10}$ to 4.4 $\log_{10}$, and *E. coli* from 6.3 $\log_{10}$ to 3.5 $\log_{10}$, even without the addition of chlorine or the use of hot water (Northcutt et al., 2005). However, Zweifel et al. (2015), who collected broiler carcasses from three abattoirs and examined them at selected stages of slaughter for indicator bacteria and *Campylobacter* spp., found that carcass washing with cold water (without any added chemical compounds) resulted in redistribution of the microbes rather than an actual

reduction in the microbial load. Another study by Smith et al. (2004) also demonstrated little effect of carcass washing on the number of *E. coli* (~0.2 $\log_{10}$ reduction) and observed an actual increase in the proportion of carcasses with *Salmonella* after washing. King et al. (2011) concluded that, while washing may remove some pathogenic bacteria from the carcasses, these microorganisms could be trapped within the skin and feather follicles and cross-contamination could occur via the wash water. To minimize cross-contamination at washing, the removal of contamination may be aided by the use of brushing equipment installed in line with the inside/outside wash or by the addition of approved chemical compounds to the washing water (FAO/WHO, 2011). For instance, carcass washing with a spray application of 20-50 ppm chlorinated water showed a reduction in the prevalence of *Salmonella*-positive broiler carcasses from 25% to 20%. Similarly, carcass washing with 1-3 washes using water containing 25-35 ppm total chlorine resulted in a reduction of the number of *Campylobacter* by about 0.5 $\log_{10}$ cfu/ml in the whole carcass rinse sample (FAO/WHO, 2011).

2.3.8 Carcass Chilling

The purpose of carcass chilling is to reduce the temperature of carcasses to 4 to 7℃ or below as quickly as possible to limit the opportunity for the growth of microorganisms (King et al., 2011; Russell, 2012b). The common methods for carcass chilling include water immersion and air-chilling (FAO/WHO, 2007; FSANZ, 2005). Immersion chilling is common in the USA and Australia and is standard in New Zealand; whereas most plants in Europe use air-chilling (FAO/WHO, 2007; FSANZ, 2005; Russell, 2012b).

The benefits and disadvantages of immersion chilling versus air-chilling have been debated for many years (Russell, 2012b). Russell (2012b) and Mead (2005) pointed out that when an immersion chilling system was operating efficiently,

carcass-associated-bacteria could be partially removed and the overall microbiological quality of carcasses could be improved. A study by Simas et al. (2011) investigated *Salmonella* spp. on broiler carcasses before and after chilling and found there was a significant reduction (78.95%) in the proportion of carcasses with *Salmonella* spp. after chilling. Similarly, Dan et al. (2013) collected poultry carcasses during the main stages of slaughtering and processing to evaluate the load and presence of microorganisms. They observed that the total bacterial counts and *E. coli* counts were reduced during the chilling process (spraying with water at 2℃ for 60-90 min) by 0.54 and 1.13 $\log_{10}$ cfu/ml, respectively. However, improperly operated spray chilling systems can lead to cross-contamination between carcasses (Byrd & McKee, 2005) and there are numerous studies demonstrating an increase in the prevalence of pathogenic bacteria on poultry carcasses after immersion-chilling (FSANZ, 2005). For example, Lillard (1990) evaluated the impact of commercial processing procedures on the bacterial contamination and cross-contamination of broiler carcasses. In that study there was a significant increase in the presence of *Salmonella* on carcasses during the immersion chilling stage. These findings may indicate that chilling potentially could be the most significant point for cross-contamination in broiler processing plants. Similarly, a study by Sarlin et al. (1998) suggested that the chilling tank was a major site for cross-contamination between *Salmonella*-negative and positive flocks. Furthermore, Sanchez et al. (2002) compared the levels and the percentage of carcasses contaminated with *Salmonella* and *Campylobacter* after immersion chilling or air-chilling. They found that these two microorganisms were less frequently found in carcasses that had been air-chilled than in those that had been immersion-chilled.

European Union countries have generally adopted the air-chilling of carcasses due to the higher risk of cross-contamination with immersion chilling (FAO/WHO, 2007; FSANZ,

2005). However, not all research articles support this conclusion. For example, Hänninen (2010) found that air and water-immersion chilling resulted in similar reductions in *Campylobacter* counts. Similarly, an analysis of factors associated with *Salmonella* contamination of broiler carcasses in EU countries did not show an association between the *Salmonella*-contamination result on the carcass and the type of chilling (EFSA, 2010). Fluckey (2003) investigated the microbiological profile of carcasses processed in a plant that used air-chilling. The results showed generic *E. coli* counts of carcass rinse samples were slightly reduced by 0.88 $\log_{10}$ cfu/ml during air-chilling, but no reduction in the numbers of *Campylobacter* or *Salmonella* was found. Miettinen et al. (2001) hypothesized that contamination of the carcasses with *L. monocytogenes* probably occurred during or after the air-chilling step. In addition, with air-chilling, there is no opportunity to use chemical intervention as it is banned in most countries where air-chilling is adopted (Russell, 2012b). In many studies examining the impact of different chilling methods, *L. monocytogenes* has been shown to be more frequently detected during or after carcass chilling (Bouayad et al., 2015; Chiarini et al., 2009; Cox et al., 1997; Escudero-Gilete et al., 2014; Miettinen et al., 2001). The psychrotrophic nature of this microorganism may help explain this phenomenon (Chiarini et al., 2009; Escudero-Gilete et al., 2007).

To minimize cross-contamination with pathogenic bacteria during immersion chilling, chemical substances are often added to the water (Lake et al., 2004). These may include chlorine, acidified sodium chlorite, chlorine dioxide, peroxyacetic acid or trisodium phosphate (Hugas & Tsigarida, 2008). The FSIS permits the addition of up to 50 ppm of chlorine to processing waters in carcass wash applications and chiller makeup water (Russell, 2012b). In Taiwan, according to the Sanitation Standard for Food Cleansers issued by the Ministry of Health and Welfare (MOHW) and the Directive of the use of

food-grade chlorine-containing disinfectants in slaughterhouses issued by BAPHIQ, approved substances for the disinfection of food (including carcasses) include: acidified sodium chlorite solutions (ASC), chlorine dioxide, hypochlorous acid, and sodium hypochlorite. After using these compounds, rinsing with potable water or blanching or cooking is required, to ensure that the final product contains no more than 1 ppm total available chlorine (BAPHIQ, 2021; MOHW, 2017).

A report examining the effects of chlorine on the prevalence of *Salmonella* after immersion chilling by FAO/WHO (2002) highlighted that there was a tendency for a reduced prevalence in carcasses treated with chlorine, as opposed to an increase when chlorine was not used in the chilling water. Similarly a study by Rivera-Perez et al. (2014) demonstrated chilling treatments could be quite efficient in eliminating bacteria and preventing bacterial multiplication if the water flow and chlorine levels were strictly controlled. Another study by Cossi et al. (2010) highlighted the importance of chlorination in the chilling tank to reduce contamination with hygiene indicator microorganisms, such as total coliforms and thermotolerant coliforms. In addition, Yang et al. (2001) inoculated *Salmonella* Typhimurium and *C. jejuni* into chilling water and onto chicken skins to examine the effects of the chlorine level in chilling water that was either 0 or 8 hours old. In the 0-h chilled water containing 10 ppm of chlorine, *C. jejuni* and *S.* Typhimurium were reduced by 3.3 and 0.7 $\log_{10}$ cfu/ml, respectively, and became non-detectable with 30 and 50 ppm of chlorine. In contrast in 8-h chilled water with 10 ppm of chlorine, the reduction of *C. jejuni* and *Salmonella* Typhimurium was <0.5 $\log_{10}$ cfu/ml and the reduction ranged from 4 to 5.5 $\log_{10}$ cfu/ml with 50 ppm of chlorine. However, these authors observed chlorination of chilled water did not effectively reduce the bacteria attached to the skin of the chickens.

For maximizing the reduction of pathogenic bacteria through the addition of chlorine to

chilling water, it is important to maintain a free available chlorine content of 1 to 5 ppm and to keep the pH of the water below 6.5, but above 5 to prevent corrosion of equipment (Russell, 2012b). Sofos et al. (2013) further pointed out that controlling the cross-contamination of carcasses during immersion chilling is also dependent on the amount of water overflowed and replaced per carcass and the ratio of carcasses to water in the chilling tank, with approximately 5 liters per bird recommended by FSANZ (2005). Moreover, as with scalding, flow direction can impact on the performance of immersion chilling, and the water flow should be counter-current and be agitated to assist the cooling and washing action (FAO/WHO, 2011; FSANZ, 2005; Russell, 2012b). In counter-current flow process, water flow is in the opposite direction to the movement of the carcasses, which allows the carcasses to come into contact with the coldest, cleanest water at the exit end of the chilling tank (Byrd & McKee, 2005). In addition, Russell (2012b) stated that organic material (blood, ingesta, digesta, fat, protein) in the chilling tank could reduce the effect of chlorine disinfection, and he recommended using multi-staged tanks (more tanks the better) to wash and dilute such organic material.

2.3.9 Portioning, packing and labeling

In general, most studies have shown that the prevalence of pathogenic bacteria at the end of processing is higher than at the start (FSANZ, 2005). During further processing, contaminated carcasses might cause cross-contamination of equipment and workers, and the level of contamination of the final cut-up products might be even higher than the contaminated carcasses from which they were prepared (FAO/WHO, 2007).

One study reported that the prevalence of *Salmonella* on poultry carcasses or portions after processing ranged between 2 and 62.5%, with the overall prevalence found in 10 countries being 18%, similar to the 23.7% found on Australian whole chicken carcasses

(FSANZ, 2005). Carcasses at portioning and packaging stages have the potential to be contaminated from knives, surfaces and hands or gloves of workers (King et al., 2011). A study by Holder et al. (1997) observed a greater contamination at sites which were touched by rubber gloves (geometric mean 4.33 ± 0.54 $\log_{10}$ cfu/cm^2) when carcasses were hung on the automatic portioning lines than at other sites (3.43 ± 0.62 $\log_{10}$ cfu/cm^2), and they recommended more frequent washing of gloves to reduce this contamination. In addition, the temperature of the cutting and packing room affects the contamination, especially when the temperature becomes favorable for bacterial growth (King et al., 2011). In Taiwan, according to the Establishment Standards for Abattoir, the temperature of the cutting and packing room must be maintained at or below 15℃ through installed air conditioners. However, another report recommended it was important to maintain an air temperature ≤ 10℃ during portioning due to the growth of *Salmonella* which may occur during a typical 8-hour shift when air temperatures are greater than 10℃. Also, the report suggested that contact surfaces should be cleaned and disinfected every 8 hours to ensure that buildup of *Salmonella* did not occur (FSANZ, 2005).

2.3.10 Other factors

In addition to the risk factors mentioned above, some other factors have also been identified that are associated with contamination during poultry processing. The first is the age of the poultry at slaughter. For example, Lawes et al. (2012) reviewed an investigation of prevalence and risk factors for *Campylobacter* in broiler flocks at slaughter and found bird age was a significant risk factor (older birds had a higher risk). Similarly, a study by Habib et al. (2012) investigated factors associated with *Campylobacter* contamination of broiler carcasses and found that the prevalence of *Campylobacter* was positively associated with broiler age. Also, Arsenault et al. (2007)

estimated the prevalence and risk factors for cecal colonization by *Salmonella* spp. and *Campylobacter* spp. in chickens and found that older birds had an increased risk of *Campylobacter* colonization. They considered this could be either related to an increase in the risk of colonization with exposure time, or an increase in the probability of detecting infection due to an increased within-flock prevalence of *Campylobacter* with time. In contrast analysis of factors associated with *Salmonella* contamination of broiler carcasses in one study demonstrated that the age of broilers did not affect the prevalence of *Salmonella* (EFSA, 2010).

The month or season of sampling or slaughtering can also influence bacterial carriage. Habib et al. (2012) observed that both the prevalence and concentration of *Campylobacter* were significantly higher in carcasses sampled during June and September in Belgium, than on carcasses sampled in January. Another study by Powell et al. (2012) demonstrated that birds processed in the summer months (June, July, August) had an increased risk of carcass contamination with *Campylobacter*. This was supported by the findings of Lawes et al. (2012). These authors suggested that the seasonal pattern of the carriage of campylobacters by poultry could be due to various environmental factors, such as temperature, humidity, sunlight, low rainfall, and season of processing. In contrast, no seasonal effect on the risk of *Salmonella* contamination of broiler carcasses has been reported (EFSA, 2010).

The slaughter capacity of the abattoir also affects contamination. An investigation in the EU showed the risk for *Salmonella*-contaminated carcasses increased with increased slaughter capacity of the abattoir. This could be linked to larger abattoirs processing more flocks and batches of birds, thus increasing the opportunity for cross-contamination (EFSA, 2010). However, a study by Cossi et al. (2010) found that the slaughter capacity of the abattoir was not associated with *Salmonella* on chicken

carcasses. In general, larger abattoirs also have more automated equipment and Tsola et al. (2008) observed that the automation of slaughter plants led to a reduction in the level of contamination. In addition, the slaughtering of a single species of poultry has been identified as a significant risk factor for increasing the prevalence of *Salmonella* spp. on broiler carcasses by Hue et al. (2011). These authors indicated that when several species were slaughtered in the same abattoir, sanitary measures adopted, such as disinfection, drainage of the scalding bath, or changing of gloves or operators between species could help minimize cross-contamination between the different species. In contrast, these practices were less frequently adopted when only broiler chickens were slaughtered.

In conclusion, there are many risk factors associated with contamination of poultry carcasses during their processing. The incidence and level of contamination are dependent on the differences in the practices and facilities between the abattoirs and the status of the processed birds/flocks/batches, as well as the operations performed during slaughtering and processing. This information can be used by slaughter plants and meat safety authorities to develop preventive measures during slaughter operations, thus yielding more wholesome products for consumers.

Although a nation-wide microbiological screening program of carcasses in Taiwanese poultry abattoirs has been undertaken since 2006, little is known about the risk factors associated with contamination of poultry carcasses during their processing. In the next chapter, the prevalence of *Salmonella* in Taiwanese broiler carcasses at slaughter and risk factors associated with the presence of *Salmonella* in batches of broiler carcasses at processing are described.

CHAPTER 3: Prevalence and Risk Factors for *Salmonella* spp. Contamination of Slaughtered Chickens in Taiwan

Preface

Salmonella is a common cause of foodborne infectious disease throughout the world. Contaminated poultry and poultry products have been identified as a major source of human salmonellosis, with contamination commonly occurring at abattoirs during the slaughtering and processing stages. Prior to the research reported in this thesis there was little information available regarding contamination of broilers at slaughter with *Salmonella* spp. in Taiwan, even though poultry meat is the most commonly consumed meat in the country. This chapter presents the results of a nationwide investigation of *Salmonella* spp. in Taiwanese broiler carcasses processed at 45 abattoirs. The prevalence of *Salmonella* spp. and risk factors associated with their presence in broiler carcasses during processing at these abattoirs were explored to highlight the role of the abattoir's environmental conditions and bird type on contamination during processing.

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Abstract

The present study was designed to estimate the prevalence of *Salmonella* contamination in Taiwanese broilers at slaughter and to identify risk factors associated with the presence of *Salmonella* in processed batches of broilers. Carcass rinse samples from 362 batches of broilers were collected from 45 chicken abattoirs in Taiwan between February 2013 and November 2014. Univariate analyses and multivariable logistic regression analyses were conducted to identify putative risk factors for contamination. *Salmonella* was detected in 32.6% (95% CI: 30.4-34.8) of individual broilers and 56.4% (95% CI: 51.1-61.5) of the sampled batches. The multivariable logistic regression model identified season (July to November) (OR = 1.95; 95% CI: 1.2-3.2) as increasing the risk of infection. Abattoirs in the southern region (Taichung and Kaohsiung) (OR = 0.45; 95% CI: 0.3-0.8); batches scalded for > 90 s (OR = 0.2; 95% CI: 0.1-0.3) and batches of commercial white broilers (BR) (OR = 0.21; 95% CI: 0.1-0.4) all had a decreased risk of contamination compared to abattoirs from the northern region, scalding < 90 s and Taiwan native chickens (TNC), respectively. This study highlights the influence of environmental conditions and poultry breed on the risk of *Salmonella* contamination of chickens during slaughter.

3.1 Introduction

Salmonella is a common cause of foodborne infectious disease throughout the world, resulting in more than 100,000 human deaths globally each year (Dougan et al., 2011; Majowicz et al., 2010; WHO, 2013). Contaminated poultry and poultry products have been identified as a major food source responsible for human salmonellosis (EFSA, 2014; FAO/WHO, 2007; Finstad et al., 2012; Mead et al., 2010; Morar et al., 2014) with contamination commonly occurring at abattoirs during the slaughtering and processing stages (Cardinale, Tall, et al., 2005; Chotinun et al., 2014; Goksoy et al., 2004; Lin et al., 2009; Rasschaert et al., 2007; Rasschaert et al., 2008; Zutter et al., 2005). Risk factors for carcass contamination with *Salmonella* have been identified as: *Salmonella* status of the flock/birds prior to slaughter; management conditions during the production of the poultry; and level of hygiene during processing (Arsenault et al., 2007; Brizio & Prentice, 2015; Cardinale, Tall, et al., 2005; FSANZ, 2005). However, currently there is little information available regarding *Salmonella* contamination in poultry in Taiwan, even though it is the second most frequently consumed meat, with an annual *per capita* consumption of 31.9 kg (42.2% of all meat consumption) (COA, 2014b).

The aims of the present study were to estimate the prevalence of *Salmonella* in Taiwanese broilers at slaughter and to identify risk factors associated with the presence of *Salmonella* in batches of broilers at processing.

3.2 Materials and methods

3.2.1 Selection of abattoirs

The study was conducted between February 2013 and November 2014. In Taiwan, there are 57 chicken abattoirs which process >100,000 birds per annum; however, 12 of these were unable to be sampled due to work force demands. Chickens from the remaining 45 abattoirs were sampled for this study (Figure 3.1). These 45 abattoirs are responsible for

processing 91.6% of all broilers slaughtered each year in Taiwan (BAPHIQ, 2015b). Chickens processed at these selected abattoirs consist primarily of two distinct types; commercial white broilers (BR) (approximately 64%), and Taiwan native chickens (TNC) (approximately 34%). The differences between TNC and BR, and the main processing steps undertaken at these abattoirs have been detailed in Lin et al. (2020). Due to the different types of chickens slaughtered and the capacity of production of each abattoir, the equipment and processes used between the sampled abattoirs differed slightly. However, all equipment and processes in these abattoirs are required to meet the sanitary, safety and animal welfare regulations (https://www.baphiq.gov.tw/en/ws.php?id=6009) set by the Bureau of Animal and Plant Health Inspection and Quarantine (BAPHIQ), Taiwan.

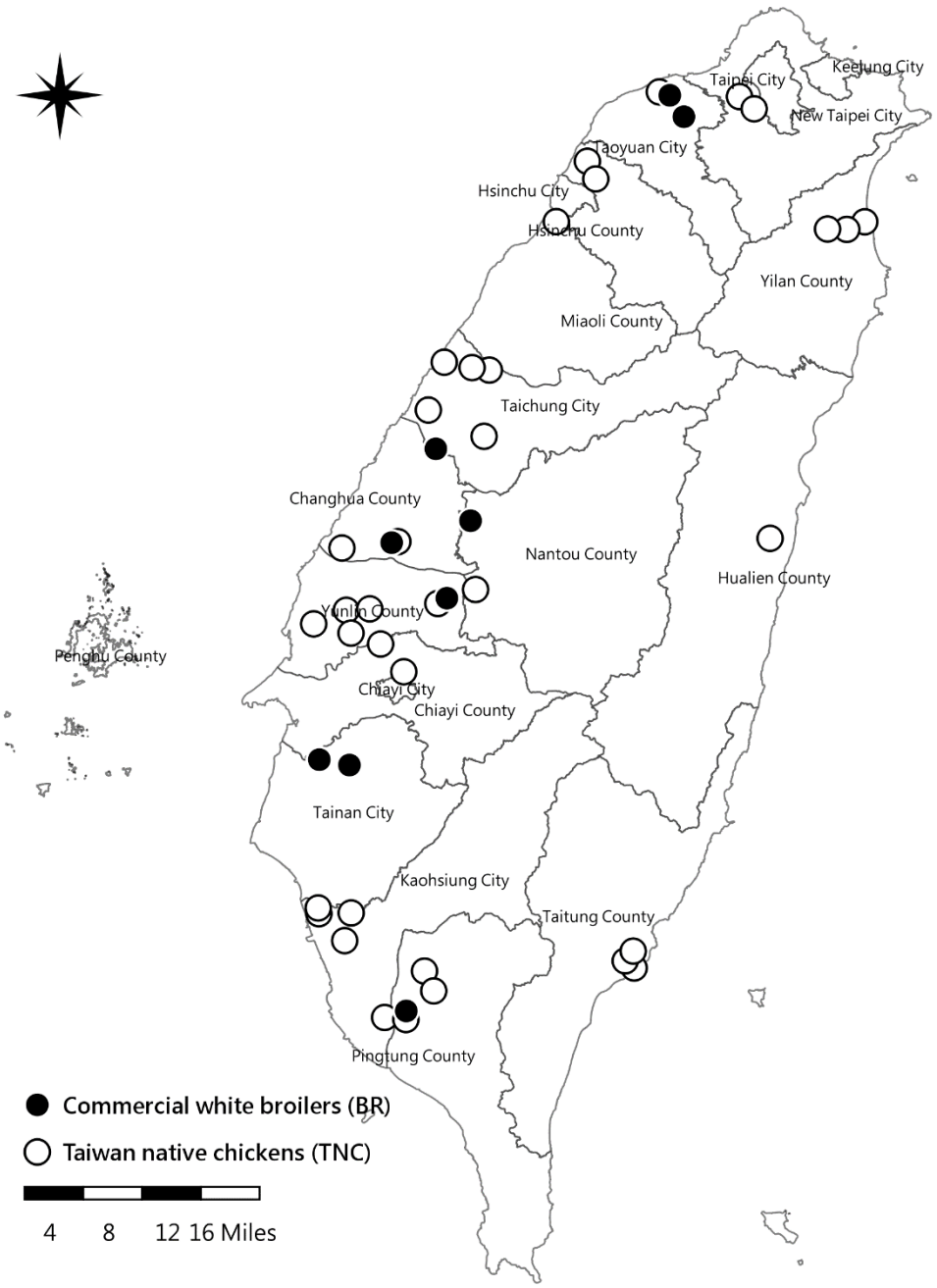

Figure 3.1 Location of the 45 abattoirs sampled in this study.

3.2.2 Sample and data collection

The number of batches sampled from each abattoir was based on the proportion of the annual number of chickens slaughtered at that abattoir compared with the national annual total of the previous year. Five carcass rinse samples were collected from each batch. A batch refers to a group of chickens from the same farm that was

slaughtered/processed during the same shift. Using Epitools (https://epitools.ausvet.com.au) with an assumed animal level (carcass) prevalence of 35%, 95% confidence intervals, and 5% precision a sample size of 350 batches of 5 birds each was deemed appropriate (1650 carcasses). Samples from a total of 362 batches (1810 carcasses) were subsequently collected.

Sampling was performed by the official meat inspection veterinarians at the respective abattoirs. Prior to sampling, the sampler used EXCEL RANDBETWEEN function to generate 5 random whole numbers between 1 and the total number of processed birds of a selected batch. The five carcasses corresponding to these randomized numbers were selected for sampling after they had passed through the chilling tank. Carcass rinse samples were collected as per the methodology outlined in Lin et al. (2020). The characteristics of each of the sampled batches were recorded for univariate analysis, including; sampling year and season, bird type, processing conditions, as well as abattoir information (Table 3.1).

Table 3.1 Univariate analyses of relationship between exposure variables and *Salmonella* status in batches of broilers at slaughter at 45 abattoirs in Taiwan

Variable	Level	Number of batches	% positive	OR (95% CI)	*p*-value
Sampling year	2014	199	55.8	0.95 (0.6-1.4)	0.81
	2013	163	57.1	1.0	
Sampling season	warm (May - Nov.)	235	60.9	1.68 (1.1-2.6)	0.02
	cool (Dec. - Apr.)	127	48.0	1.0	
Branch of BAPHIQ [a]	Southern	264	52.3	0.53 (0.3-0.9)	0.01
	Northern	98	67.3	1.0	
Slaughter speed (birds/hour)	> 2100	211	41.2	0.20 (0.1-0.3)	< 0.01
	≤ 2100	151	77.5	1.0	
Abattoir age (years)	> 9	233	43.8	0.21 (0.1-0.3)	< 0.01
	≤ 9	129	79.1	1.0	
Number of birds slaughtered annually	≥ 1million	310	52.6	0.30 (0.1-0.6)	< 0.01
	< 1million	52	78.8	1.0	
Bird type	BR	177	37.9	0.21 (0.1-0.3)	< 0.01
	TNC	185	74.1	1.0	
Total number of workers	> 40	190	38.4	0.20 (0.1-0.3)	< 0.01
	≤ 40	172	76.2	1.0	
Certified Agricultural Standards	Yes	224	42.0	0.18 (0.1-0.3)	< 0.01
	No	138	79.7	1.0	
Certified ISO 22000	Yes	161	48.4	0.56 (0.4-0.9)	0.01
	No	201	62.7	1.0	
Time in lairage (minutes)	> 120	68	63.2	1.42 (0.8-2.4)	0.20
	≤ 120	294	54.8	1.0	
Types of scalding	Tunnel	221	48.0	0.40 (0.3-0.6)	< 0.01
	Tank	141	69.5	1.0	
Time of scalding (seconds)	> 90	127	33.1	0.22 (0.1-0.4)	< 0.01
	≤ 90	235	68.9	1.0	
Temperature of scalding (°C)	≥ 62	156	71.2	3.00 (2.0-4.7)	< 0.01
	< 62	206	45.1	1.0	
Type of plucking process	tunnel, or tank & tunnel	204	39.7	0.19 (0.1-0.3)	< 0.01
	Tank	158	77.8	1.0	
Time of plucking (seconds)	> 60	112	66.1	1.80 (1.1-2.9)	0.01
	≤ 60	250	52.0	1.0	
Evisceration type	Manual	124	75.8	3.65 (2.2-5.9)	< 0.01
	Automated	238	46.2	1.0	
Types of viscera hanging	detached from carcass	93	47.3	0.61 (0.4-1.0)	0.04
	attached to carcass	269	59.5	1.0	

Continued on following page

Table 3.1 Univariate analyses of relationship between exposure variables and *Salmonella* status in batches of broilers at slaughter at 45 abattoirs in Taiwan (Continued)

Variable	Level	Number of batches	% positive	OR (95% CI)	*p*-value
Total number of chilling tanks	> 3	71	60.6	1.24 (0.7-2.1)	0.43
	≤ 3	291	55.3	1.0	
Concentration of chlorine in chilling tank water (ppm)	≥ 30	193	43.0	0.30 (0.2-0.5)	< 0.01
	< 30	169	71.6	1.0	
Clean with hot water	Yes	148	43.9	0.42 (0.3-0.6)	< 0.01
	No	214	65.0	1.0	

OR, Odds ratio; CI, confidence interval.

[a] Branch of BAPHIQ where the abattoir is located. The northern region includes the abattoirs belong to the BAPHIQ Branches of Keelung and Hsinchu; and the southern region includes the abattoirs belong to the BAPHIQ Branches of Taichung and Kaohsiung.

3.2.3 Bacteriology

Bacteriology was adopted as per the methodology outlined in Lin et al. (2020). Briefly, samples were processed according to the methods described in the U.S. Department of Agriculture/Food Safety and Inspection Service (USDA/FSIS) Microbiology Laboratory Guidebook (FSIS, 2014) and recommended by the Association of Official Agricultural Chemists (AOAC) International (Andrews, 1998). Samples involved primary enrichment by the addition of 30 mL of BPW to 30 mL of poultry carcass rinse fluid sample, and then all samples were enriched in BPW at a 1:10 dilution and by incubating at 35°C for 20-24 h, following which a 50 µl aliquot of the primary enrichment culture was inoculated into 5 mL of Rappaport-Vassiliadis broth (Oxoid) for secondary (selective) enrichment and incubated at 42°C for 24 h (Andrews, 1998). A loopful of Rappaport-Vassiliadis culture was streaked onto Xylose Lysine Desoxycholate (Difco, BD), Hektoen enteric agar (Difco, BD) and *Salmonella* identification agar (CHROmagar Microbiology, Paris) and incubated at 35°C for 24 h. Colonies with typical *Salmonella* characteristics were confirmed with API 20E (bioMe´rieux) biochemical assays. Isolates identified as *Salmonella* were serotyped by

slide and tube agglutination tests with *Salmonella* polyvalent O and H antisera (Difco, BD) according to the Kauffmann–White scheme (Grimont & Weill, 2007). One colony from each sample was serotyped. Samples with one or more colonies with typical *Salmonella* characteristics were classified as positive. The prevalence was reported at the individual bird and batch levels. A batch was classified as positive when one or more samples from the individual birds that made up that batch were *Salmonella* positive.

3.2.4 Statistical analysis

Initially, univariate analyses using the Pearson χ^2 test were conducted and variables with a $p \leq 0.2$ were offered to a multivariable logistic regression model (Mickey & Greenland, 1989). Because of the likely presence of additional variation due to the abattoirs, abattoir was incorporated as a random effect in the initial logistic-normal multiple regression model (Curtis et al., 1993). The goodness-of-fit for the random effects was evaluated by comparing the deviance and the change in the degrees of freedom (Hosmer & Lemeshow, 2000). As the abattoir random effect was not significant, a standard logistic regression analysis was undertaken. The final logistic model was developed using a backward elimination process and examination of the Wald statistic for each variable. Two-way interaction terms among the explanatory variables were examined after identification of the reduced set main effects. Each interaction was added sequentially to the model and the significance assessed. The suitability of the model was assessed using the Hosmer and Lemeshow test (Hosmer & Lemeshow, 2000). Data were analyzed using SPSS (Version 21.0; IBM Corp, Armonk, New York, USA), Egret (Version 2.0.3, Cytel, Inc., Cambridge, Massachusetts, USA), and Statistix (Version 9, Analytical Software, Tallahassee, Florida, USA) statistical software and a p-value < 0.05 was considered statistically significant in the final model

(Ansari-Lari et al., 2014).

3.3 Results

3.3.1 Prevalence of *Salmonella*

Of the 1810 carcass rinse samples (from 362 batches) collected, 1808 were included in the analysis (two samples were spilt during transport and hence were unsuitable for culture). *Salmonella* was detected in 589 of the 1808 individual samples (32.6%, 95% CI: 30.4-34.8, with 155/883 of BR and 434/925 of TNC positive). The prevalence was significantly different between samples collected from BR and TNC carcasses (17.6%, 95% CI: 15.1-20.2 and 46.9%, 95% CI: 43.7-50.1; respectively) ($p < 0.001$). *Salmonella* were detected in 204 of the 362 batches collected (56.4%, 95% CI: 51.1-61.5, with 67/177 of BR and 137/185 of TNC positive). The prevalence was also significantly different between batches taken from BR and TNC (37.9%, 95% CI: 30.7-45.4 and 74.1%, 95% CI: 67.1-80.2; respectively) ($p < 0.001$).

3.3.2 Distribution of *Salmonella* serotypes

Of the 589 *Salmonella* isolates 578 were characterized into 24 serotypes, with the remaining 11 isolates (1.9%) non-typeable (Table 3.2). The most common serotypes detected were *S.* Albany (n = 187, 31.8%, 95% CI: 28.0-35.7, of which 34 were from BR and 153 from TNC), *S.* Enteritidis (n = 80, 13.6%, 95% CI: 10.9-16.6, of which 41 were from BR and 39 from TNC) and *S.* Typhimurium (n = 57, 9.7%, 95% CI: 7.4-12.4, of which 19 were from BR and 38 from TNC).

Table 3.2 The serotypes of *Salmonella* isolates from all 45 abattoirs sampled in Taiwan

Serotype	Count (n = 589)	% isolates (95% CI)
Albany	187	31.7 (28.0-35.7)
Enteritidis	80	13.6 (10.9-16.6)
Typhimurium	57	9.7 (7.4-12.4)
Schwarzengrund	44	7.5 (5.5-9.9)
Montevideo	38	6.5 (4.6-8.7)
Tennessee	38	6.5 (4.6-8.7)
Hadar	35	5.9 (4.2-8.2)
Livingstone	24	4.1 (2.6-6.0)
Newport	16	2.7 (1.6-4.4)
1,4,[5],12:i:-	11	1.9 (0.9-3.3)
Livingstone var. O14+	8	1.4 (0.6-2.7)
Agona	7	1.2 (0.5-2.4)
Derby	6	1.0 (0.4-2.2)
Cremieu	5	0.8 (0.3-2.0)
Virchow	5	0.8 (0.3-2.0)
Cerro	4	0.7 (0.2-1.7)
Kentucky	4	0.7 (0.2-1.7)
Potsdam	2	0.3 (0-1.2)
Stanley	2	0.3 (0-1.2)
Bardo	1	0.2 (0-0.9)
Haardt	1	0.2 (0-0.9)
Mbandaka	1	0.2 (0-0.9)
Muenster	1	0.2 (0-0.9)
Vejle	1	0.2 (0-0.9)
Non-typeable	11	1.9 (0.9-3.3)

3.3.3 Risk factors analysis

Eighteen of the 21 variables analyzed showed an association ($p < 0.2$) with *Salmonella* isolation (Table 3.1). Of these, four remained in the final logistic regression model: season of sampling; branch of BAPHIQ to which the abattoir belonged; duration of scalding; and bird type (Table 3.3). The odds for the presence of *Salmonella* were higher in batches sampled in the warm season (July to November) (OR = 1.98; 95% CI: 1.2-3.3) than in batches sampled in the cooler season. Contaminated batches were less likely to be present in abattoirs located within the southern region (BAPHIQ Branches of

Taichung and Kaohsiung) (OR = 0.44; 95% CI: 0.2-0.8) than those from the northern region (BAPHIQ Branches of Keelung and Hsinchu) of Taiwan. Similarly, fewer batches sampled from abattoirs with scalding times > 90 s were positive for *Salmonella* (OR = 0.42; 95% CI: 0.2-0.9) compared to shorter scalding times (≤ 90 s). Fewer batches of BR (OR = 0.36; 95% CI: 0.2-0.6) were contaminated with *Salmonella* than batches of TNC (Table 3.3). The model was shown to be a good fit of the data (Hosmer and Lemeshow goodness-of-fit test: χ^2 = 7.611, d.f. = 8, p = 0.472).

Table 3.3 Final multivariable logistic regression model for contamination of carcasses with *Salmonella* spp. in 362 batches of broilers slaughtered in 45 abattoirs in Taiwan

Variables	β	*Salmonella*-positive batches (%)	OR (95% CI)	*p*-value
Sampling season	0.681			
warm		60.9	1.98 (1.2-3.3)	0.01
cool		48	1	
Abattoir location	-0.814			
southern		52.3	0.44 (0.2-0.8)	0.01
northern		67.3	1	
Time of scalding	-0.87			
> 90 s		33.1	0.42 (0.2-0.9)	0.02
≤ 90 s		68.9	1	
Bird type	-1.016			
BR		37.9	0.36 (0.2-0.6)	< 0.01
TNC		74.1	1	
Bird type * Time of scalding	-1.641		0.19 (0.1-0.6)	< 0.01
Constant	1.505			

OR, Odds ratio; CI, confidence interval.

Hosmer and Lemeshow goodness-of-fit test: χ^2 = 7.611, d.f. = 8, p = 0.472

3.4 Discussion

3.4.1 Prevalence of *Salmonella*

At the individual bird level, 32.6% (95% CI: 30.4-34.8) of broilers were contaminated

with *Salmonella* spp. with significantly more TNC (46.9%) contaminated than BR (17.6%). There is little published information about contamination of TNC as they are rarely produced or consumed in other countries. The prevalence in BR carcasses in this study was higher than that reported in New Zealand (2%) (Lake et al., 2004), the United States of America (3.9%) (FSIS, 2015), and Thailand (9%) (Padungtod & Kaneene, 2006), although it was similar to that reported in European Union (EU) countries (up to 22.7%) (EFSA, 2014), but lower than that reported in the Republic of Korea (42.7%) (Bae et al., 2013). However, caution needs to be made when comparing results with other studies, due to differences in sampling and isolation methods. Chen et al. (2004) used similar methods to study rinse samples from BR carcasses from 14 abattoirs in Taiwan to investigate the prevalence of pathogenic microorganisms. In their study the prevalence of *Salmonella* spp. were 4.5, 0.7 and 1.7% in 2000, 2001 and 2002, respectively. The current findings indicate a higher contamination level in 2013 and 2014 in Taiwan. Unfortunately Chen et al. (2004) did not record the characteristics of the carcass samples or information about the abattoirs, so it is not clear why the *Salmonella* prevalence between the two studies is substantially different.

3.4.2 Distribution of *Salmonella* serotypes

In the present study, the predominant serotype, *S.* Albany (31.8%), was the same as that reported by Lin et al. (2008) who isolated *Salmonella* spp. from the liver, gall bladder and cecal contents of BR and TNC in Taiwan. Similarly, Lin et al. (2020) reported *S.* Albany as the predominant (41.7%) serotype detected in samples from BR and TNC in Taiwan. The high level of carcass contamination with *S.* Albany in this study may indicate cross contamination with fecal material from *Salmonella* infected birds during processing. However, the predominant serotypes of *Salmonella* on chicken carcasses vary between studies and countries. For example, the prevalent serotypes detected from

chicken carcasses in the EU in decreasing order were *S.* Infantis, *S.* Enteritidis, and *S.* Kentucky (EFSA, 2010); *S.* Sofia, *S.* Typhimurium, and *S.* Infantis in Australia (FSANZ, 2010); *S.* Paratyphi B, *S.* Hvittingfoss, and *S.* Muenster in Colombia (Rodriguez et al., 2015); and *S.* Typhimurium, *S.* Hadar, and *S.* Rissen in Korea (Lee et al., 2016). In contrast, the most prevalent serotypes detected in the present study were: *S.* Albany (31.7%), *S.* Enteritidis (13.6%), and *S.* Typhimurium (9.7%) (Table 3.2). These three serotypes accounted for 64% of human salmonellosis infections in Taiwan in 2013 and 2014 (Chiou et al., 2019). This finding indicates that the *Salmonella* serotypes affecting humans in Taiwan are consistent with those found at the sampled abattoirs, highlighting that contaminated chicken meat is one source of human salmonellosis. However, the confirmation of sources of salmonellosis in humans should be informed by more detailed strain identification, such as whole-genome sequencing or elucidation of exposure factors (Koutsoumanis et al., 2019).

3.4.3 Risk factors analysis

Four main risk factors were identified as being associated with carcass contamination of broilers. The present study found an increased prevalence during the warm season (July to November), which is consistent with results of other international studies (Ayaz et al., 2010; Cohen et al., 2007; Huang et al., 2016; Williams et al., 2014; Zdragas et al., 2012). This is likely due to the mesophilic nature of salmonellae, with optimum growth occurring between 35 and 43°C (Ellermeier & Slauch, 2006; FAO/WHO, 2007), and hot, humid seasons providing conditions conducive for salmonellae growth and survival in the environment (Huang et al., 2016). In contrast, no seasonal effect on the risk of *Salmonella* contamination of chicken carcass samples was observed from an EU survey, but abattoir management procedures (not investigated) could have masked an association at the batch level (EFSA, 2010). A six-year study investigating the levels of

contamination with *Salmonella* of raw chickens at retail outlets in the United Kingdom found a significant peak in the first quarter of each year (January to March); however, no reason was proposed as to why this might have occurred (Wilson, 2002).

Abattoirs under the supervision of the southern branches of BAPHIQ (Taichung and Kaohsiung) had fewer *Salmonella* isolations than from the northern abattoirs in this study. This may be associated with the duration of transport from farms to the abattoirs. Chicken farms are predominantly located in the south of Taiwan and most poultry slaughtered at the northern abattoirs are sourced from farms located in south Taiwan, increasing transportation distance and time (COA, 2014a). Extended transportation times could increase the likelihood of birds experiencing stress, fecal excretion and ultimately higher levels of cross contamination prior to arriving in lairage (FAO/WHO, 2002). A study by Mainali et al. (2009) also found that longer transport times were associated with an increased prevalence of *Salmonella* in neck skin samples of broiler chickens. Due to fasting prior to transportation, it is possible that longer transport times could increase the likelihood of birds consuming contaminated materials (e.g. feces) during transport.

During processing, poultry carcasses are immersed in scald tanks to loosen feathers and facilitate plucking (FSANZ, 2005). In general, when scalding is undertaken properly, lower levels of pathogenic (FAO/WHO, 2007) and indicator bacteria (Zweifel et al., 2015) are present on carcasses. The temperature used for scalding in Taiwan is usually between 43 and 66°C, depending upon the type and size of chickens slaughtered and the duration of scalding. In this study chickens were scalded for between 20 and 140 s, and shorter scalding times generally corresponded to higher scalding temperatures. Not surprisingly a longer duration of scalding (> 90 s) was associated with a decreased risk of *Salmonella* contamination. Yang et al. (2001) also observed reduced numbers of

Salmonella both on chicken skin and in the scalding water when the duration of scalding was increased. Along with extending the duration of scalding, higher scalding temperatures are deemed to provide a beneficial reduction in the abundance of bacteria present on carcasses (FSANZ, 2005). However, in the univariate analysis a high scalding temperature (≥ 62°C) was associated with an increased odds of *Salmonella* detection from carcasses. This may be linked to higher scalding temperatures resulting in a greater propensity for tearing of the carcass skin and damage to the epidermis during plucking which subsequently makes it easier for *Salmonella* to adhere to carcasses (FSANZ, 2005; Löhren, 2012; Zweifel et al., 2015). A study conducted by Kim et al. (1993) further found that chicken carcass skins scalded at 52 and 56℃ retained most of the epidermis, whereas skins scalded at 60℃ began to lose most of the epidermal layers during scalding and the dermal surface was exposed after plucking. They observed that the number of *Salmonella* attached to skins processed at 60°C, as measured by scanning electron microscopy, was 1.1 to 1.3 logs higher than when processed at 52 and 56℃, respectively. Furthermore, Russell (2012b) pointed out that the fat under the chicken skin is unsaturated, and becomes liquefied when carcasses are placed in a scalding tank at a temperature of 54.5℃ for only 2 min. Liquefied fat can drain out from under the skin as carcasses are transported to the chill tank where the temperature of carcasses will reduce to 4-7℃ gradually from > 35℃ over a 30 min period. During this time liquefied fat can also drain out into the chilling water. Therefore, scalding at a higher temperature can result in the chilling water containing greater amounts of fat, reducing the effectiveness of the chlorine disinfectant (Russell, 2012b), and potentially allowing *Salmonella* to survive the chilling process. However, the observed result in the univariate analysis indicates that the effect of scalding temperature was confounded by other variables, and the results from the multivariable

model are more appropriate.

Of the chickens slaughtered in Taiwan for human consumption, the majority are BR (60.4% - 186 million/year) or TNC (33.5% - 103 million/year) with the remaining being culled layers (BAPHIQ, 2015b). The present study showed a lower contamination in BR which may arise from a lower susceptibility in this type of bird. A similar result was reported in Taiwan by Lin et al. (2009) who found a lower isolation of *Salmonella* from the cecal contents and liver and gall bladder samples of BR (43.7%) than TNC (62.7%). Arsenault et al. (2007) detected that the age of processing of the chickens influenced the prevalence of cecal colonization with *Salmonella* spp. and *Campylobacter* spp., with older birds having a higher prevalence. They concluded that this could be due to an increase in the risk of colonization over time through exposure to *Salmonella*-infected chickens. Their findings support those of the current study, with TNC being older than BR (12-15 weeks compared with 5-6 weeks, respectively) (COA, 2014a). However, the lower contamination in BR than TNC may also be due to other uncontrolled confounding variables. For example, TNC are slaughtered at an older age and if the incidence rate for new *Salmonella* infections is constant with age, than this would inevitably result in a higher prevalence in older birds. Furthermore, *Salmonella* contamination may be associated with abattoir processing methods, as well as nutrition, husbandry, intercurrent disease and vaccination of the sampled flocks that potentially could contribute to the observed outcome. It is suggested that future studies should collect and analyze data for these potential effects. A further limitation of the current study was that batches presented to each abattoir were only sampled on one day at one processing point in the abattoir. In the future birds presented to abattoirs should be sampled at multiple processing points to understand the impact processing stage has on the prevalence of *Salmonella* and on multiple days to determine variation in the

prevalence with time.

The BR industry in Taiwan is based on birds bred for a high feed conversion rate so that birds reach market weight sooner at a more uniform weight. Brizio and Prentice (2015) reported that if birds were of an even weight range (size) there was reduced contamination levels during evisceration. Similarly, Malher et al. (2011) found that a similar weight range of birds in a batch could reduce the risk of contamination of broiler carcasses by enteric bacteria carried by the birds. In contrast, the marketing size of TNC varies even within the same batch, and Nunes (2013) reported that flocks with non-standardized sizes had a greater proportion of carcasses with fecal and bile contamination during automatic evisceration operations than batches containing uniform sized birds.

3.5 Conclusions

This study detected a high proportion of broiler carcasses in Taiwan contaminated with *Salmonella* spp. at the individual (32.6%) and batch level (56.4%) with more TNC contaminated than BR. Serotyping highlighted similarities in isolates cultured in this study from those commonly isolated from humans in Taiwan, supporting the belief that contaminated chicken meat is one source of *Salmonella* infection of humans in the country. Analysis of the risk factors indicated that season, the location of the abattoir, duration of scalding, and bird type were associated with *Salmonella* contamination of chicken carcasses at abattoirs. These findings can be used to implement targeted measures to better control cross contamination of carcasses with *Salmonella* spp. in the future.

3.6 Declaration of Competing Interest

The authors report no declarations of interest.

3.7 Acknowledgments

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CHAPTER 4: Detection of Chicken Carcasses Contaminated with *Salmonella Enterica* Serovar in the Abattoir Environment of Taiwan

Preface

Chapter three described a nationwide study of the prevalence of *Salmonella* spp. in Taiwanese broiler carcasses at slaughter, including an analysis of risk factors associated with the presence of *Salmonella* spp. in batches of broiler carcasses at processing. However, a limitation of the preceding study was that batches presented to each abattoir were only sampled at one processing point in the abattoir. In this chapter the results of sampling batches of chickens from the same farms at multiple processing points at six abattoirs in Taiwan are reported. This study was designed to identify when and where *Salmonella* cross-contamination occurred in chickens processed at these abattoirs. Understanding where contamination occurs in an abattoir is critical to developing preventive measures to reduce carcass contamination which should improve public health through reduced foodborne transmission of *Salmonella* spp..

This manuscript was presented as a poster at the annual Poster Day of the School of Veterinary and Life Sciences, Murdoch University, Perth, Australia on the 6 November 2015 and was awarded the overall best poster for the College of Veterinary Medicine.

The text of this chapter is identical to that in the manuscript published in '***International Journal of Food Microbiology***' except for the reference list which has been combined with references of other chapters and incorporated as one list at the end of the thesis.

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Abstract

Although a nation-wide microbiological screening program of chicken carcasses after chilling in Taiwanese chicken abattoirs has been undertaken since 2006, little is known regarding the potential sources of the *Salmonella* during the slaughter process. The present study provides data on the detection and serotypes of *Salmonella* isolated from broilers during processing and from the environment in six abattoirs in Taiwan. Overall, *Salmonella* were detected in 156 of 622 samples (25.1%; 95% CI: 21.7-28.7) collected. The prevalence of *Salmonella* varied between sampling sites with 5.8, 17.6, 31.3 and 35.5% of cloacal swabs, environmental samples prior to processing, environmental samples during processing and carcass rinse fluid, respectively, being positive ($\chi^2 = 51.3$, $p < 0.0001$). A total of 15 serotypes were identified from the 156 *Salmonella* isolates with *S.* Albany (41.7%) *S.* Schwarzengrund (20.5%), *S.* Kentucky (12.8%) and *S.* Tennessee (5.1%) being the most commonly isolated serotypes. Characterization of 156 isolates by Pulsed-Field Gel Electrophoresis (PFGE) identified 50 PFGE types. Typing confirmed the presence of the same PFGE type at multiple stages during processing including plucking, evisceration, chilling and post-chilling. The abattoir environment and intestinal contents of chickens are important sources of *Salmonella* in broiler chicken abattoirs, with the same PFGE types detected at different stages of processing both before and during slaughtering. It is concluded that *Salmonella* isolates present in the environment and intestinal contents of processed birds survived in the abattoir environment resulting in subsequent carcass contamination along the processing chain including plucking, evisceration, chilling and post-chilling.

4.1 Introduction

Salmonella are one of the leading causes of food-borne illness in humans worldwide (EFSA, 2014; WHO, 2013). Salmonellosis usually results in self-limiting acute gastroenteritis, with clinical symptoms of diarrhea, fever, and abdominal pain occurring 12 to 72 h after infection (WHO, 2013). In some instances, particularly in young, old, and/or immunocompromised patients, these symptoms can become severe and life-threatening (CDC, 2015b; Ellermeier & Slauch, 2006; WHO, 2013). Majowicz et al. (2010) estimated that *Salmonella* spp. cause 93.8 million cases of gastroenteritis and 115,000 deaths globally each year. The Centers for Disease Control and Prevention (CDC) estimate that approximately 1.2 million cases of illness with 450 deaths occur annually in the USA (CDC, 2015b), with the annual cost of these infections estimated at US$3.7 billion (CDC, 2014). In Taiwan, *Salmonella* spp. were responsible for 5.6% of all food-borne outbreaks between 1986 and 1995 (Pan et al., 1997). The annual cost for salmonellosis-related hospitalization has been estimated at US$3.2 million in Taiwan; however, this is likely an underestimate as it did not include costs associated with outpatients, post-hospital care and associated productivity losses (Chen et al., 2012).

Fresh chicken meat is a major food source of human salmonellosis (EFSA, 2014; FAO/WHO, 2007; Mead et al., 2010). Contamination has been demonstrated to mainly occur during the slaughter process (Goksoy et al., 2004; Rasschaert et al., 2008; Zutter et al., 2005), with the most critical processing steps being plucking, evisceration and chilling (Keener et al., 2004). Molecular genotyping methods, such as PFGE, have been used to investigate the relationships between *Salmonella* isolates obtained at abattoirs with those from human cases of salmonellosis and potentially contaminated sites during the slaughter process (Arguello et al., 2013; Botteldoorn et al., 2004; De Busser et al., 2011; van Hoek et al., 2012).

Poultry is the second most commonly consumed meat in Taiwan, with an annual per capita consumption of 31.9 kg (42.2% of all meat consumption) (COA, 2014b). According to a nation-wide Taiwanese study (unpublished) conducted by the Bureau of Animal and Plant Health Inspection and Quarantine (BAPHIQ), the prevalence of *Salmonella* spp. in carcass rinse samples collected after chilling in Taiwanese chicken abattoirs in 2013 was 32.6% (95% CI: 30.4-34.8). However, little is known regarding the sources of the *Salmonella* during the slaughter process in Taiwan. Consequently this study was designed to investigate the sources of *Salmonella* contamination, and when and where *Salmonella* cross-contamination occurred in chicken abattoirs in Taiwan. Understanding where contamination occurs is critical to developing preventive measures, such as using the recommended concentrations and application times of disinfectants (Kudirkienė et al., 2011), to reduce carcass contamination at abattoirs to increase product wholesomeness and reduce human disease.

4.2 Materials and methods

4.2.1 Selection of abattoirs

In 2014 the annual chicken production of Taiwan was approximately 281 million birds, which were processed at 84 abattoirs approved by the Bureau of Animal and Plant Health Inspection and Quarantine (BAPHIQ). Of these chickens slaughtered, the majority were commercial white broilers (BR, 196 million) or Taiwan native chickens (TNC, 75 million) (BAPHIQ, 2015b). The TNC represents a number of locally developed slow-growing breeds favored by Taiwanese consumers (Cheng et al., 2008). Genetically, they are not native at all, but have been crossed with foreign breeds, notably French chickens around 1980 (Lee, 2006) with the characteristics of a single comb, red to black feathers, and black shanks (Chao & Lee, 2001). The TNC takes approximately 80-110 days to achieve a market live weight of 2-2.5 kg. In contrast, the

BR requires only 35-40 days to achieve a live weight of approximately 1.5 kg (Cheng et al., 2008; Chumngoen et al., 2016; Huang et al., 2007).

Samples for this study were collected from six abattoirs in Taiwan. These abattoirs were selected based on the different type of broilers processed, and were representative of the slaughter speeds, capacities, evisceration systems and geographical locations of abattoirs in Taiwan (Table 4.1). The main processing steps of these abattoirs include holding, ante-mortem inspection, unloading and hanging, stunning, bleeding, scalding and plucking, venting and evisceration, post-mortem inspection, carcass washing and chilling, followed by packing and labeling with an inspection certificate. All equipment and processes in the abattoirs are required to meet the sanitary, safety and animal welfare regulations set by the BAPHIQ. Annual numbers of birds processed at these abattoirs varied from 150,000 to 21,000,000 broilers, and their total annual production was around 46 million broilers representing approximately 16% of the national production (2014 slaughter data) (BAPHIQ, 2015b).

Table 4.1 Information on the six abattoirs sampled

Abattoir[$]	Chicken type[#]	Location in Taiwan	Evisceration type	Chlorination in chilling tank	Slaughter speed (head/h)	Annual production
A	BR	North	Automated	Yes	6,300	17,000,000
B	BR	South	Automated	Yes	8,400	21,000,000
C	BR	South	Automated	Yes	4,000	4,500,000
D	TNC	Central	Automated	Yes	2,000	2,000,000
E	TNC	Central	Manual	No	2,000	1,500,000
F	TNC	Central	Manual	No	50	150,000

[$]Abattoir A is located in Taoyuan City, northern Taiwan; abattoir B is located in Tainan City, southern Taiwan; abattoir C is located in Pingtung County, southern Taiwan; abattoir D and E are located in Yunlin County, central Taiwan; abattoir F is located in Changhua County, central Taiwan.
[#]BR: commercial white broiler; TNC: Taiwan native chicken.

4.2.2 Sample and data collection

Samples were collected for this study in July and August 2014. Sampling was

performed by the official meat inspection veterinarians at each abattoir. All samples for an individual abattoir were collected on the same day but different abattoirs were sampled on different days. Samples were collected from the environment, carcass rinse fluid samples and cloacal swabs (Table 4.2). Pre-slaughter environmental samples were collected 1 h prior to the commencement of slaughter. Samples from the birds and further environmental samples were collected during the processing of the first batch of broilers at each abattoir. Sterile gloves were worn by operators during sampling and were changed between samples. Sponges, water samples and chicken rinse fluid samples were stored at 4℃ and transported to the Agricultural Technology Research Institute in Taiwan for microbiological analysis within 24 h of collection.

Table 4.2 Results of the samples collected at different processing stages and the frequency of detection of *Salmonella* spp. in six abattoirs

Sample stages	Number of samples (+ve/total samples)						Number positive/total collected (%; 95% CI)
	A	B	C	D	E	F	
Environment-before processing							22/125 (17.6; 11.4-25.4)
Plucking machine	3/3	2/3	0/3	1/3	2/3	3/3	11/18 (61.1; 35.7-82.7)
Evisceration machine/table	0/3	0/3	0/3	1/3	1/3	0/3	2/18 (11.1; 1.4-34.7)
Gloves of a carcass trimmer	0/3	0/3	0/3	2/3	1/2	0/3	3/17 (17.6; 3.8-43.4)
Shackles/conveyer belt	0/3	0/3	0/3	1/3	0/3	3/3	4/18 (22.2; 6.4-47.6)
Chilling tank inside surface	0/0	0/9	0/3	1/6	0/3	0/3	1/24 (4.2; 0.1-21.1)
Chilling tank water	0/3	1/12	0/3	0/6	0/3	0/3	1/30 (3.3; 0.1-17.2)
Environment-during processing							41/131 (31.3; 23.5-40.0)
Plucking machine	2/3	0/3	1/3	3/3	1/3	0/3	7/18 (38.9; 17.3-64.3)
Evisceration machine/table	0/3	0/3	0/3	0/3	0/3	3/3	3/18 (16.7; 3.6-41.4)
Gloves of a carcass trimmer	1/2	1/3	0/3	2/3	0/3	3/3	7/17 (41.2; 18.4-67.1)
Shackles/conveyer belt	2/3	3/3	0/3	3/3	0/3	3/3	11/18 (61.1; 35.7-82.7)
Chilling tank inside surface	0/0	1/9	0/3	1/6	0/3	1/3	3/24 (12.5; 2.7-32.4)
Chilling tank water	0/9	3/9	0/6	4/6	2/3	1/3	10/36 (27.8; 14.2-45.2)
Carcass							86/246 (35.0; 29.0-41.3)
After plucking	0/5	4/5	1/5	5/5	5/5	4/5	19/30 (63.3; 43.9-80.1)
After evisceration	0/10	5/5	1/5	9/10	2/5	1/5	18/40 (45.0; 29.3-61.5)
In chilling tank	0/5	2/16	0/10	5/5	5/10	7/10	19/56 (33.9; 21.8-47.8)
After chilling tank	0/20	0/20	0/20	15/20	7/20	8/20	30/120 (25.0; 17.5-33.7)
Intestinal contents							7/120 (5.8; 2.4-11.6)
Cloacal swabs	0/20	0/20	2/20	0/20	0/20	5/20	7/120 (5.8; 2.4-11.6)
Number positive/total collected (%; 95% CI)	8/95 (8.4; 3.7-15.9)	22/129 (17.1; 11.0-24.7)	5/99 (5.1; 1.7-11.4)	53/108 (49.1; 39.3-58.9)	26/95 (27.4; 18.7-37.5)	42/96 (43.8; 33.6-54.3)	156/622 (25.1 21.7-28.7)

4.2.2.1 Environmental samples

Environmental surface samples were collected before and during slaughtering along the slaughter line, including from the plucking machine, evisceration machine/table, carcass trimmer gloves, shackles or conveyer belt before the chilling tank, and the chilling tank. A reference template (10 x 10 cm) was placed over the selected location and samples obtained by swabbing 10x horizontally and 10x vertically within the template using a sterile sponge (Whirl-Pak Speci-Sponge, NASCO, Fort Atkinson, Wis.) moistened with 10 ml of 0.1% buffered peptone water (BPW, United States Biological). After swabbing, sponges were immediately placed into sterile bags. A sample of 500 ml of water from the chilling tanks was collected into sterile bottles both before and during slaughtering operations. At the time of collection, the temperature of the chilling tanks was recorded, and the amount of free residual chloride measured with a Pocket Colorimeter™ II (Hach, Loveland, Colorado).

4.2.2.2 Cloacal swabs

Samples of intestinal contents (approximately 1 g) were aseptically collected by the meat inspectors with swabs of the cloaca of randomly selected broilers during post-mortem inspection. These swabs were placed into 50 ml sterile tubes.

4.2.2.3 Carcass rinse samples

Randomly selected whole ca**rc**asses were sampled at four points along the slaughter chain: after plucking; after evisceration; in the chilling tank; and after the chilling tank. Chicken carcasses were aseptically placed in a 3500 ml sterile “Stomacher-type” bag, and 400 ml of sterile 0.1% BPW added. The bag was closed and shaken thoroughly with a rocking motion for 1 min at approximately 35 forward-and-backward swings per minute to wash the interior and exterior surfaces of the carcass. The carcass was then removed aseptically from the bag and the rinse fluid transferred to a 500 ml sterile

bottle.

4.2.3 Bacteriology

4.2.3.1 *Salmonella* isolation

Samples were processed according to the methods described in the U.S. Department of Agriculture/Food Safety and Inspection Service (USDA/FSIS) Microbiology Laboratory Guidebook (FSIS, 2014) and recommended by the Association of Official Agricultural Chemists (AOAC) International (Andrews, 1998). Samples were prepared by the addition of: (1) 50 ml of BPW to the pre-moistened environmental sponge sample; (2) 30 ml of BPW to 30 ml of poultry carcass rinse fluid or chilling tank water sample; or (3) 2 ml of BPW to 1 g of intestinal contents. All samples were enriched in BPW at a 1:10 dilution and by incubating at 35°C for 20-24 h, following which a 50 µl aliquot of the growth media was inoculated into 5 ml of Rappaport-Vassiliadis broth (Oxoid) and incubated at 42°C for 24 h (Andrews, 1998). A loopful of Rappaport-Vassiliadis culture was streaked onto Xylose Lysine Desoxycholate (Difco, BD), Hektoen enteric agar (Difco, BD) and *Salmonella* identification agar (CHROmagar Microbiology, Paris). Colonies with typical *Salmonella* characteristics were confirmed with API 20E (bioMe'rieux) biochemical assays. Isolates identified as *Salmonella* were serotyped by slide and tube agglutination tests with *Salmonella* polyvalent O and H antisera (Difco, BD) according to the Kauffmann-White scheme (Grimont and Weill, 2007). One colony from each sample was serotyped. Samples with one or more colonies with typical *Salmonella* characteristics were classified as positive.

4.2.3.2 Pulsed-field gel electrophoresis (PFGE)

Salmonella isolates were typed by PFGE according to the "Standard Operating Procedure for PulseNet PFGE of *E. coli* O157:H7, *E. coli* Non-O157 (STEC), *Salmonella* serotypes, *Shigella sonnei* and *Shigella flexneri*" (CDC, 2013a). Briefly,

chromosomal DNA was digested with 50 U of *XbaI* (NEB, MA) for 1.5-2 h at 37°C. Electrophoresis was carried out with 0.5 x Tris-borate-EDTA buffer at 14°C for 19 h using a CHEF Mapper electrophoresis system (Bio-Rad, Hercules, CA). Pulse times were ramped from 2.2 to 63.8 s during an 18 h run at 6.0 V/cm. *Salmonella* Braenderup H9812 was used as a molecular size marker. Gels were stained with ethidium bromide, and DNA bands visualized with UV transillumination (UVP). The PFGE patterns were analyzed with Bionumerics software (Applied Maths, Kortrijk, Belgium), using UPGMA (unweighted pair-group method with an arithmetic mean). The relatedness of the PFGE profiles was estimated based on the presence or absence of shared bands. Isolates with the same pattern from the same abattoir were considered to be the same strain/type.

4.2.4 Statistical analysis

The unit of study was the individual sample. The results of the prevalence of *Salmonella* spp. were compared between abattoirs and steps of processing within abattoirs. In each analysis, specific comparisons were made using the chi-square test for independence (or Fisher's exact test when any cell of a 2 x 2 table was < 5), and a p-value < 0.05 was considered statistically significant.

4.3 Results

4.3.1 Prevalence of *Salmonella*

Salmonella were detected in 156 of 622 samples collected (25.1%; 95% CI: 21.7-28.7). The prevalence detected was significantly different between abattoirs (χ^2 = 90.8, p < 0.0001) (Table 4.2). Overall, *Salmonella* prevalence was significantly different between sample types (χ^2 = 42.9, p < 0.0001), with the highest prevalence detected in carcass rinse samples (Table 4.2).

4.3.1.1 Environmental samples

Before processing of birds commenced, 22 of 125 (17.6%; 95% CI: 11.4-25.4) environmental samples were positive for *Salmonella*. The proportion of *Salmonella*-positive environmental samples before processing from the abattoirs are presented in Table 4.2. Overall there was no significant difference in prevalence between the six abattoirs (χ^2 = 9.9, p = 0.0769). However, among the different stages of processing, the proportion of *Salmonella*-positive environmental samples ranged from 1 of 30 (3.3%; 95% CI: 0.1-17.2) for the chilling tank water, 1 of 24 (4.2%; 95% CI: 0.1-21.1) for the inside surface of the chilling tank, 2 of 18 (11.1%; 95% CI: 1.4-34.7) for the evisceration machine/table, 3 of 17 (17.6%; 95% CI: 3.8-43.4) for the gloves of carcass trimmers, 4 of 18 (22.2%: 95% CI: 6.4-47.6) for the shackles/conveyer belt to 11 of 18 (61.1%; 95% CI: 35.7-82.7) for the inside surface of the plucking machine (overall χ^2 = 31.5, p < 0.0001).

During processing, 31.3% (95% CI: 23.5-40.0) of 131 environmental samples were positive for *Salmonella*. The proportion positive was significantly different between abattoirs (χ^2 = 22.6, p = 0.0004) ranging from 12.5 to 61.1% (abattoirs C and F, respectively). The proportion of *Salmonella*-positive environmental samples for all abattoirs varied from 12.5% (95% CI: 2.7-32.4) for the inside surface of the chilling tank, 16.7% (95% CI: 3.6-41.4) for the evisceration machine/table, 27.8% (95% CI: 14.2-45.2) for the water of the chilling tank, 38.9% (95% CI: 17.3-64.3) for the inside surface of the plucking machine, 41.2% (95% CI: 18.4-67.1) for the gloves of carcass trimmers to 61.1% (95% CI: 35.7-82.7) for the shackles or conveyer belt (χ^2 = 14.6, p = 0.0120). The proportion of *Salmonella*-positive environmental samples collected during processing (31.3%) was significantly higher than that prior to processing (17.6%) (χ^2 = 6.5, p = 0.011).

4.3.1.2 Carcass rinse fluid samples

Salmonella were detected in 86 of 246 (35.0%; 95% CI: 29.0-41.3) carcass rinse fluid samples (range 0% for abattoir A to 85.0% for D; overall χ^2 = 90.6, p < 0.0001). The prevalence was significantly different between sampling sites (χ^2 = 17.7, p = 0.0005) with the highest at the first sampling point (after plucking: 63.3%, 95% CI: 43.9-80.1). The prevalence then decreased sequentially in carcasses along the chain (45.0, 95% CI: 29.3-61.5 after evisceration, 33.9, 95% CI: 21.8-47.8 in the chilling tank and 25.0, 95% CI: 17.5-33.7 after chilling).

No significant difference was observed in the prevalence of *Salmonella*-positive carcass samples before (63.3%; 95% CI: 43.9-80.1) and after evisceration (45%; 95% CI: 29.3-61.5) (χ^2 = 2.3, p = 0.1284). However, the prevalence before chilling (samples collected after plucking and evisceration) (52.9%; 95% CI: 40.6-64.9) was significantly higher than that after chilling (25.0%; 95% CI: 17.5-33.7) (χ^2 = 15.0, p = 0.0001).

Salmonella spp. were not isolated from any carcass samples collected after chilling from the abattoirs that process BR (A, B, and C) (Table 4.2). In contrast 50% (95% CI: 36.8-63.2) of the samples were positive in the abattoirs that processed TNC (D, E, and F) (χ^2 = 37.4, p < 0.0001) (Table 4.2).

The prevalence in carcass rinse samples was significantly higher than in the environmental samples collected prior to processing (χ^2 = 12.1, p = 0.0005). However, no difference was found between the prevalence of all carcass rinse samples and all of the environmental samples collected during processing (χ^2 = 0.5, p = 0.4738).

4.3.1.3 Cloacal swabs

Salmonella were detected in 7 of 120 (5.8%; 95% CI: 2.4-11.6) cloacal samples - an assessment of the *Salmonella* status of the live birds (Cardinale, Tall, et al., 2005). However, cloacal samples collected from birds were positive at only two abattoirs (C -

prevalence of 10% 95% CI: 1.2-31.7; and F - 25% 95% CI: 8.7-49.1) (Table 4.2). The prevalence in birds sampled at abattoir F was significantly higher (χ^2 = 21.1, p = 0.0003) than those from abattoirs A, B, D and E where all 20 birds tested were negative (0%; 95% CI: 0-16.8). There was no significant difference in the cloacal prevalence of BR (3.3%; 95% CI: 0.4-11.5) and TNC (8.3%; 95% CI: 2.8-18.4) (χ^2 = 1.4, p = 0.2195).

The prevalence in intestinal contents was significantly lower than carcass rinse samples (χ^2 = 36.1, p < 0.0001) and environmental samples during processing (χ^2 = 26.3, p < 0.0001).

4.3.2 Distribution of *Salmonella* serotypes and PFGE types

Fifteen serotypes were identified from the 156 *Salmonella* isolates (Table 4.3). The most prevalent serotypes were *S.* Albany (41.7%; 95% CI: 33.8-49.8), *S.* Schwarzengrund (20.5%; 95% CI: 14.5-27.7), *S.* Kentucky (12.8%; 95% CI: 8.0-19.1) and *S.* Tennessee (5.1%; 95% CI: 2.2-9.9). Serotype *S.* Schwarzengrund was detected at all six abattoirs and *S.* Albany at four abattoirs, whereas *S.* Kentucky, *S.* Muenster, *S.* Newport, and *S.* Typhimurium were detected at two abattoirs and the remaining serotypes in only one abattoir. The predominant serotype in the respective abattoirs were *S.* Albany for A, D, and F; *S.* Kentucky for B; *S.* Schwarzengrund for C; and *S.* Tennessee for E.

Of the 15 serotypes isolated, only two, *S.* Albany and *S.* Schwarzengrund, were detected in cloacal samples, and only six (*S.* Albany, *S.* Hadar, *S.* Livingstone var. O14+, *S.* Muenster, *S.* Newport and *S.* Schwarzengrund) were detected from environmental samples collected before processing the birds. In contrast, 12 serotypes were found in environmental samples collected during processing and 13 from carcass samples.

The isolates were characterized into 50 PFGE types with 24 isolates (15.4%) untypable (14 isolates of *S.* Kentucky and 3 of *S.* Lindenberg from abattoir B, 2 of *S.* Livingstone

for D, 5 of *S.* Livingstone var. O14+ for F) (Fig. 4.1). *Salmonella* Schwarzengrund strain S-4-D and *S.* Albany strain A-1-F were the most prevalent (14 isolates of each type) and the most widely distributed during processing (8 and 9 stages involved for each PFGE type, respectively).

Table 4.3 Serotypes of *Salmonella* detected at the six abattoirs sampled

Serotype	Count (n = 156)	% isolates	Abattoir
Albany	65	41.7	A,D,E,F
Schwarzengrund	32	20.5	A,B,C,D,E,F
Kentucky	20	12.8	B,D
Tennessee	8	5.1	E
Livingstone var. O14+	5	3.2	F
Muenster	5	3.2	A,B
Newport	5	3.2	A,E
Haardt	3	1.9	D
Lindenberg	3	1.9	B
Enteritidis	2	1.3	D
Montevideo	2	1.3	D
Typhimurium	2	1.3	C,D
Livingstone	2	1.3	D
Hadar	1	0.6	A
Havana	1	0.6	E

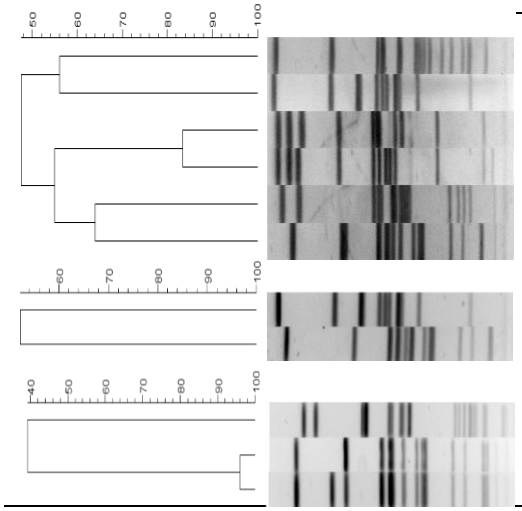
Samples collected from the carcasses and the environment during processing contained the same types (including 8 PFGE types; Mu-1-B, A-3-D, A-5-D, S-4-D, A-1-E, A-1-F, A-4-F, and S-1-F) as those detected prior to processing. Similarly, types S-1-C and A-5-F collected from intestinal contents of birds at abattoirs C and F were subsequently found in environmental and carcass samples at those abattoirs.

Seven PFGE types (A-1-A, A-6-D, K-1-D, S-3-D, Te-1-E, N-1-E, and A-3-F) were not found in the environment before processing nor in cloacal samples, but they were detected in environmental or carcass samples at two or more stages during processing (Fig. 4.1). Moreover, 14 PFGE types were detected on carcass samples, which could be

traced to environmental sources before and during processing. Furthermore, *S.* Albany strain A-3-D was also found at the same stage (inside surface of the chilling tank) before and during processing as with *S.* Schwarzengrund strain S-4-D (plucking machine) (Fig. 4.1). Six PFGE types (A-3-D, A-6-D, Te-1-E, A-1-F, A-3-F, and A-5-F) were found on carcasses in the chilling tank and after chilling and in water samples from the chilling tanks. Finally, certain PFGE types (A-2-D, A-4-D, Haa-1-D, Mo-1-D, Mo-2-D, S-2-D, A-4-E, S-2-E, S-4-E, Hav-1-E, and A-2F) were only found in the final stage (after chilling).

PFGE patterns of *Salmonella* isolates at each processing stage

Xbal



Abattoir	PFGE pattern[$] (number of isolates)	Number of *Salmonella* isolates at each sample stage																
		Before processing						During processing										
		PM	EM	GT	SC	CTW	CT	CS	PM	CAP	EM	CAE	GT	SC	CTW	CICT	CT	CACT
A	N-1-A (1)	1																
A	Mu-1-A (1)								1									
A	A-1-A (3)								1				1	1				
A	A-2-A (1)													1				
A	Had-1-A (1)	1																
A	S-1-A (1)	1																
B	Mu-1-B (4)	2										1					1	
B	S-1-B (1)					1												
C	Ty-1-C (1)									1								
C	S-1-C (3)							1	1			1						
C	S-2-C (1)							1										

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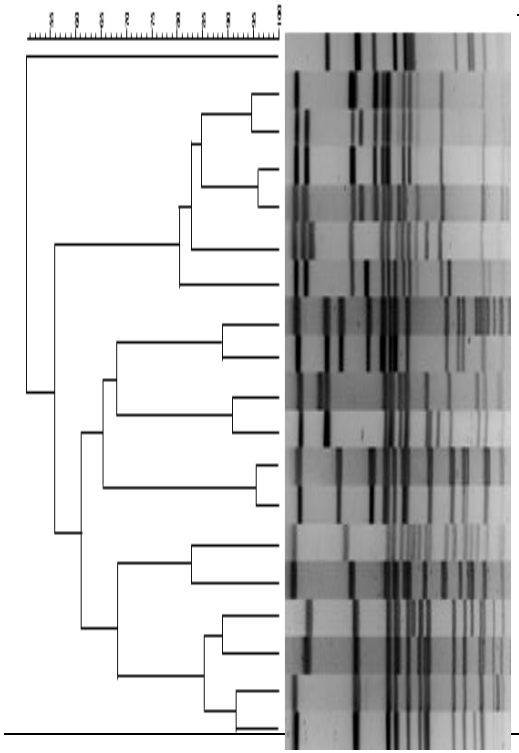
Fig 4.1 PFGE patterns of *Salmonella* isolates at each processing stage

PFGE patterns of *Salmonella* isolates at each processing stage (Continued)

Xbal



Abattoir	PFGE pattern[$] (number of isolates)	Number of *Salmonella* isolates at each sample stage																
		Before processing						During processing										
		PM	EM	GT	SC	CTW	CT	CS	PM	CAP	EM	CAE	GT	SC	CTW	CICT	CT	CACT
D	Ty-1-D (1)								1									
D	A-1-D (1)													1				
D	A-2-D (1)																	1
D	A-3-D (9)				1		1					1			1	4	1	
D	A-4-D (1)																	1
D	A-5-D (2)			1								1						
D	A-6-D (5)												1		1			3
D	K-1-D (5)								1	1		2						1
D	K-2-D (1)													1				
D	E-1-D (1)															1		
D	E-2-D (1)														1			
D	Haa-1-D (2)																	2
D	Haa-2-D (1)														1			
D	Mo-1-D (1)																	1
D	Mo-2-D (1)																	1
D	S-1-D (1)									1								
D	S-2-D (1)																	1
D	S-3-D (2)									1		1						
D	S-4-D (14)	1	1	1					1	1		3		2				4

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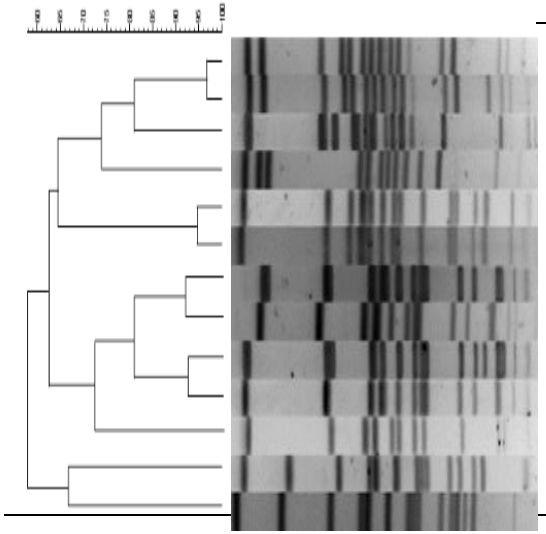
Fig. 4.1 (continued)

PFGE patterns of *Salmonella* isolates at each processing stage (Continued)

Xbal

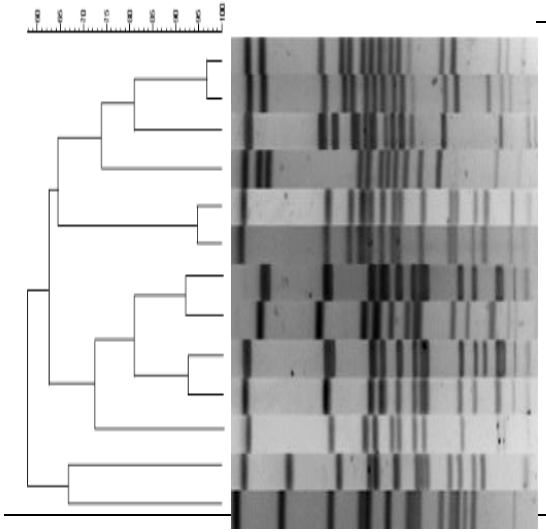


Abattoir	PFGE pattern[$] (number of isolates)	Number of *Salmonella* isolates at each sample stage														
		Before processing						During processing								
		PM	EM	GT	SC	CTW	CT	CS	PM	CAP	EM	CAE	GT	SC	CTW	CICT	CT	CACT
E	A-1-E (4)	2	1															1
E	A-2-E (1)									1								
E	A-3-E (1)									1								
E	A-4-E (1)																	1
E	Te-1-E (4)														2			2
E	Te-2-E (4)															4		
E	S-1-E (1)			1														
E	S-2-E (1)																	1
E	S-3-E (3)									3								
E	S-4-E (1)																	1
E	Hav-1-E (1)																	1
E	N-1-E (3)								1			2						
E	N-2-E(1)															1		

Continued on following page

Fig. 4.1 (continued)

PFGE patterns of *Salmonella* isolates at each processing stage (Continued)

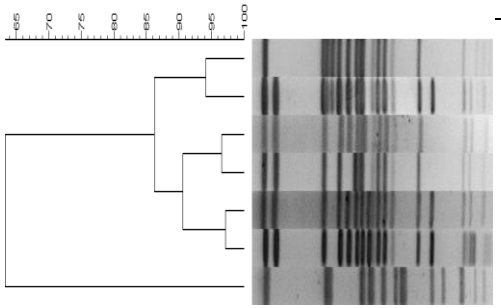
Xbal	Abattoir	PFGE pattern[$] (number of isolates)	Number of *Salmonella* isolates at each sample stage															
			Before processing						During processing									
			PM	EM	GT	SC	CTW	CT	CS	PM	CAP	EM	CAE	GT	SC	CTW	CICT	CT	CACT
	E	A-1-E (4)	2	1															1
	E	A-2-E (1)									1								
	E	A-3-E (1)									1								
	E	A-4-E (1)																	1
	E	Te-1-E (4)														2			2
	E	Te-2-E (4)															4		
	E	S-1-E (1)			1														
	E	S-2-E (1)																	1
	E	S-3-E (3)									3								
	E	S-4-E (1)																	1
	E	Hav-1-E (1)																	1
	E	N-1-E (3)								1			2						
	E	N-2-E(1)															1		

Continued on following page

Fig. 4.1 (continued)

PFGE patterns of *Salmonella* isolates at each processing stage (Continued)

Xbal



Abattoir	PFGE pattern[$] (number of isolates)	Number of *Salmonella* isolates at each sample stage																
		Before processing						During processing										
		PM	EM	GT	SC	CTW	CT	CS	PM	CAP	EM	CAE	GT	SC	CTW	CICT	CT	CACT
F	A-1-F (14)	1								2	2		1	1	1	2	1	3
F	A-2-F (1)																	1
F	A-3-F (9)									1		1	1	1		2		3
F	A-4-F (2)				1								1					
F	A-5-F (8)							5			1					1		1
F	A-6-F (1)															1		
F	S-1-F (2)	1								1								
B	K-UT[#]-B (14)									4		3	1	1	3	2		
B	Lin-UT-B (3)											1		2				
D	Liv-UT-D (2)									1			1					
F	Liv14-UT-F (5)	1			2									1		1		

[$]PFGE patterns are indicated by numerical and abattoir sampled suffixes after a capital letter indicating the name of the serotype (e.g., Hav-1-E refers to the serotype Havana collected from abattoir E).

[#]UT, PFGE typing untyped.

PFGE pattern detected in isolates from this stage.

PM: Plucking machine; EM: Evisceration machine/table; GT: Gloves of a carcass trimmer; SC: Shackles/conveyer belt; CTW: Chilling tank water; CT: Chilling tank inside surface; **CS: Cloacal** swabs; CAP: Carcass rinse samples after plucking; CAE: Carcass rinse sample after evisceration; CICT: Carcass rinse samples in chilling tank; CACT: Carcass rinse sample after the chilling tank.

Fig. 4.1 (continued)

4.4 Discussion

4.4.1 Prevalence of *Salmonella*

All of the abattoirs included in this study cleaned and disinfected the environment/equipment after the conclusion of each day's slaughtering/processing. The purpose of this was to remove contamination and to minimize subsequent contamination of birds slaughtered on the next processing day. However, *Salmonella* positive environmental samples were detected at five of the six abattoirs after cleaning and prior to processing birds, indicating that the cleaning/disinfection processes were not sufficient to eliminate environmental contamination with *Salmonella*. Similar results were reported by Kudirkienė et al. (2011), who believed that this could be due to a failure of adequate cleaning through application of lower concentrations or shorter application times of disinfectants than those recommended. Furthermore, it is evident from the current study that environmental contamination present at the start of a day's processing could result in subsequent carcass contamination along the processing chain. Thorough cleaning and disinfection procedures in abattoirs have been shown to significantly reduce the risk of *Salmonella* contamination of carcasses (Cardinale, Tall, et al., 2005; Heyndrickx et al., 2002; White et al., 1997); however, regular review of these processes is required to ensure their effectiveness at removal of environmental sources of bacteria.

The highest proportion of contamination before processing was detected in the plucking machine, which was similar to the findings reported in the studies of Rasschaert et al. (2007) and Olsen et al. (2003). This may be associated with the structure of the rubber fingers on the plucking machine (rounded) allowing residues of feathers and organic matter to remain after cleaning (Rasschaert et al., 2007). In this study feathers were observed on the rubber fingers after cleaning and prior to slaughter of the next batch of

birds, and residual organic matter and feathers would increase the ability of *Salmonella* to survive the disinfection procedure (Kudirkienė et al., 2011). Others have also reported that the surface of the rubber fingers becomes roughened with age, allowing bacteria to colonize crevices on the surface and to multiply overnight (Fries, 2002). If the fingers are not disinfected properly, transfer of bacteria can occur when the machine is next used (Fries, 2002; FSANZ, 2005; King et al., 2011). It is recommended that broken rubber fingers should be replaced prior to the next day's processing and at least the last half of the plucking equipment is disinfected to minimize subsequent microbial contamination (NZFSA, 2017).

The highest prevalence of *Salmonella* on the carcass samples was observed after plucking, indicating that the most important stage responsible for microbial contamination of chicken carcasses is defeathering. This is supported by many reports and studies which have highlighted that de-feathering is a major source of cross-contamination (Byrd & McKee, 2005; FAO/WHO, 2009b; FSANZ, 2005; King et al., 2011; Morar et al., 2014). Furthermore, Rigby et al. (1980) observed that live bacteria were present on feathers even after scalding, and these contaminated feathers were likely to be an important means of introducing bacteria during de-feathering. Moreover, the feather follicles in the skin at this stage are opened and the rubber fingers used in de-feathering can drive microorganisms into the skin tissue and feather follicles, which may decrease the wash-off effect of subsequent carcass washes (Byrd & McKee, 2005; Sofos et al., 2013).

In addition to de-feathering, evisceration has also been considered a major stage for cross-contamination of carcasses due to the risk of contact with intestinal contents (Byrd & McKee, 2005). This was supported in the current study with 45% of carcasses contaminated with *Salmonella* spp. after evisceration (Table 4.2). Brizio and Prentice

(2015) reported that one of the biggest problems in processing poultry is carcass contamination by the leakage of crop and intestinal contents during the evisceration process. This leakage might not only cause contamination of the birds being processed, but could also contaminate the slaughter equipment and lead to extensive cross-contamination of carcasses of subsequently slaughtered birds (Russell, 2012b; Sofos et al., 2013; Van Immerseel et al., 2009). This might explain the finding in the current study where no *Salmonella* were detected in samples of the evisceration table prior to processing in abattoir F, whereas all three samples collected during evisceration at the same abattoir were positive (Table 4.2).

However, the prevalence of *Salmonella*-positive carcass samples decreased slightly, but not significantly, from 63.3% before evisceration to 45% after evisceration (Table 4.2). A similar result was reported in a USA study (Morris & Wells, 1970) that reported evisceration had only a slight effect in the prevalence of *Salmonella* on poultry carcasses. In contrast most studies summarized by FAO/WHO (2002) showed a two to five fold increase in the proportion of *Salmonella*-contaminated carcasses after evisceration. In addition, it is noteworthy that abattoir D, where TNCs are processed using automated evisceration, had a higher contamination of carcass samples (9 of 10 positive) after evisceration than those collected from the TNC abattoir E and F which used manual evisceration (3 of 10 positive) (Table 4.2). This result is in agreement with Nunes (2013) who reported that hand-eviscerated carcasses were less likely to be contaminated than carcasses eviscerated automatically, and concluded that flocks containing birds of various sizes contributed to increasing the degree of fecal and bile contamination during automatic evisceration. Similarly, Brizio and Prentice (2015) recommended that standardizing the weight of slaughtered birds in a batch and adjusting the period of pre-slaughter fasting could reduce contamination levels during

evisceration. In this study, although the market age of TNSs was 12 to 15 weeks old, within the same batch of TNCs presented, the size of birds varied.

In the present study, a decreasing trend in the prevalence of *Salmonella* was observed in carcass samples at subsequent selected processing steps (Table 4.2). Furthermore, a significantly lower prevalence was found in the samples collected from carcasses after chilling as compared with samples collected prior to chilling (Table 4.2). Two previous studies have shown this decrease may be associated with chlorination of the chill water (Demirok et al., 2013; Lillard, 1990). In the present study in the four abattoirs (A, B, C and D) that chlorinated the chill water, the prevalence of *Salmonella*-positive carcass samples decreased significantly from 50.0% (95% CI: 35.5-64.5) before chilling to 18.8% (95% CI: 10.9-29.0) after chilling (data not shown). In contrast in abattoirs E and F, which did not use chlorinated chill water, the proportion of positive carcass samples decreased from 60% (95% CI: 36.1-80.9) before chilling to 37.5% (95% CI: 22.7-54.2) after chilling (data not shown). Bilgili et al. (2002) and Demirok et al. (2013) proposed that washing in the immersion chiller may also contribute to the reduction in numbers. All six abattoirs in the current study employed counter current flow, continuous overflow, and air agitation which may assist this washing effect.

In this study the prevalence of *Salmonella* contaminated carcasses following chilling overall was 25%; however, significant differences were detected among the different type of chickens slaughtered: 0% (95% CI: 0-6.0) for BRs processed at slaughterhouses A, B, and C; and 50% (95% CI: 36.8-63.2) for the TNCs processed at slaughterhouses D, E, and F (data not shown). The prevalence of *Salmonella* contaminated BR carcasses in this study was similar to that reported in New Zealand (2%) (King et al., 2011), European Union countries (range 0 to 22.7%) (EFSA, 2014), and the USA (3.9%) (FSIS, 2015), and lower than that reported in Thailand (9%) (Padungtod & Kaneene, 2006),

Korea (42.7%) (Bae et al., 2013) and another nation-wide study conducted by the BAPHIQ in 2013 (unpublished) (17.6%; 95% CI: 15.1-20.2). However, when comparing results of different studies, care needs to be taken due to different sampling and isolation methods used. In contrast the prevalence of contamination of TNC carcasses in this study was similar to that of the BAPHIQ's study in 2013 (46.9%; 95% CI: 43.7-50.1). Unfortunately there is an absence of species-specific data on the prevalence and origin of microbial pathogens within TNCs from other countries.

Finally, in this study the proportion of *Salmonella*-positive environmental samples during processing was higher than that prior to processing, indicating increased contamination during processing ($p < 0.05$). This coincided with a higher prevalence in carcass rinse fluid samples compared with cloacal swabs and environmental samples before processing commenced. Such observations corroborate the occurrence of cross-contamination during processing (Chotinun et al., 2014; Goksoy et al., 2004; Rasschaert et al., 2007; Rasschaert et al., 2008; Zutter et al., 2005).

4.4.2 Distribution of *Salmonella* serotypes and PFGE types

In the present study, the two most common serotypes detected were *S.* Albany and *S.* Schwarzengrund (Table 4.3). These results agree with those of Lin et al. (2008) who isolated these and other *Salmonella* spp. from the liver, gall bladder and cecal contents of boilers in Taiwan. Only two isolates of *S.* Enteritidis and *S.* Typhimurium were detected in the present study, even though these serotypes have been reported to be the predominant serotypes involved in human infections in Taiwan (Chu et al., 2009; Torpdahl et al., 2013). Cardinale, Perrier Gros-Claude, et al. (2005) also observed that the serotypes present in poultry were different to those isolated from clinically affected people in developed countries. These findings may indicate that poultry meat is not the only source of human salmonellosis.

Salmonella molecular genotyping methods provide valuable epidemiological information about the nature of the contamination encountered at the slaughterhouse (Arguello et al., 2013). According to the results of the PFGE analysis from the current research, only six of fifteen serotypes (Albany, Hadar, Livingstone var. O14+, Muenster, Newport, and Schwarzengrund) were detected from environmental samples collected before processing, whereas>10 serotypes were found in environmental samples collected during processing and from carcass samples (Fig. 4.1). It is likely that some carcasses contained multiple subtypes or even serotypes of *Salmonella*. This also indicates that some *Salmonella* serotypes can survive the cleaning and disinfection process. Eight PFGE types collected from carcasses and the environment during slaughtering were also detected pre-processing. This might indicate that contamination originated from *Salmonella* in the slaughter environment which survived the cleaning and disinfection process. Moreover, two types (S-1-C and A-5-F) from the cloacal samples were subsequently found in environmental and carcass samples, highlighting the likelihood that processing birds from *Salmonella*-infected flocks could result in the subsequent contamination of carcasses (Fig. 4.1). This finding was similar to that reported by others (Corry et al., 2002; Nógrády et al., 2008; Olsen et al., 2003), and demonstrates that *Salmonella*-infected flocks are an important source of post-slaughter contamination. To eliminate the potential for cross-contamination to *Salmonella* free flocks from infected flocks, *Salmonella*-negative flocks should be slaughtered first; however, this does require pre-slaughter testing of birds (Evers, 2004; Van Immerseel et al., 2009). This practice has been adopted in the Netherlands and was shown to reduce the prevalence of contaminated flocks after slaughter by 9% (Evers, 2004).

Seven PFGE types were present in environmental or carcass samples at two or more stages during processing but were not found in the environment before processing or

from cloacal samples (Fig. 4.1). These findings indicate the occurrence of cross-contamination at different stages during processing. In addition, 14 PFGE types were found on carcass samples, which could be traced to environmental sources before and during processing. This and other studies (Corry et al., 2002; Lillard, 1990; Olsen et al., 2003; Rasschaert et al., 2007; Rasschaert et al., 2008; Rigby et al., 1980; Zutter et al., 2005) have demonstrated that equipment can be a source of cross-contamination during processing. Identical strains were found at the same stage before and during processing (A-3-D from inside the surface of the chilling tank and S-4-D from the plucking machine) indicating the potential for *Salmonella* to survive on equipment after cleaning. In addition, six PFGE types (A-3-D, A-6-D, Te-1-E, A-1-F, A-3-F, and A-5-F) were found on carcasses in the chilling tank and after chilling, and in water samples from the chilling tanks. Similar findings have been observed in other studies (Lillard, 1990; Lopes et al., 2007), highlighting the potential for contamination through direct contact between carcasses in the chilling tank or via the water.

Certain PFGE types were only found in the final sampling stage (after chilling), indicating that these strains may have survived in the environment or on carcasses but were not detected by the sampling and isolation methods adopted. However, the low number of samples collected may also have contributed to this finding and it is important that future studies expand both the number of samples collected and the points of sampling throughout the slaughter process. The results of the cloacal samples are likely to be indicative of the carriage of *Salmonella* by the birds and their source flocks; however, further samples are required to confirm this as it is possible that not all birds from a flock were carrying *Salmonella*. It is recommended that regular sampling to estimate the occurrence of *Salmonella* on processed carcasses is undertaken annually to monitor the serovars present and to identify any temporal change in contamination

levels.

4.5 Conclusions

This study provides data regarding the sources of *Salmonella* contamination, and cross-contamination of carcasses with *Salmonella* in broiler and traditional native chickens post-slaughter in Taiwan. The findings highlight that contamination of carcasses with *Salmonella* can occur at several stages along the processing line. The results show that environmental samples collected before and during processing, as well as carcass samples, had a high prevalence of *Salmonella* contamination. In addition, characterization of the isolates by PFGE indicated that *Salmonella* isolates were surviving in the abattoir environment, as well as being present in the intestinal contents of processed birds resulting in subsequent carcass contamination. This information can be used by abattoirs to develop preventive measures to yield a more wholesome product for consumers, such as using the recommended concentrations and application times of disinfectants before slaughter operations, and using counter current flow, continuous overflow and air agitation in the chilling tank during the slaughter operations.

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CHAPTER 5: Contamination of Chicken Carcasses and the Abattoir Environment with *Listeria monocytogenes* in Taiwan

Preface

Listeriosis is a relatively rare disease in humans compared with salmonellosis; however, it is an important disease as it has the highest case fatality rate of all foodborne acquired infections. *Listeria monocytogenes* occurrence in poultry meat has been reported to originate from infected flocks or from cross-contamination during processing. This chapter describes the results of testing chicken carcasses processed at six abattoirs in Taiwan for *L. monocytogenes*. Samples were collected at different processing stages to identify the stages where contamination was occurring so that targeted interventions could be developed and applied to minimize contamination with *L. monocytogenes*, resulting in less risk to public health.

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Abstract

1. The following study provides the first data on the detection and types of *Listeria monocytogenes* isolated from broiler chickens during processing and from six Taiwanese abattoir environments.
2. *Listeria monocytogenes* was not detected in any cloacal (n = 120) or environmental (n = 256) samples collected before and during processing, indicating that faecal material and the environment of abattoirs were not important sources of *L. monocytogenes* for poultry carcases. However, 28 of 246 (11.4%; 95% CI: 7.7-16.0) rinse samples collected from carcases post-evisceration from three abattoirs were positive for *L. monocytogenes*.
3. The only serotypes detected were 1/2a (82.1%; 95% CI: 63.1-93.9) and 1/2b (14.3%; 95% CI: 4.0-32.7), with 3.6% (95% CI: 0.1-18.3) non-typable isolates.
4. Characterisation by Pulsed Field Gel Electrophoresis (PFGE) identified five PFGE types, confirming cross-contamination with *L. monocytogenes* during evisceration, chilling and post-chilling.
5. These findings highlight the potential for cross-contamination to occur through direct contact between carcases, especially whilst in the chilling tanks.

5.1 Introduction

In the European Union (EU) and the USA, the annual incidence of listeriosis in 2012 and 2013 was reported as 0.41 and 0.26 per 100,000 population, respectively with a case fatality rate of 17.8 and 16.3%, respectively (CDC, 2015a; EFSA, 2014). In contrast, listeriosis has rarely been reported in Taiwan, with only 48 cases identified in a Taiwanese hospital between 1996 and 2008 (Huang et al., 2011). However, this study detected an increase in the average annual incidence from 0.029 (1996-2004) to 0.118 cases per 1,000 hospital admissions during 2005-2008, with 28% of admissions dying by day 14 of hospitalisation (Huang et al., 2011). A more recent study reported the annual incidence of invasive listeriosis at four medical centres in Taiwan from 2010-2012 was > 0.125 cases per 1,000 hospital admissions (Huang et al., 2015). Subsequently, the Taiwan Centre of Disease Control listed listeriosis as a notifiable disease on 1 January, 2018 in an effort to improve its control (TCDC, 2018).

Worldwide, listeriosis outbreaks have been associated with the consumption of raw milk, soft-ripened cheeses, ice cream, vegetables, fruits, seafood, and meat and meat products (Batt, 2014; Cartwright et al., 2013; EFSA, 2014; FAO/WHO, 2004; Meloni, 2014). Whilst chicken meat has never been identified as the source for *L. monocytogenes* outbreaks internationally (Aury et al., 2011; Bouayad et al., 2015; Rørvik et al., 2003; Rothrock et al., 2017), ready-to-eat and undercooked chicken have been linked to sporadic cases of human listeriosis worldwide (Kerr et al., 1988; Rothrock et al., 2017; Schwartz et al., 1988), with a single outbreak linked to turkey meat in the USA (Olsen et al., 2005).

Internationally, the prevalence of *L. monocytogenes* in chicken carcases or fresh chicken meat has been reported to range from 25.7 to 70% (Cox et al., 1997; Praakle-Amin et al., 2006; Rørvik et al., 2003; Uyttendaele et al., 1997). It has been stated that *L.*

monocytogenes in poultry meat originates from infected flocks (Ryser & Buchanan, 2012) or from environmental contamination during processing (Franco et al., 1995; Lawrence & Gilmour, 1994; Loura et al., 2005; Sakaridis et al., 2011).

Serotyping is usually the first level of *L. monocytogenes* subtyping and is based on antibodies that specifically react with somatic (O) lipopolysaccharides (LPS) on the external surface of the bacterial outer membrane and flagellin (H) antigens (Gasanov et al., 2005). A total of 13 different serotypes have been distinguished by serotyping, namely 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and serovar 7 (Batt, 2014). Serotypes 1/2a, 1/2b, and 4b are responsible for 98% of cases of human listeriosis (Zeinali et al., 2016) and serotypes 1/2a, 1/2b, 1/2c, 3b and 4b are commonly found in chickens and their products (Jamshidi & Zeinali, 2019; Maung et al., 2019; Oliveira et al., 2018; Zeinali et al., 2016; Zhang et al., 2007). In addition to serotyping, pulsed field gel electrophoresis (PFGE) is commonly used to investigate the source and relationship of strains of *Listeria* isolated from chicken carcases (Ojeniyi et al., 1996). A study in Chile demonstrated that strains of *L. monocytogenes* from poultry products had similar PFGE profiles as strains isolated from human cases of listeriosis, highlighting the potential risk for human health from contaminated carcases (Foerster et al., 2013).

In Taiwan, broiler meat is an important protein source for humans, with an annual *per capita* consumption of 31.9 kg, representing 42.2% of all meat consumed (COA, 2014b). Wong et al. (1990) demonstrated that 50% of chicken carcases from local markets in Taiwan were contaminated with *L. monocytogenes.* An earlier study of rinse samples collected from chicken carcases sourced from 14 Taiwanese slaughterhouses reported a prevalence of *L. monocytogenes* of 2.8, 4.4 and 0.6% in 2000, 2001 and 2002, respectively (Chen et al., 2004). However, there is no recent data available regarding the sources of *L. monocytogenes* or the potential pathways for contamination within

chicken abattoirs in Taiwan. Consequently, the focus of the following study was to identify the source(s) of *L. monocytogenes* contamination and pathways for cross-contamination within abattoirs processing broiler chickens in Taiwan.

5.2 Materials and methods

5.2.1 Selection of abattoirs

Samples for this study were collected from six representative abattoirs (coded A to F) in Taiwan as outlined in Lin et al. (2020). The main processing steps of these abattoirs included holding, *ante-mortem* inspection, unloading and hanging, stunning, bleeding, scalding and plucking, venting and evisceration, *post-mortem* inspection, carcase washing and chilling, followed by packing and labelling with an inspection certificate. Abattoirs A, B, C and D use automated evisceration whilst manual evisceration is used in abattoirs E and F. The annual numbers of birds processed at these abattoirs varied from 150,000 to 21,000,000 broilers, and total annual production was around 46 million broilers, representing approximately 16% of the national production (2014 slaughter data; BAPHIQ, 2015b).

5.2.2 Sample and data collection

Samples were collected as per the methodology outlined in Lin et al. (2020). Briefly, samples were collected in July and August 2014. Sampling was performed by the official meat inspection veterinarians at each abattoir. All samples (environment, carcase rinse fluid samples and cloacal swabs - Table 5.1) from an individual abattoir were collected on the same day with different abattoirs sampled on different days. The differences in sampling sites between abattoirs were due to availability of inspectors and processing arrangements between abattoirs. Pre-slaughter environmental samples were collected one hour prior to the commencement of slaughter, and samples from the birds and further environmental samples were collected during the processing of the first

batch of broilers at each abattoir. All samples were stored at 4℃ and transported to the Agricultural Technology Research Institute in Taiwan for microbiological analysis within 24 hours of collection.

Environmental surface samples were collected before and during slaughtering along the processing line, including from the plucking machine, evisceration machine (automated) or table (manual), carcase trimmer gloves, shackles or conveyer belt before the chilling tank, and from the chilling tank. A reference template (10 × 10 cm) was placed over the selected location and samples obtained by swabbing 10× horizontally and 10× vertically within the template using a sterile sponge (Whirl-Pak Speci-Sponge, NASCO, Fort Atkinson, Wis.) moistened with 10 ml of 0.1% buffered peptone water (BPW, United States Biological). After swabbing, sponges were immediately placed into sterile bags.

A sample of 500 ml of water from the chilling tanks was collected into sterile bottles both before and during slaughtering operations. At the time of collection, the temperature of the chilling tanks was recorded and the amount of free residual chloride measured with a Pocket Colorimeter™ II (Hach, Loveland, Colorado).

Table 5.1 Results of the samples collected at different processing stages and the frequency of detection of *L. monocytogenes* spp. in six abattoirs (A-F)

Sample stages	Number of samples (+ve/total samples) at each abattoir						Number positive/total collected (%; 95% CI)
	A	B	C	D	E	F	
Environment-before processing							**0/125 (0; 0-2.9)**
Plucking machine	0/3	0/3	0/3	0/3	0/3	0/3	0/18 (0; 0-18.5)
Evisceration machine/table	0/3	0/3	0/3	0/3	0/3	0/3	0/18 (0; 0-18.5)
Gloves of a carcase trimmer	0/3	0/3	0/3	0/3	0/2	0/3	0/17 (0; 0-19.5)
Shackles/conveyer belt	0/3	0/3	0/3	0/3	0/3	0/3	0/18 (0; 0-18.5)
Chilling tank inside surface	0/0	0/9	0/3	0/6	0/3	0/3	0/24 (0; 0-14.2)
Chilling tank water	0/3	0/12	0/3	0/6	0/3	0/3	0/30 (0; 0-11.6)
Environment-during processing							**0/131 (0; 0-2.8)**
Plucking machine	0/3	0/3	0/3	0/3	0/3	0/3	0/18 (0; 0-18.5)
Evisceration machine/table	0/3	0/3	0/3	0/3	0/3	0/3	0/18 (0; 0-18.5)
Gloves of a carcase trimmer	0/2	0/3	0/3	0/3	0/3	0/3	0/17 (0; 0-19.5)
Shackles/conveyer belt	0/3	0/3	0/3	0/3	0/3	0/3	0/18 (0; 0-18.5)
Chilling tank inside surface	0/0	0/9	0/3	0/6	0/3	0/3	0/24 (0; 0-14.2)
Chilling tank water	0/9	0/9	0/6	0/6	0/3	0/3	0/36 (0; 0-9.7)
Carcase							**28/246 (11.4; 7.7-16.0)**
After plucking	0/5	0/5	0/5	0/5	0/5	0/5	0/30 (0; 0-11.6)
After evisceration	0/10	0/5	0/5	0/10	5/5	0/5	5/40 (12.5; 4.2-26.8)
In chilling tank	5/5	1/16	0/10	0/5	2/10	0/10	8/56 (14.3; 6.4-26.2)
After chilling tank	10/20	0/20	0/20	0/20	5/20	0/20	15/120 (12.5; 7.2-19.8)
Intestinal contents							**0/120 (0; 0-3.0)**
Cloacal swabs	0/20	0/20	0/20	0/20	0/20	0/20	0/120 (0; 0-3.0)
Number positive/total number collected (%; 95% CI)	**15/95 (15.8; 9.1-24.7)**	**1/129 (0.8; 0.0-4.2)**	**0/99 (0; 0-3.7)**	**0/108 (0; 0-3.4)**	**12/95 (12.6; 6.7-21.0)**	**0/96 (0; 0-3.8)**	**28/622 (4.5; 3.0-6.4)**

Swabs of the cloaca of randomly selected broilers were aseptically collected during *post-mortem* inspection and placed into 50 ml sterile tubes. Randomly selected whole carcases were sampled at four points along the processing line, immediately after plucking, immediately after evisceration, in and after the chilling tank. The handling of samples was as described by Lin et al. (2020).

5.2.3 *Listeria monocytogenes* isolation

Samples were processed according to the methods described in the USDA/FSIS Microbiology Laboratory Guidebook (FSIS, 2013) and recommended by AOAC International (Andrews, 1998). In summary, 100 ml of carcase rinse fluid was filtered through a 0.45 μm hydrophobic grid membrane (Millipore, Merck, Germany). The filter was transferred aseptically in a whirl pak filter bag and 200 ml of modified University of Vermont broth (UVM; Oxoid) added, and then incubated at 30 ± 2°C for 20-24 h for enrichment. A 0.1 ml aliquot of the UVM enrichment was then transferred to 10 ml of Fraser broth (FB; Oxoid) and incubated at 35 ± 2°C for 26 ± 2 h. The FB enrichment was then streaked onto modified Oxford agar (MOX; Oxoid) incubated at 35 ± 2°C for 26 ± 2 h, and Chromagar *Listeria* agar (CL; CHROMAgar, Paris, France) incubated at 35 ± 2°C for 24-48 h. One colony showing typical *L. monocytogenes* characteristics (small, 1-2 mm; surrounded by a zone of darkening due to aesculin hydrolysis on MOX; blue colonies surrounded by a limpid ring on CL) (Gasanov et al., 2005) were aseptically transferred from positive plates to trypticase soy sheep blood agar (BA; TPM, Taiwan) plate and incubated at 35 ± 2°C for 22 ± 4 h. After incubation, colonies with typical characteristics (surrounded by a small zone of β-haemolysis) (Cassiday et al., 1990) were processed to expedite the final identification by using the API *Listeria* test system (bioMe´rieux) for screening *L. monocytogenes*. Isolates identified as *L. monocytogenes* were serotyped by slide and tube agglutination tests with O and H

antisera (Denka Seiken), according to the methods described in the U.S. Food and Drug Administration Bacteriological Analytical Manual (Bennett & Weaver, 2001).

5.2.4 Pulsed-field gel electrophoresis (PFGE)

Listeria monocytogenes isolates were typed by PFGE according to the Standard Operating Procedure for PulseNet PFGE of *Listeria monocytogenes* (CDC, 2013b). Briefly, chromosomal DNA was digested with 40U *Asc*I (NEB, MA) for 3 h at 37°C. Electrophoresis was carried out with 0.5 x Tris-borate-EDTA buffer at 14°C for 19 h using a CHEF Mapper electrophoresis system (Bio-Rad, Hercules, CA). Pulse times were ramped from 4 to 40 s during an 18 h run at 6 V/cm. *Salmonella* Braenderup H9812 was used as a molecular size marker. Gels were stained with ethidium bromide, and DNA bands visualised with UV transillumination (UVP). The PFGE patterns were analysed by Bionumerics software (Applied Maths, Kortrijk, Belgium), using the unweighted pair–group method with an arithmetic mean (UPGMA). The relatedness of the PFGE profiles was estimated based on the presence or absence of bands. Isolates with the same pattern from the same abattoir were considered to be the same strain/type.

5.2.5 Statistical analysis

The unit of study was the individual sample. The prevalences of *L. monocytogenes* was compared between abattoirs and processing stage within abattoirs. In each analysis, specific comparisons were made using the chi-square test for independence or Fisher's exact test and a $P < 0.05$ was considered statistically significant.

5.3 Results

5.3.1 Prevalence of *L. monocytogenes*

Listeria monocytogenes was detected in 28 of 622 samples (4.5%; 95% CI: 3.0-6.4) with the bacterium only detected in carcase samples from abattoirs A, B and E. All cloacal (n = 120) and environmental samples (n = 256) collected from the six abattoirs

were negative for *L. monocytogenes* (Table 5.1).

Listeria monocytogenes was detected in 28 of 246 (11.4%; 95% CI: 7.7-16.0) carcase rinse samples. The bacterium was detected in 37.5% (95% CI: 22.7-54.2) of the rinse samples from abattoir A, 2.2% (95% CI: 0.1-11.5) from B, 30.0% (95% CI: 16.6-46.5) from E, and not in any samples from each of abattoirs C, D and F (0%; 95% CI: 0-8.8). The prevalence in samples from abattoirs A and E was significantly higher than that in abattoirs B, C, D and F.

Along the processing line, *L. monocytogenes* was detected overall on 0% (95% CI: 0-11.6) of carcases immediately after plucking, 12.5% (95% CI: 4.2-26.8) after evisceration, 14.3% (95% CI: 6.4-26.2) in the chilling tank, and 12.5% (95% CI: 7.2-19.8) after chilling. Prevalence immediately after plucking was significantly lower than at the other three stages; however, there were no differences in prevalence between the three latter stages (*i.e.* after evisceration, in the chilling tank and after chilling).

Of the carcase samples collected after the carcases had been chilled, *L. monocytogenes* was only detected from abattoirs A (50%; 95% CI: 27.2-72.8) and E (25%; 95% CI: 8.7-41.9).

5.3.2 Distribution of *L. monocytogenes* serotypes and PFGE types

Two serotypes were identified in this study: 1/2a (82.1%; 95% CI: 63.1-93.9) and 1/2b (14.3%; 95% CI: 4.0-32.7) with one isolate (3.6%; 95% CI: 4.0-0.1-18.3) non-typable (Table 5.2). Serotype 1/2a was detected in carcase samples collected from abattoirs A, B and E, whereas serotype 1/2b and the non-typable isolates were only detected in samples from abattoir E.

Table 5.2 Serotypes of *L. monocytogenes* detected at six abattoirs in Taiwan

Serotype	Count (n = 28)	% isolates (95% CI)	Abattoir code
1/2a	23	82.1 (63.1-93.9)	A, B, E
1/2b	4	14.3 (4.0-32.7)	E
Non-typable	1	3.6 (0.1-18.3)	E

All 28 isolates were characterised into five distinct PFGE patterns: Lm-1, Lm-2, Lm-3, Lm4, and Lm-5 (Table 5.3). The pattern Lm-1 was detected at three abattoirs (A, B and E), whereas patterns Lm-2, Lm-3, Lm-4, and Lm-5 were only detected in samples from abattoir E.

The PFGE pattern of *L. monocytogenes* isolates at each processing stage is summarised in Table 5.4. In abattoir A, five isolates with PFGE pattern Lm-1 were isolated from samples collected from carcases in the chilling tank, and 10 isolates with the same pattern were detected after carcases had traversed the chilling tanks. In abattoir B, one isolate with PFGE pattern Lm-1 was detected in rinse samples from carcases in the chilling tank. In abattoir E, seven isolates with PFGE pattern Lm-1 were detected at three stages, *i.e.* after evisceration (n = 1), in the chilling tank (n = 2) and after chilling (n = 4). The patterns Lm-2 (n = 1), Lm-4 (n = 2) and Lm-5 (n = 1) were detected in samples collected after evisceration, and pattern Lm-3 was found in a single carcase sample after the chilling tank.

Table 5.3 PFGE patterns of *L. monocytogenes* isolated from poultry abattoirs in Taiwan and the processing stage at which they were detected

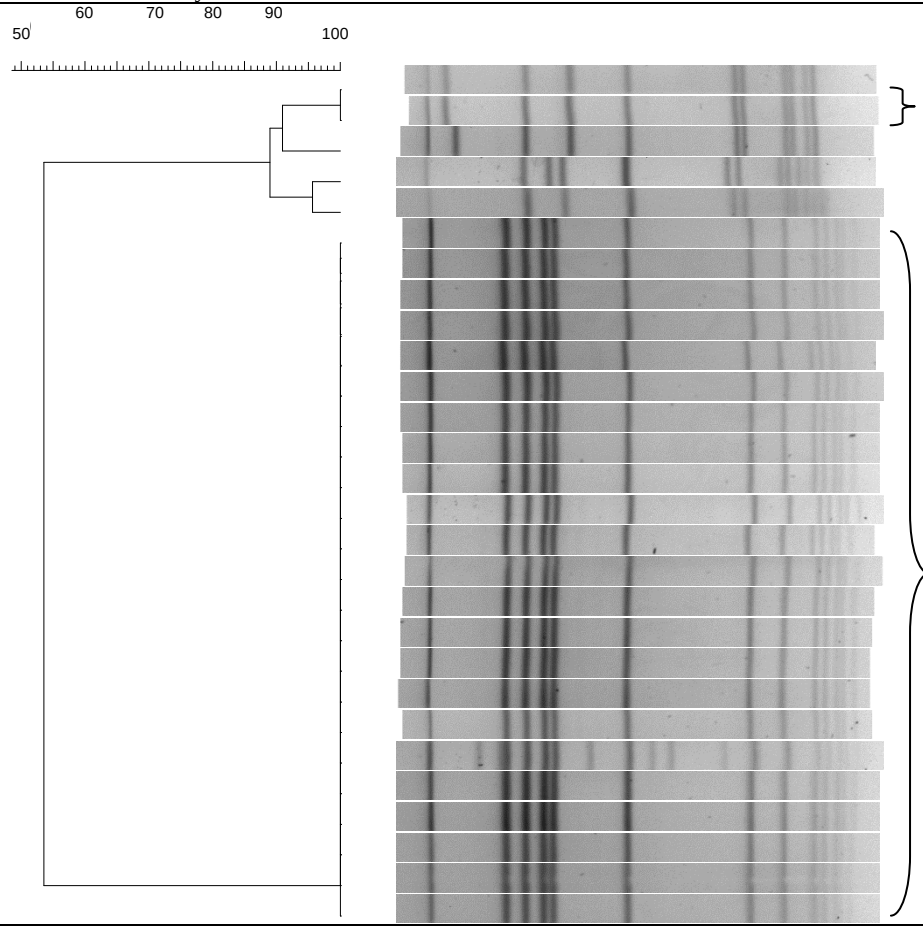
Similarity / *Asc*I	PFGE Patterns	Abattoirs	Sample stages	Serotype
	Lm-4	E	After evisceration	1/2b
		E	After evisceration	1/2b
	Lm-2	E	After evisceration	1/2b
	Lm-5	E	After evisceration	Non-typable
	Lm-3	E	After chilling tank	1/2b
		A	In chilling tank	1/2a
		A	In chilling tank	1/2a
		A	In chilling tank	1/2a
		A	In chilling tank	1/2a
		A	In chilling tank	1/2a
		A	After chilling tank	1/2a
		A	After chilling tank	1/2a
		A	After chilling tank	1/2a
		A	After chilling tank	1/2a
		A	After chilling tank	1/2a
		A	After chilling tank	1/2a
	Lm-1	A	After chilling tank	1/2a
		A	After chilling tank	1/2a
		A	After chilling tank	1/2a
		A	After chilling tank	1/2a
		B	In chilling tank	1/2a
		E	After evisceration	1/2a
		E	In chilling tank	1/2a
		E	In chilling tank	1/2a
		E	After chilling tank	1/2a
		E	After chilling tank	1/2a
		E	After chilling tank	1/2a
		E	After chilling tank	1/2a

Table 5.4 The number of *L. monocytogenes* isolates and their PFGE patterns found at each processing stage in the six abattoirs processing poultry in Taiwan

Abattoir	PFGE pattern (number of isolates)	Number of *L. monocytogenes* isolates at each sampling stage																
		Before processing						During processing										
		Plucking machine	Evisceration machine/table	Gloves of a carcase trimmer	Shackles/conveyer belt	Chilling tank water	Chilling tank inside surface	Cloacal swabs	Plucking machine	Carcase rinse samples after plucking	Evisceration machine/table	Carcase rinse sample after evisceration	Gloves of a carcase trimmer	Shackles/conveyer belt	Chilling tank water	Carcase rinse samples in chilling tank	Chilling tank inside surface	Carcase rinse sample after the chilling tank
A	Lm-1 (15)															5		10
B	Lm-1 (1)															1		
E	Lm-1 (7)											1				2		4
E	Lm-2 (1)											1						
E	Lm-3 (1)																	1
E	Lm-4 (2)											2						
E	Lm-5 (1)											1						

PFGE pattern and number of isolates detected from this stage

5.4 Discussion

In the current study no *L. monocytogenes* were cultured from cloacal swabs or from any environmental samples from the six abattoirs. The bacterium was only isolated from carcase samples collected after evisceration, in the chilling tank, and after traversing the chilling tank. This outcome implies that either the environment in the sampled broiler abattoirs did not support the persistence of *L. monocytogenes* or the bacterium was present below the detectable threshold of the adopted methodology. A study conducted by Chasseignaux et al. (2002) evaluated environmental factors associated with *L. monocytogenes* contamination on surfaces in poultry and pork processing plants and concluded that a smooth surface, such as stainless steel or tiles, maintained at a temperature above 10°C, offered protection against contamination with *L. monocytogenes*. In contrast a granular, stripped or damaged surface in resin or plastic under comparable conditions facilitated the survival of the bacterium. The findings of the current study matched those of Chasseignaux et al. (2002) whereby the majority of the surfaces sampled in the current study (plucking machine, evisceration machine/table, shackles, and chilling tank) were constructed from stainless steel. In addition, the current study was conducted during the Taiwan summer and room temperatures ranged from 11 to 32.7°C in the six abattoirs (data not shown). This finding was further supported by the psychrotrophic nature of *L. monocytogenes* (Pearson & Marth, 1990) and over-competition by other microflora at higher temperatures likely restricted the opportunity for *Listeria* to persist and be detected (Chasseignaux et al., 2002). These results indicated that the environmental conditions within the six abattoirs were not conducive to *L. monocytogenes* proliferation.

In the present study, no *L. monocytogenes* were detected from samples (n = 120) collected from the cloaca of processed poultry. Other studies have similarly failed to

detect *L. monocytogenes* or found a low prevalence (< 0.2%) in caecal samples from processed broilers, indicating that faecal material does not appear to be an important zoonosis for poultry (Cox et al., 1997; Rørvik et al., 2003). Although no *L. monocytogenes* were detected in cloacal samples, the bacteria were subsequently detected from carcases over a range of processing stages in three abattoirs (A, B and E). It was noteworthy that the prevalence in carcases sampled after the chilling tank at abattoir A and E (37.5%; 22.7-54.2) was significantly higher (χ^2: 16.08, df: 1, p = 0.0001) than for cloacal samples collected from birds processed at these abattoirs. Cox et al. (1997) similarly failed to detect *L. monocytogenes* from 115 caecal samples collected from birds processed at two slaughterhouses, although 27 of 105 (23.5%) carcases were subsequently found to be contaminated after the final chilling process. These results support the hypothesis that, even though there appears to be a very low prevalence of *L. monocytogenes* in the cloaca of birds at slaughter (below detection thresholds), there was still sufficient *L. monocytogenes* present to cause subsequent cross-contamination of carcases during processing. However, the failure to isolate *L. monocytogenes* from any of the water samples suggested proliferation did not occur in the chilling tank water, and direct contact between carcases in the chilling tank may facilitate cross-contamination.

Alternately, additional pathways for *L. monocytogenes* contamination within a poultry abattoir may include airborne transmission. Lues et al. (2007) have reported high counts of airborne *L. monocytogenes* (and other microorganisms) in a chicken abattoir. Similarly, Liang et al. (2013) identified bioaerosols containing *L. monocytogenes* could be distributed throughout a chicken abattoir by air movement, such as from the live-bird holding area to the slaughtering area and the evisceration, cutting, and packing areas. Whilst airborne transmission was not investigated as a possible route of contamination

in the present study, distribution of *L. monocytogenes via* bioaerosols throughout the abattoir environment could explain the pattern of contamination detected in samples. The significant level of cross-contamination occurring during processing highlighted the need for increased attention to be applied to determining and controlling both direct and indirect cross-contamination pathways during processing.

A study in Algeria, which compared the prevalence of *L. monocytogenes* on the neck skin of broilers during processing at three abattoirs, reported an overall prevalence of 8.9% from 212 samples, which increased from 0.1% at the evisceration stage to 17% at the end of processing (Bouayad et al., 2015). Similarly, Ojeniyi et al. (1996) investigated the prevalence of *L. monocytogenes* on the neck skin of broilers in seven Danish abattoirs and found prevalence of 22.5% for carcase samples collected before the spin chiller, compared with 45% in carcase samples after the spin chiller. These and the current findings highlight the occurrence of cross-contamination after evisceration and during chilling. It was notable that all samples from carcases after evisceration from abattoir E (n = 5) and from the chilling tank in abattoir A (n = 5) were positive for *L. monocytogenes.* Whilst prevalence increased as a result of traversing the chilling tank, no water samples from these tanks were culture positive.

In this study *L. monocytogenes* positive samples were detected immediately after evisceration only from abattoir E, which used a manual evisceration process. In contrast, no *L. monocytogenes* were detected from all four abattoirs (A, B, C and D) with automatic evisceration, as well as abattoir F which uses manual evisceration. In contrast to the current findings, the study conducted by Chiarini et al. (2009) reported a similar prevalence in abattoirs with automatic and manual evisceration processes (both 11.1%). In addition, during sampling in abattoir E, it was observed that the drainage hole of the evisceration table was often blocked by portions of viscera, which caused the

carcase-washing water to mix with intestinal contents. Furthermore, due to the slower speed of manual evisceration processing, multiple carcases were commonly piled together on the evisceration table, increasing the risk of cross-contamination. It is likely that these carcases were directly contaminated with the water/intestinal content mix, resulting in contamination with *L. monocytogenes* in all carcases sampled after evisceration (n = 5) in abattoir E. This finding implied that cross-contamination came from the contents of digestive tracts, rather than from the cloaca. This was supported by the findings of Adzitey et al. (2013) who reported a higher detection rate of *L. monocytogenes* in duck intestinal contents compared to cloacal contents.

In abattoirs A and B, *L. monocytogenes* isolates were only detected in carcase samples during and after the chilling tank phase. The psychrotrophic nature of the microorganism may help explain this phenomenon (Chiarini et al., 2009; Escudero-Gilete et al., 2007) as the temperature of the chilling tank was 2°C for abattoir A and 0.4°C for abattoir B, whereas the temperatures of other areas in these abattoirs varied from 11 to 32.7°C. Reiter et al. (2005) similarly found that *L. monocytogenes* were tolerant of low temperatures and were capable of surviving low-temperature processes. This ability allows *L. monocytogenes* to multiply in a low-temperature environment where other competitive microflora cannot grow (Chasseignaux et al., 2002). In addition, all carcase samples from the chilling tank in abattoir A (n = 5) were positive for *L. monocytogenes*. The possible mechanisms for this might have arisen from bird-to-bird contact during immersion chilling. Bacteria that were not fully attached to the skin of the broiler carcase could also become detached during the immersion chilling agitation, resulting in cross-contamination of other carcases in the chilling tank (Demirok et al., 2013). The results of the current study agreed with other studies where, *L. monocytogenes* were only detected in carcase samples at the end of

processing (Barbalho et al., 2005; Bouayad et al., 2015) which suggested that contamination occurred during or after the chilling step (Chiarini et al., 2009; Cox et al., 1997; Escudero-Gilete et al., 2007; Miettinen et al., 2001; Ojeniyi et al., 1996). Furthermore, Russell (2012c) stated that bacterial reduction (both numbers and prevalence) can best be accomplished during the chilling stage compared to at any other stage of processing in chicken abattoirs. All six abattoirs in the present study employed counter current flow, continuous overflow and air agitation during chilling, which was expected to improve both the effect of washing and chilling. However, the data indicated that the washing effect during chilling in abattoirs A and E was ineffective resulting in a high contamination rate of carcases (37.5%; 22.7-54.2) after chilling.

In the present study, no *L. monocytogenes* were detected in any of the water samples from the chilling tank during processing (n = 36). These results differed from those of Reiter et al. (2005) and Chiarini et al. (2009), who found that 12 of 30 samples and one of 8 chilling water samples were *L. monocytogenes*-positive, respectively. However, it was possible that the bacterium was present below the detectable threshold of the methodology used in the current study. Although 500 ml of chilling tank water were collected, only 30 ml was cultured. In future studies a larger volume of water should be cultured or a concentrating filtration method adopted to increase the sensitivity of the detection method.

The prevalence of *L. monocytogenes* on broiler carcases after chilling in the six abattoirs (12.5%; 7.2-19.8), with two of them adopting manual evisceration, was higher than in an earlier study conducted in Taiwan by Chen et al. (2004) who took rinse fluid samples from chicken carcases collected from all 14 Taiwanese slaughterhouses with automatic evisceration from 2000 to 2002 and reported a prevalence from 0.6 to 4.4%. The difference in the results from these two studies appears to echo the effects of the

different eviscerating methods described previously. The current findings were similar to those reported from a study of neck-skin samples in Algeria (8.5%; Bouayad et al., 2015), and lower than those reported from studies in the USA (25.7% with 100 ml UVM rinse fluid samples; Cox et al., 1997), Denmark (45% with neck-skin samples; Ojeniyi et al., 1996), and Norway (50% with carcase swabs samples; Rørvik et al., 2003). However, care needs to be taken in comparing these results due to the different sampling schemes and isolation methods used.

In the present study, the only *L. monocytogenes* serotypes detected were 1/2a and 1/2b. These are recognised as the predominant serotypes involved in human infections (48 patients) at a Taiwanese hospital from 1996 to 2008 (Huang et al., 2011). This indicated that the serotypes affecting humans in Taiwan were consistent with those detected at abattoirs. These findings were in agreement with other international studies (Batt, 2014; Ryser & Buchanan, 2012) where serotypes 1/2a and 1/2b were linked to the majority of listeriosis outbreaks in livestock, as well as sporadic cases in humans and other animals.

The PFGE technique is useful for tracing contamination during processing to enhance knowledge about the environmental conditions where *L. monocytogenes* can survive and develop within an abattoir (Chasseignaux et al., 2001; Lomonaco & Nucera, 2014). In the current study, PFGE pattern Lm-1 was detected from samples collected from carcases in and after chilling from abattoirs A and E. These findings highlighted the potential for contamination through direct contact between carcases in the chilling tank and after chilling, or potentially through tank water (Chiarini et al., 2009; Reiter et al., 2005). Furthermore, finding this PFGE pattern in samples after evisceration from abattoir E highlighted the potential for cross-contamination after evisceration.

Cox et al. (1997) proposed that the presence of *Listeria* spp. on poultry carcases might be influenced by the presence of asymptomatically infected birds, ineffective cleaning

of equipment, contaminated worker's hands or gloves, or the inability of some sanitising treatments to eliminate this microorganism. However, the entry point of *L. monocytogenes* to these abattoirs could not be established in the current study, due to the microorganism only being present in carcase samples, with no detection from the cloacal or environmental samples. It is noteworthy that all samples collected from carcases immediately after evisceration in abattoir E (n = 5) were positive for *L. monocytogenes*, which was likely due to direct contamination from the chiller water or intestinal contents present on the evisceration table. Further studies are required to focus on how *L. monocytogenes* are introduced to these broiler abattoirs and should include further sampling of cloacal and other digestive tract contents.

Lm-1 was detected in samples from abattoirs A, B and E, even though these abattoirs are located in different regions of the country and were sampled on different days with birds originating from different farms. This agreed with the results of Boerlin et al. (1997), Chasseignaux et al. (2001) and Bouayad et al. (2015), who postulated that some clones were widely spread through the movement of poultry *via* trade channels. The PFGE patterns, Lm-2, Lm-3, Lm-4 and Lm-5, were only found in a single stage in abattoir E, *i.e.* immediately after evisceration or after chilling, which indicated that these strains may have survived in the environment or on carcases at other stages, but were not detected by the sampling and isolation methods adopted. The low number of samples collected may have contributed to this finding and it is important that future studies expand both the number of samples collected and the points of sampling throughout the slaughter process.

The present study provides the first data regarding the sources of *L. monocytogenes* contamination and the potential for cross-contamination of carcases in broiler chickens post-slaughter in Taiwan. As all cloacal and environmental samples collected from the

six abattoirs were negative it is likely that faecal material and the abattoir environment are not important sources of *L. monocytogenes* for poultry carcases. Moreover, *L. monocytogenes* isolates were only detected in carcase samples after evisceration, which indicated the potential for contamination through direct contact between carcases during processing, or *via* contaminated water from the chilling tank. However, the route of entrance of *L. monocytogenes* to these abattoirs could not be established and further research is required to determine the source of *L. monocytogenes* for these abattoirs. Although the prevalence of *L. monocytogenes* on broiler carcases in Taiwan is not high, the serotypes detected were the same as those causing the majority of human listeriosis cases in the country, as well as in the rest of the world. This highlighted the potential danger of contamination of broilers with *L. monocytogenes* and requires close monitoring to minimise public health risks.

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5.6 Disclosure of potential conflicts of interest

No potential conflict of interest was reported by the author(s).

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CHAPTER 6: Identifying the Source of *Salmonella* spp. Contamination at a Chicken Abattoir in Taiwan Using Biomapping and PFGE

Preface

The preceding three chapters described investigations into the presence of *Salmonella* spp. and *Listeria monocytogenes* on chicken carcasses processed at 45 abattoirs throughout Taiwan, including sampling of carcasses at multiple processing stages at six abattoirs. However, these studies involved culturing samples collected on only a single working day/visit per abattoir. Although these studies generated valuable results on the prevalence of *Salmonella* spp. and *L. monocytogenes* on chicken carcasses at different processing stages in different abattoirs in Taiwan, they do not provide data on the change in contamination at individual processing sites with time or investigate the role that infection/contamination on-farm has on subsequent contamination of carcasses. This chapter presents the results of environmental sampling on-farm and intensive repeat sampling of chicken carcasses originating from 12 farms processed at a single abattoir over 12 consecutive processing days. Distribution biomaps were developed in combination with PFGE profiles of *Salmonella* isolates cultured from samples, to reveal where in the abattoir processing line *Salmonella* contamination of broiler carcasses occurred. These data were used to investigate and identify the potential sources of *Salmonella* contamination.

The manuscript outlined in this chapter is currently under review for publication in '***Preventive Veterinary Medicine***'.

Abstract

The present study used biomapping to determine the frequency of serotypes and Pulsed-Field Gel Electrophoresis (PFGE) subtypes of *Salmonella* isolated from broiler carcasses during processing at one abattoir in Taiwan, as well as from source broiler farms. Overall, *Salmonella* were detected in 209 of 576 samples (36.3%; 95% CI: 32.4-40.4) of carcasses from birds sourced from 12 poultry farms slaughtered on consecutive processing days. On-farm samples were also collected from 7 of these farms with 6 of 21 (28.6%; 95% CI: 11.3-52.2) staff footwear samples positive along with 8 of 70 (11.4%; 95% CI: 5.1-21.3) cloacal swabs collected from the same 7 batches of chickens as those subsequently sampled at the abattoir. The percentage of *Salmonella*-positive samples varied between sampling sites in the abattoir with 83.3, 22.9, 35.4, 34.4, 19.8 and 21.9% samples positive at post exsanguination, post plucking, post evisceration, post inside-outside bird washer (IOBW), post wash tank, and post air-chilling, respectively (overall χ^2 = 119.44, $p < 0.0001$). A total of 16 *Salmonella* serotypes were identified from the 223 isolates, with *S.* Enteritidis (35.0%), *S.* Albany (21.1%), and *S.* Brancaster (12.6%) being the most common. Characterization of the 223 isolates by PFGE identified 57 PFGE types. Typing detected the presence of the same PFGE type on the source farms of the processed birds and in carcass samples collected along the slaughter line, indicating that *Salmonella*-infected flocks are likely to be important sources for *Salmonella* contamination of carcasses at the abattoir. In addition, 14 strains predominated in the abattoir, being detected at multiple sites along the processing line. The results of this study highlight that biomapping, along with identification of the PFGE profiles of *Salmonella* isolates, can be used to identify cross-contamination points within an abattoir as well as trace potential sources of contamination.

6.1 Introduction

Salmonella are one of the most common food-borne pathogens of humans, being responsible for 95.1 million cases of enterocolitis with 50,771 deaths worldwide in 2017 (Lee et al., 2021). Contaminated poultry and poultry products have been identified as a major food source of human salmonellosis (EFSA, 2014; FAO/WHO, 2007; Mead et al., 2010). This contamination has been demonstrated to mainly occur during the slaughter process (Goksoy et al., 2004; Rasschaert et al., 2008), with *Salmonella*-infected flocks and the abattoir environment being important sources (Lin et al., 2020). Moreover, several authors have used Pulsed-Field Gel Electrophoresis (PFGE) techniques to further investigate the sources of *Salmonella* contamination and the contamination cycles of the slaughter process (Dias et al., 2016; Lee et al., 2019; Lin et al., 2020).

To reduce contamination by *Salmonella* in poultry and poultry products, many countries have implemented national programs for *Salmonella* monitoring (Ferrari et al., 2019); however, most monitoring programs involve collection of samples from a limited number of sites in individual abattoirs on only one or a few processing days/visits (Goksoy et al., 2004; Lee et al., 2019; Lin et al., 2020; Meloni et al., 2017). Consequently, interpretation of results is challenging, leading to difficulties in evaluating the role individual processing practices may have on carcass contamination in the abattoir environment. Russell (2012a) used biomapping of *Salmonella* isolates cultured from broiler carcasses at abattoirs over a continuous 12-day-period to assess which processes were effective in reducing the level of *Salmonella* and which processes required improvement.

The aim of this study was to combine *Salmonella* biomapping and PFGE to reveal where in an abattoir processing line *Salmonella* contamination of broiler carcasses occurred and to investigate the potential sources of this contamination, to highlight

where interventions either could be most effectively applied or required improving.

6.2 Materials and methods

6.2.1 Selection of the abattoir

This study was carried out in a chicken abattoir located in central Taiwan, which processes approximately 40,000 broiler chickens per day (average line speed of 5,000 per hour). Broilers are slaughtered at around 34 days old when they weigh approximately 2.2 kg. The main processing steps are holding, ante-mortem inspection, unloading and hanging, stunning, exsanguinating, scalding and plucking, venting and evisceration, post-mortem inspection, carcass wash (by inside-outside bird washer, and then by traversing a carcass washing tank) and air-chilling, followed by packing and labeling with an inspection certificate. All equipment and processes in the abattoir are required to meet the sanitary, safety and animal welfare regulations set by the Taiwan Bureau of Animal and Plant Health Inspection and Quarantine (BAPHIQ).

6.2.2 Sample collection

6.2.2.1 Cloacal swabs and samples from footwear in the source farms

To determine the *Salmonella* status of the source farms, one week prior to processing, cloacal swabs were collected from the same batch of birds that was subsequently sampled at the abattoir, along with swabs collected from plastic covers placed over the footwear (overboots) worn by workers in the shed from which these birds were sourced. Seven source farms, with an average batch/shed size of 34,000 chickens (range 19,000 – 46,000), were sampled corresponding to batches of broilers that were subsequently slaughtered and processed on days 1, 4, 8, 9, 10, 11, and 12 of the abattoir sampling. On the other five days (2, 3, 5, 6, and 7) of the study only carcasses sourced from five farms (average batch/shed size 45,000 birds; range 27,000 – 61,000) were sampled at the abattoir and no corresponding on-farm samples were able to be collected. All farms

adopted an all-in all-out practice for each shed, so the birds sampled on the 12 consecutive processing days were representative of the whole shed/batch. Sampling was performed by staff of the Agricultural Technology Research Institute (ATRI) in Taiwan. Ten cloacal swabs (Transystem™, COPAN, USA) were collected from randomly selected live chickens from the seven farms one week prior to the batch being processed, with each swab placed into 50 ml sterile tubes containing transport media (Amies agar gel). In addition, three pairs of overboots were collected from each farm at this time. This involved placing sterile overboots on the footwear of staff and requiring the wearer to walk around the poultry house containing the poultry batch that was to be subsequently sampled following the European Food Safety Authority guidelines (European Commission No 200/2012) (EC, 2012). To ensure that all sections of the poultry house were sampled, the poultry house was arbitrarily divided into thirds. One set of footwear covers was used to sample each third of the house, with the wearer required to take at least 100 steps in a zig-zag manner to ensure adequate coverage of the section. After collection, the cloacal swabs and footwear covers were put in a sterile bag and stored at 4℃ and transported to ATRI for bacterial culture within 24 hours of collection.

6.2.2.2 Carcass rinse samples in the abattoir

Sample collection at the abattoir occurred between 18 April and 4 May 2018 (over a period of 12 consecutive working days) and was performed by the official meat inspection veterinarians at the abattoir. The 12 farms/batches of interest (birds from the seven farms which had both live bird cloacal and footwear covers sampled, and birds from five farms that were not sampled on-farm) were the first, and often only (6 farms), batch processed on each of the respective sampling days at the abattoir to avoid the potential risk of cross-contamination from any other batches processed on the same day.

Carcass samples were collected at six sites (A-F) along the processing line to determine the influence of the different processes on contamination: (A) post exsanguination, (B) post plucking, (C) post evisceration, (D) post inside-outside bird washer (IOBW), (E) post wash tank, and (F) post air-chilling.

Eight whole carcasses from each batch were randomly selected and sampled at each of six sites on the 12 consecutive working days (576 individual carcasses sampled in total). Each carcass was aseptically placed into a 3,500 ml sterile "Stomacher-type" bag, and 400 ml of sterile 0.1% Buffered Peptone Water (BPW, BD Difco, NJ, USA) added. The bag was closed and shaken thoroughly with a rocking motion for 1 min at approximately 35 forward-and-backward swings per minute to wash the interior and exterior surfaces of the carcass. The carcass was then removed aseptically from the bag and the rinse fluid transferred to a 500 ml sterile bottle. Sterile gloves were worn by operators during sampling and were changed between carcasses. The chicken rinse samples were stored at 4℃ and transported to ATRI for bacterial culture within 24 hours of collection. After sampling, the randomly selected carcasses were placed back into the processing line. Due to the large number of carcasses processed from the 12 farms/batches of interest (~38,500) it was considered highly unlikely that the same carcass was sampled at multiple points along the processing line.

6.2.3 Bacteriology

6.2.3.1 *Salmonella* isolation

Samples were processed according to the methods described in the USDA/FSIS Microbiology Laboratory Guidebook (FSIS, 2014), European Food Safety Authority guidelines (European Commission No 200/2012) (EC, 2012) and modified ISO 6579-1:2017 method (Anonymous, 2017). Samples involved primary enrichment by the addition of: (1) 30 ml of double concentration (0.2%) BPW to 30 ml of poultry carcass

rinse fluid sample; (2) 5 ml of 0.1% BPW to the tube containing the cloacal swab; and (3) a sterile sponge (Nasco Whirl-Pak Speci-Sponge Bags) pre-moistened with 30 ml of 0.1% BPW and used to aseptically swab the entire surface of each pair of footwear covers. All samples were incubated at 35°C for 20-24 h, following which a 100 µl aliquot of each of the primary enrichment cultures was inoculated into 10 ml of Rappaport-Vassiliadis broth (Oxoid) for secondary (selective) enrichment and incubated at 42°C for 24 h (Andrews, 1998). A loopful of Rappaport-Vassiliadis culture was then streaked onto Xylose Lysine Desoxycholate (XLD; Difco, BD), Hektoen enteric (HE) agar (Difco, BD) and *Salmonella* identification (SI) agar (CHROmagar Microbiology, Paris) and incubated at 35°C for 24 hours. Colonies with typical *Salmonella* characteristics, including colonies that were pink on XLD agar plates, blue-green to blue with or without black centers on HE agar plates and mauve color on SI agar, were confirmed with API 20E (bioMe´rieux) biochemical assays. Samples with one or more colonies identified as *Salmonella* were classified as positive. Isolates identified as *Salmonella* were serotyped by slide and tube agglutination tests with *Salmonella* polyvalent O and H antisera (Difco, BD) according to the Kauffmann–White scheme (Grimont & Weill, 2007). Due to resource constraints only one colony from each positive sample was serotyped.

6.2.3.2 Pulsed-field gel electrophoresis (PFGE)

Salmonella isolates were typed by PFGE according to the "Standard Operating Procedure for PulseNet PFGE of *Escherichia coli* O157:H7, *Escherichia coli* non-O157 (STEC), *Salmonella* serotypes, *Shigella sonnei* and *Shigella flexneri*" (CDC, 2013a) and as per the methodology outlined in Lin et al. (2020). Briefly, a pure culture of each isolate was obtained by inoculating one colony onto blood agar. One colony was then selected and inoculated onto Tryptone Soy Agar and the pure bacterial cultures treated

to release chromosomal DNA, before digesting with 50 U of *Xba*I (NEB, MA) for 1.5-2 h at 37°C. Electrophoresis was carried out with 0.5 x Tris-borate-EDTA buffer at 14°C for 19 h using a CHEF Mapper electrophoresis system (Bio-Rad, Hercules, CA). Pulse times were ramped from 2.2 to 63.8 s during an 18 h run at 6.0 V/cm. The genomic DNA of *Salmonella* Braenderup H9812 was digested by *Xba*I and used as a molecular size marker. Gels were stained with U-Safe Nucleic Acid Gel Staining Dye (Bio-Genesis, Taiwan), and DNA bands visualized with UV transillumination (UVP). The PFGE patterns were analyzed with BioNumerics software (Applied Maths, Kortrijk, Belgium), using the unweighted pair group method with an arithmetic mean (UPGMA). The relatedness of the PFGE profiles was estimated based on the presence or absence of shared bands. Isolates with the same pattern from the farms and the abattoir were considered to be the same PFGE type.

6.2.4 Statistical analysis

The unit of study was the individual sample. The results of the percentage of *Salmonella*-positive carcass samples were compared between sampling sites at the abattoir. In each analysis, specific comparisons were made using the chi-square test for independence, and a p-value < 0.05 was considered statistically significant. Data were analyzed using Statistix 9 (Analytical Software, Tallahassee, Florida).

6.3 Results

6.3.1 The percentage of *Salmonella*-positive cloacal and footwear covers swab samples from the broiler source farms

Three of the seven farms contained birds that were culture-positive for *Salmonella* from cloacal swabs (8/30) and footwear samples (6/9), but no *Salmonella* were found from 40 cloacal swabs and 12 footwear samples from the remaining 4 farms (Table 6.1). Overall, 6 of 21 (28.6%; 95% CI: 11.3-52.2) footwear samples and 8 of 70 (11.4%; 95% CI:

5.1-21.3) cloacal swabs collected from live chickens at the source farms were positive for *Salmonella* (Table 6.1). No significant difference was observed in the percentage of *Salmonella*-positive results from footwear and cloacal samples (χ^2 = 3.65, p = 0.056).

Table 6.1 Summary of the samples collected and the frequency of *Salmonella* spp. detection from the source farms

Slaughtering day	Number of samples (+ve/total samples) collected from source farms	
	Cloacal swabs	**Overboots (footwear covers)**
D01	0/10	0/3
D02	NS[#]	NS
D03	NS	NS
D04	1/10	2/3
D05	NS	NS
D06	NS	NS
D07	NS	NS
D08	3/10	1/3
D09	0/10	0/3
D10	0/10	0/3
D11	0/10	0/3
D12	4/10	3/3
Total	**8/70**	**6/21**
+%	**11.4**	**28.6**
95% CI	**5.1-21.3**	**11.3-52.2**

[#]NS: not sampled

6.3.2 The percentage of *Salmonella*-positive carcass samples in the abattoir

Salmonella were detected in 209 of 576 (36.3%; 95% CI: 32.4-40.4) carcass rinse fluid samples (Table 6.2). The change in the percentage of *Salmonella*-positive samples along the processing chain is displayed in Figure 6.1. The percentage of *Salmonella*-positive

carcass samples was significantly different between sampling sites over the 12 day-period (overall χ^2 = 119.44, $p < 0.0001$). The highest percentage of positive samples (83.3%, 95% CI: 74.4-90.2) was detected at the first sampling site (A: Post exsanguination). The percentage of positive samples decreased significantly to 22.9% (95% CI: 15.0-32.6; χ^2 = 70.36, $p < 0.0001$) at the second sampling site (B: Post plucking) (Table 6.2). However, no significant difference was observed in the percentage of *Salmonella*-positive carcass samples between post plucking (22.9%; 95% CI: 15.0-32.6) and after evisceration (C: Post evisceration, 35.4%; 95% CI: 25.9-45.8) (χ^2 = 3.63, p = 0.057). After the carcasses passed through the fourth site (D: Post IOBW), the percentage decreased slightly to 34.4% (95% CI: 25.0-44.8; χ^2 = 0.02, p = 0.88). The lowest percentage of positive samples (19.8%, 95% CI: 12.4-29.2) was detected after the carcasses passed through the wash tank (E: Post wash tank), and this was significantly lower than at post IOBW (χ^2 = 5.17, p = 0.02). The percentage of positive samples then increased slightly, but not significantly (χ^2 = 0.13, p = 0.72), to 21.9% (95% CI: 14.1-31.5) at the final sampling site (F: Post air-chilling) (Table 6.2).

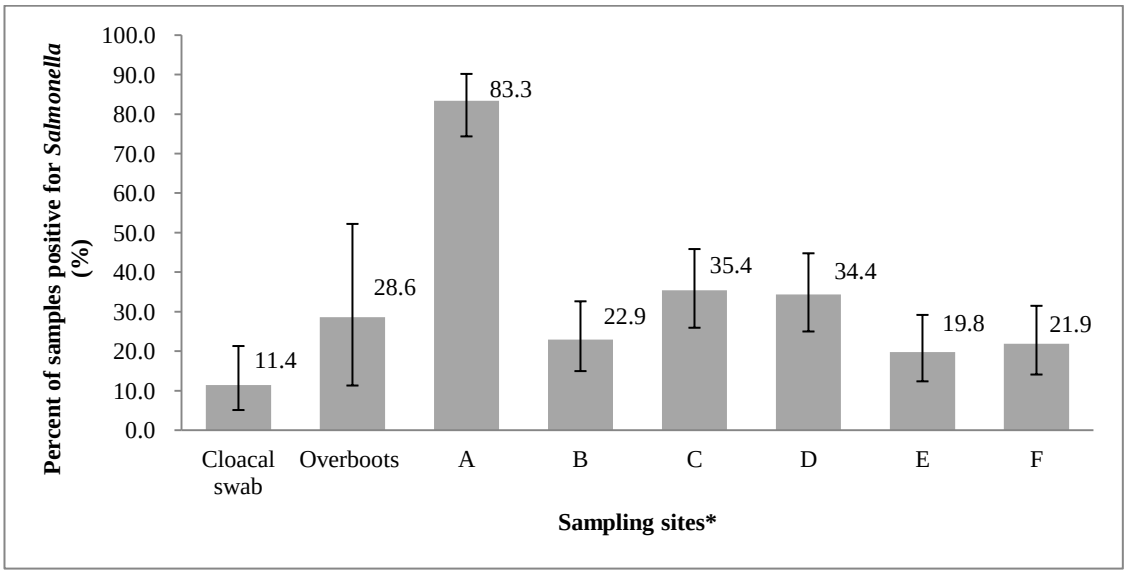

Figure 6.1 The percentage of *Salmonella*-positive samples at different sites at the selected farms and abattoir.
*Cloacal swab and overboot samples were only available from 7 source farms. Carcass samples were sampled from 12 flocks at the abattoir from different sampling sites: A. Post exsanguination; B. Post plucking; C. Post evisceration; D. Post inside-outside bird washer (IOBW); E. Post wash tank; and F. Post air-chilling. Error bars represent 95% confidence intervals.

The overall percentage of *Salmonella*-positive cloacal samples from the seven source farms (11.4%) was significantly (χ^2 = 66.48, p < 0.0001) lower than the carcass rinse samples from the same flocks immediately post exsanguination (site A) (83.9%; 95% CI: 71.7-92.4).

Table 6.2 Overview of the frequencies of *Salmonella* spp. detected at different sampling sites in the abattoir

Slaughtering day	Number of positive samples taken at each site in the abattoir*						Total	% positive	95% CI
	A	B	C	D	E	F			
D01	3	0	0	1	1	0	**5**	**10.4**	**3.5-22.7**
D02	4	2	2	1	0	0	**9**	**18.8**	**8.9-32.6**
D03	6	1	1	2	1	0	**11**	**22.9**	**12.0-37.3**
D04	7	3	4	3	0	3	**20**	**41.7**	**27.6-56.8**
D05	8	1	1	1	1	1	**13**	**27.1**	**15.3-41.8**
D06	7	3	5	4	1	2	**22**	**45.8**	**31.4-60.8**
D07	8	4	7	6	6	5	**36**	**75.0**	**60.4-86.4**
D08	6	0	4	2	0	2	**14**	**29.2**	**17.0-44.1**
D09	7	3	3	5	0	4	**22**	**45.8**	**31.4-60.8**
D10	8	1	0	1	0	0	**10**	**20.8**	**10.5-35.0**
D11	8	0	2	0	4	1	**15**	**31.3**	**18.7-46.3**
D12	8	4	5	7	5	3	**32**	**66.7**	**51.6-79.6**
Total	**80**	**22**	**34**	**33**	**19**	**21**	**209**	**36.3**	**32.4-40.4**
% positive	**83.3**	**22.9**	**35.4**	**34.4**	**19.8**	**21.9**	**36.3**		
95% CI	**74.4-90.2**	**15.0-32.6**	**25.9-45.8**	**25.0-44.8**	**12.4-29.2**	**14.1-31.5**	**32.4-40.4**		

*Eight carcass samples from each sampling site were collected on each slaughtering day. Sites represent: A. Post exsanguination, B. Post plucking, C. Post evisceration, D. Post inside-outside bird washer (IOBW), E. Post wash tank, and F. Post air-chilling.

6.3.3 Distribution of *Salmonella* serotypes and PFGE types

Of the 223 *Salmonella* isolates, a total of 16 unique serotypes were identified, with only one untypable isolate (0.5%) (Table 6.3). Overall, the most prevalent serotypes were *S.* Enteritidis (35.0%; 95% CI: 28.7-41.6), *S.* Albany (21.1%; 95% CI: 15.9-27.0), *S.* Brancaster (12.6%; 95% CI: 8.5-17.6), *S.* Schwarzengrund (8.1%; 95% CI: 4.9-12.5) and *S.* Anatum (6.7%; 95% CI: 3.8-10.9). Five serotypes (*S.* Anatum, *S.* Albany, *S.* Enteritidis, *S.* Give and *S.* Schwarzengrund) that had been detected on-farm were also found in samples collected at the abattoir (Table 6.3).

The 223 isolates were characterized into 57 PFGE types with, again, 1 isolate untypable. *Salmonella* Enteritidis strain E-01 was the most prevalent (76 isolates) followed by *S.* Albany strain Alb-10 (14 isolates) and *S.* Albany strain Alb-07, *S.* Anatum strain An-01 and *S.* Brancaster strain B-01 (all with 13 isolates each) (Figures 6.2 and 6.3).

Three PFGE types (Alb-11 on day 12; An-01 on day 12; E-01 on day 4) were detected in samples collected from both the source farms and the carcasses of broilers from those farms (Figure 6.2). In addition, 10 PFGE types (Alb-07 on days 5, 9, 11; Alb-10 on days 2, 6, 10; Alb-13 on day 12; B-01 on day 8; B-02 on day 6; B-04 on day 6) were detected on carcasses at site A and then were also found on carcasses from the same batch/farm at subsequent sites in the abattoir on the same day of sampling (Figure 6.2). In contrast, 3 PFGE types (E-01 on days 3, 6, 7, 9, 11, 12; N-01 on day 8; T-02 on day 5), although not detected at site A, were subsequently detected at two or more sites on the same day of processing (Figure 6.2). Although the data used to generate the biomaps displayed in Figures 6.2 and 6.3 are the same, they are presented in a different format. Figure 6.2 highlights when the *Salmonella* are introduced and helps identify the possible sources of contamination on the slaughtering day. In contrast in Figure 6.3 the processing stages resulting in on-going contamination are identified, and this figure highlights the need to

implement interventions to specific processing steps to minimize this contamination.

Nine PFGE types (Alb-07; Alb-10; B-01; B-07; L-01; M-01; Sc-03; Sc-04; T-02) were detected at site A on all 12 sampling days (Figure 6.3). PFGE type *Salmonella* Enteritidis strain E-01 was repeatedly detected at the same sampling sites on different sampling days (Figure 6.3).

6.4 Discussion

This study investigated the change in the prevalence of *Salmonella* in broilers from the farm through the abattoir processing steps (Figure 6.1). The results from sampling site A reflect the status of *Salmonella* in/on chickens when they arrive at the abattoir, indicating these birds were harboring high levels of contamination (Finstad et al., 2012). The percentage of *Salmonella*-positive carcass rinse samples at site A (83.3%), the first sampling point in the abattoir, was significantly higher than the cloacal swabs collected from batch "mates" from the originating flocks (11.4%) (Figure 6.1). Similar findings were also reported by Rigby et al. (1980), who found that contamination of the feathers of birds with *Salmonella* was more common than intestinal carriage. The detection of a significantly lower prevalence of *Salmonella* (22.9%) in samples collected from carcasses post plucking (site B), compared with site A (83.3%), suggests that the hygienic practices implemented between these steps, i.e. scalding and plucking, are effective at reducing, but not eliminating, contamination.

Table 6.3 Number of *Salmonella* serotypes detected on-farm and at each slaughtering day at the abattoir

***Salmonella* Serotype (abbreviation)**	**On Farm**	**Slaughtering day**												**Total**	**%**	**95% CI**
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**			
Enteritidis (E)	2	1		2	12		5	27		13	1	5	10	**78**	**35.0%**	**28.7-41.6**
Albany (Alb)	1	1	3	2	3	3	5	1	2	4	3	6	13	**47**	**21.1%**	**15.9-27.0**
Brancaster (B)		1	1	1		1	10	7	4	1	2			**28**	**12.6%**	**8.5-17.6**
Schwarzengrund (Sc)	3	1	2	1	2	1				3	4	1		**18**	**8.1%**	**4.9-12.5**
Anatum (An)	7												8	**15**	**6.7%**	**3.8-10.9**
Newport (N)									7	1				**8**	**3.6%**	**1.6-6.9**
Livingstone (L)			1	3		2								**6**	**2.7%**	**1.0-5.8**
Typhimurium (T)						4	1	1						**6**	**2.7%**	**1.0-5.8**
Give (G)	1		1	2					1					**5**	**2.2%**	**0.7-5.2**
Hadar (H)						1						2		**3**	**1.3%**	**0.3-3.9**
Muenster (M)					1		1					1		**3**	**1.3%**	**0.3-3.9**
Agona (Ag)		1												**1**	**0.4%**	**0.0-2.5**
Alachua (Ala)					1									**1**	**0.4%**	**0.0-2.5**
Chester (Ch)						1								**1**	**0.4%**	**0.0-2.5**
Corvallis (Co)					1									**1**	**0.4%**	**0.0-2.5**
Weltevreden (W)													1	**1**	**0.4%**	**0.0-2.5**
Untypable (UT)			1											**1**	**0.4%**	**0.0-2.5**
Total	**14**	**5**	**9**	**11**	**20**	**13**	**22**	**36**	**14**	**22**	**10**	**15**	**32**	**223**		

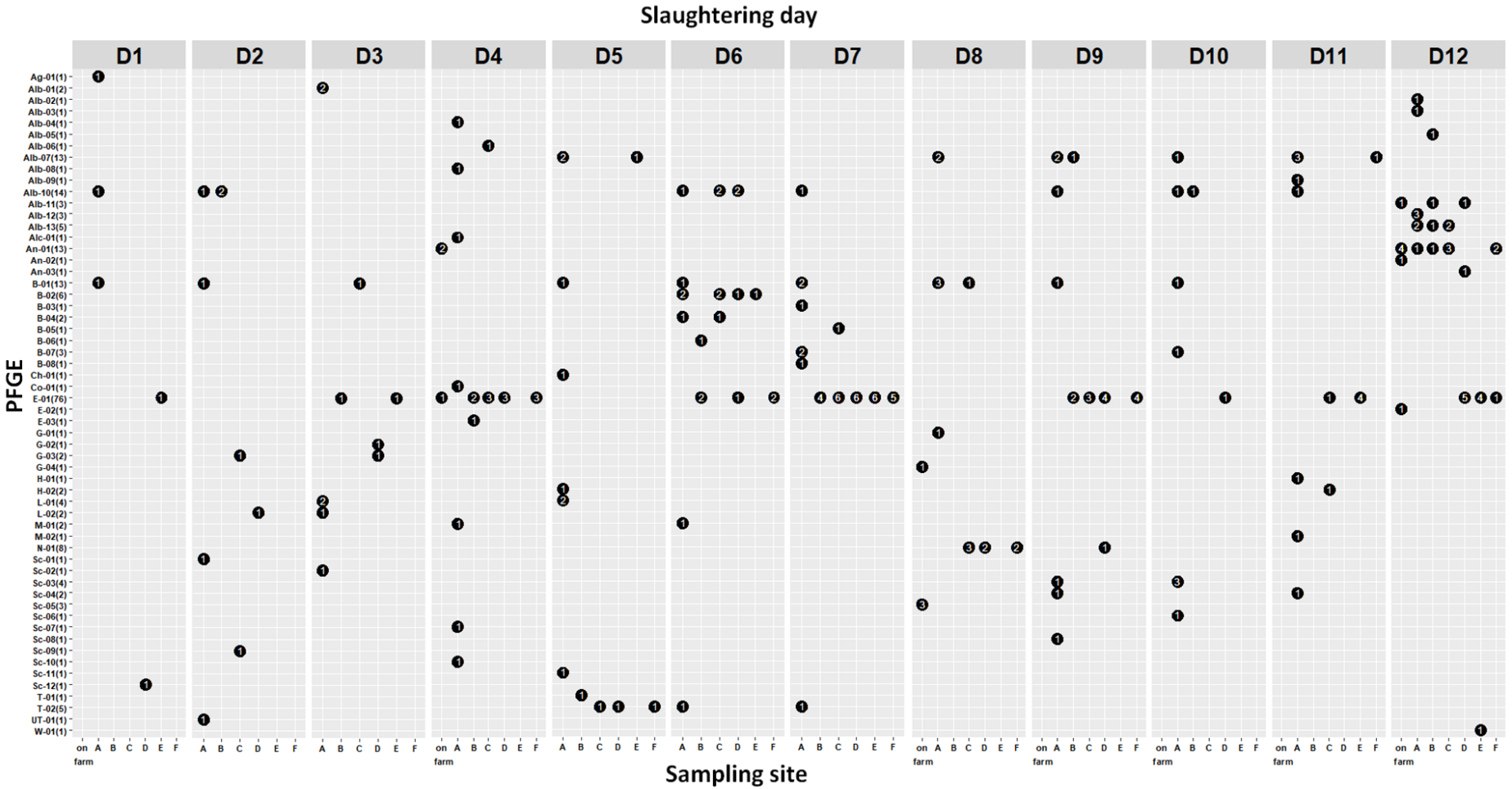

Figure 6.2 The PFGE patterns of *Salmonella* isolates on each slaughtering day and at each sampling site in the abattoir. PFGE patterns are indicated by numerical sampled suffixes after a capital letter indicating the abbreviated name of the serotype and the numbers in brackets and in circles indicate the number of strains with this pattern.

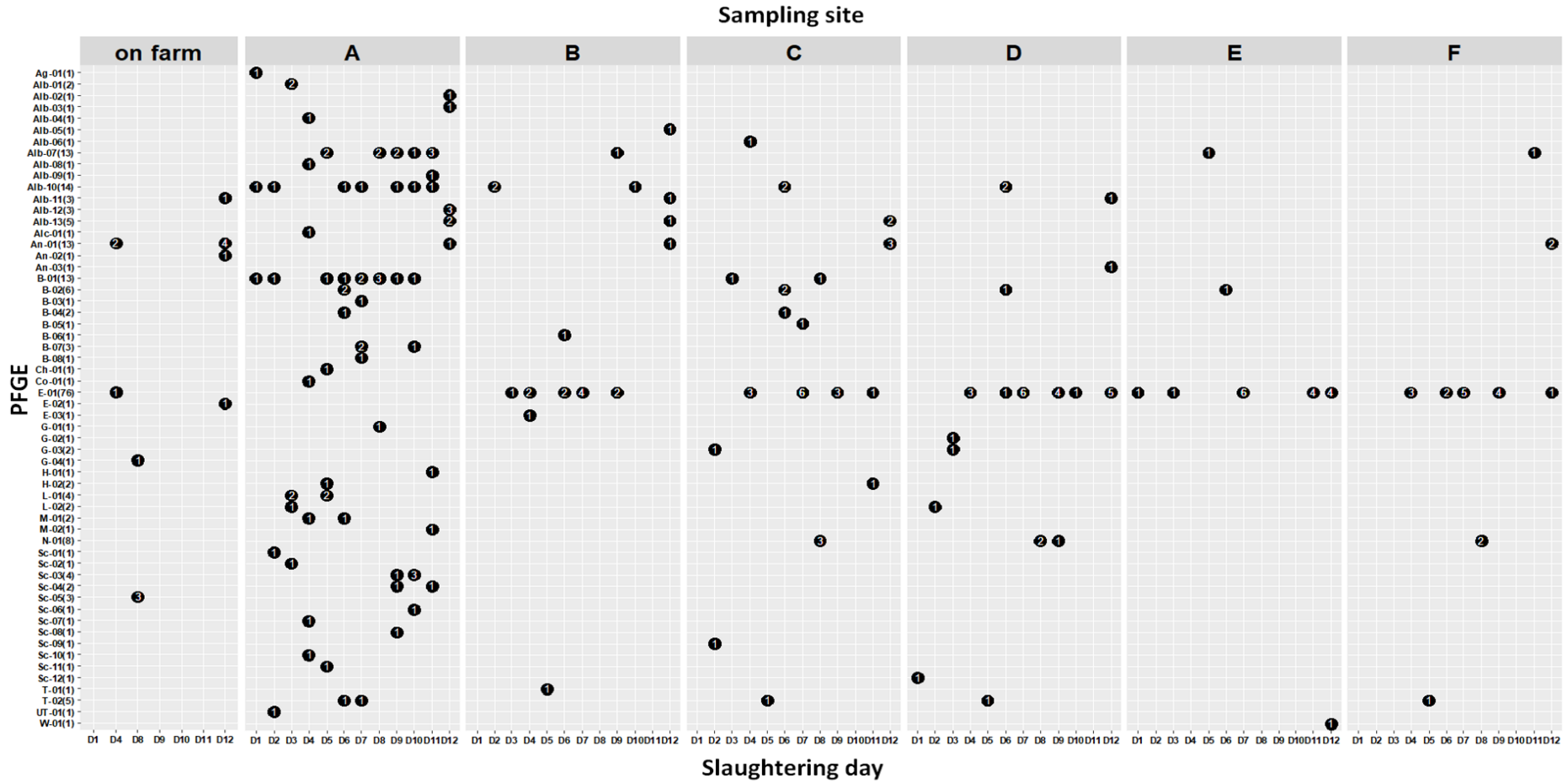

Figure 6.3 The PFGE patterns of *Salmonella* isolates at each sampling site and on each slaughtering day in the abattoir. PFGE patterns are indicated by numerical sampled suffixes after a capital letter indicating the abbreviated name of the serotype and the numbers in brackets and in circles indicate the number of strains with this pattern.

Molecular genotyping of *Salmonella* isolates by PFGE provides valuable epidemiological information about the nature of contamination encountered in the abattoir environment (Lin et al., 2020). This technique can also be used to improve the accuracy of identifying sources of carcass contamination (Arguello et al., 2013). In the present study, 57 unique *Salmonella* PFGE types were identified from multiple sampling sites throughout the 12 day-sampling period.

On multiple occasions the biomap (Figure 6.2) demonstrated the presence of the same PFGE type at different processing stages on the same slaughtering day. This finding supports the hypothesis that some PFGE types were introduced into the abattoir environment on the day of slaughter, with subsequent cross-contamination occurring along the processing chain. This result also highlights that the hygienic practices adopted within the abattoir failed to prevent these cross-contaminations. This biomapping approach can also be applied to track the possible source of contamination of introduced strains and therefore identify possible contamination routes.

Three PFGE types (Alb-11 on day 12; An-01 on day 12; and E-01 on day 4) from birds sampled on the supply farms were subsequently found on samples of carcasses from the same source batch at most processing steps in the abattoir (Figure 6.2). This finding was similar to that reported by others (Corry et al., 2002; Lin et al., 2020; Nógrády et al., 2008; Olsen et al., 2003), and highlights that *Salmonella*-infected/contaminated flocks are an important source of post-slaughter carcass contamination. This was further supported by the finding that 10 PFGE types (Alb-07 on days 5, 9, 11; Alb-10 on days 2, 6, 10; Alb-13 on day 12; B-01 on day 8; B-02 on day 6; and B-04 on day 6) were detected at both site A and subsequent processing steps on the same slaughtering day (Figure 6.2). The FAO/WHO (2002) reported that chickens could be infected with *Salmonella* either on the originating farms or be contaminated during transportation to

the abattoir.

In contrast 3 PFGE types (E-01 on days 3, 6, 7, 9, 11, 12; N-01 on day 8; T-02 on day 5) were not found at site A, but were detected from carcasses sampled at two or more subsequent processing sites (Figure 6.2). This contamination may have resulted from either direct contact between carcasses or have come from the environment after it had been contaminated by carcasses processed on previous days. This is supported by the finding that the same PFGE types were detected at the same sampling site in the abattoir on different slaughtering days (Figure 6.3), indicating that these strains may have survived the cleaning and disinfection processes undertaken after the conclusion of each day's processing/slaughtering. Unfortunately, no environmental sampling was performed in this study. It is recommended that future studies include environmental sampling at the abattoir after the completion of end-of-processing cleaning and disinfection to determine the effectiveness of such procedures.

Nine PFGE types (Alb-07; Alb-10; B-01; B-07; L-01; M-01; Sc-03; Sc-04; T-02) were detected at site A and these were detected for 2 to 8 days (Figure 6.3) implying introduction from new batches processed or persistence in the environment, even after daily cleaning was complete. As the other flocks that were processed on the 12 study days but which were not included in the sampling originated from multiple farms from different regions of Taiwan, it is likely that some PFGE types are widely distributed in the country's broiler industry. These findings are similar to those of Bouayad et al. (2015) and Lin et al. (2021), who reported that the ubiquitous nature of some PFGE types of *Listeria monocytogenes* in Algeria and in Taiwan, respectively, was likely due to the movement of poultry via routine trade channels.

The detection of the PFGE type *Salmonella* Enteritidis strain E-01 at the same sampling sites on different days (Figure 6.3) is of particular interest, especially since this PFGE

type was never detected at site A. This finding could indicate that this type was not introduced from the source farms of the sampled broilers and increased detection within the abattoir environment/equipment suggests inadequate cleaning/disinfection between processing days (Lin et al., 2020). However, the failure to detect this type at the initial sampling site (A) may also be a result of selecting only one colony from each positive sample for typing due to financial and personnel constraints. It is possible that multiple serotypes and/or PFGE types were present in individual samples. Although the approach adopted in this study would not have affected the overall proportion of positive samples, the percentage of specific serotypes or PFGE profiles may have been affected by the sampling methodology. It has been suggested that, for similar investigations with *L. monocytogenes*, 3 colonies should be selected for typing per sample (Nucera et al., 2010) and it is recommended that this approach is undertaken in future studies with *Salmonella*.

The high humidity or moisture content in the environment of poultry abattoirs favors the formation of *Salmonella* spp. biofilms, and this is considered an important environmental adaptation for the bacterium as it provides protection against the action of detergents or sanitizing agents (Chuah et al., 2018; Dantas et al., 2020). These findings also highlight the critical need for implementing practices to reduce cross-contamination, including continuous spraying of equipment and carcasses, during plucking (FAO/WHO, 2011; FSANZ, 2005), along with cleaning and sanitation operations to eliminate potential persistent cross-contamination sites which can harbor the bacterium (Williams et al., 2011).

Seventy-five isolates of E-01 strain were detected after feather plucking (sites B to F) over a period of 12 consecutive working days, accounting for 58.1% (75/129) of all *Salmonella* isolated after plucking (Figure 6.3). If this predominant strain could be

eliminated from the abattoir environment, contamination of carcasses with *Salmonella* in this abattoir would be significantly reduced. At post air-chilling (site F), a total of 21 isolates (5 PFGE types) of *Salmonella* were detected, of which 15 were E-01 strain (71.4%) (Figure 6.3). Again, if this PFGE type could be eliminated, the overall percentage of the final product contaminated with *Salmonella* could be reduced from 21.9% (21/96) to 6.3% (6/96). This emphasizes again the crucial importance of implementing effective cleaning and sanitation throughout the abattoir and should be the main focus for the control of such pathogens in the poultry meat processing line (Iannetti et al., 2020).

This study used *Salmonella* biomapping to assess the change in the percentage of *Salmonella*-positive samples from the farm and along the processing line and to reveal which steps of the process could either reduce carcass contamination or need to be re-evaluated (improved). Identification of the PFGE profiles of the *Salmonella* isolates enables confirmation of the route of carcass cross-contamination in the abattoir and tracing of the source of contamination. This information is invaluable for the poultry industry to improve meat hygiene and food-safety.

The predominant serotypes detected in the present study were: *S.* Enteritidis (35.0%), *S.* Albany (21.1%), *S.* Brancaster (12.6%), and *S.* Schwarzengrund (8.1%) (Table 6.1). Except for *S.* Brancaster, the other three types were also the most common serotypes detected in the research of Lin et al. (2008) who isolated these and other *Salmonella* spp. from the liver, gall bladder and cecal contents of broilers in Taiwan. In addition, *S.* Enteritidis and *S.* Albany are the two primary serotypes associated with human salmonellosis infections in Taiwan (Chiou et al., 2019). This finding demonstrates that the *Salmonella* serotypes affecting humans in Taiwan are consistent with those found at abattoirs, supporting the belief that contaminated poultry meat is one source of human

salmonellosis.

It is noteworthy that, although *S.* Brancaster was the fourth most common serotype detected in this study (12.6% of all serotypes), it has not been detected in other studies from Taiwan, including a large survey of broiler carcasses conducted by BAPHIQ involving 589 *Salmonella* isolates from 45 abattoirs during 2013 to 2014 (data unpublished). Similarly, *S.* Brancaster was not detected by Lin et al. (2008) who reported on 570 *Salmonella* isolates from 3 abattoirs and 2 retail stores during 2006 to 2007, nor in the study of Lin et al. (2020) of 156 *Salmonella* isolates from 6 abattoirs in 2014. These results may indicate that *S.* Brancaster could be an emerging *Salmonella* enterica serovar in Taiwan and needs further monitoring as this serovar has been associated with human salmonellosis through eating contaminated poultry meat in Senegal (Cardinale, Perrier Gros-Claude, et al., 2005).

6.5 Conclusions

Overall, this study utilized *Salmonella* biomapping (Figure 6.1) developed by Russell (2012a) to assess the change in the percentage of *Salmonella*-positive samples along the processing line at one abattoir in Taiwan, and to evaluate those stages early in the processing chain where cross-contamination is most likely compared to later stages which are generally more effective at reducing contamination. Distribution biomaps (Figures 6.2 & 6.3) were developed and, when combined with the PFGE profiles of the *Salmonella* isolates, highlighted the potential route of carcass cross-contamination in the abattoir. It is recommended that these methods are used in all abattoirs to trace the potential source of contamination. This study also confirmed that processing birds from *Salmonella*-infected/contaminated flocks could result in the subsequent contamination of carcasses with several persistent strains predominating in the abattoir. This information can assist the poultry industry to accurately find out the *Salmonella*

contamination problems in abattoirs and to develop preventive and control interventions to solve them, resulting in enhanced meat hygiene and food safety.

6.6 Acknowledgments

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Chapter 7: General Discussion

Foodborne diseases are considered a major human health issue resulting in morbidity and mortality worldwide (Gul et al., 2016). Of the bacterial foodborne pathogens, *Salmonella* is the leading cause of foodborne disease in Taiwan (Lai et al., 2020) and throughout the world (Antunes et al., 2016), and *L. monocytogenes* is recognized to have the highest case fatality rate of all foodborne acquired infections in Taiwan (Huang et al., 2015) and in the EU (EFSA, 2014). Poultry meat is currently the most consumed and affordable meat in the world, highlighting the importance of this commodity to food security (Antunes et al., 2016; Chai et al., 2016; Machado Junior et al., 2020). However; apparently healthy poultry can carry potential foodborne pathogens, and meat contaminated with these pathogens is a major source of human infection (Machado Junior et al., 2020). Furthermore, contaminated poultry and poultry products have been identified as the main food sources that cause human salmonellosis and listeriosis (Finstad et al., 2012; Mead et al., 2010; Morar et al., 2014; Olsen et al., 2005), with these contaminations usually occurring in abattoirs during the slaughtering and processing stages (Cardinale, Tall, et al., 2005; Cox et al., 1997; Rørvik et al., 2003; Rasschaert et al., 2007). Efforts involving surveillance and biosecurity have resulted in a significant reduction in the number of foodborne diseases in Europe, highlighting the importance of effective control measures, particularly during poultry processing (Machado Junior et al., 2020).

Since 2018 poultry has become the most commonly consumed meat in Taiwan (COA, 2020b). In addition, the number of officially approved poultry abattoirs in Taiwan has increased from 19 in 2002 to 115 in 2019 (BAPHIQ, 2020) due to the progression in meat safety management policies (Juan, 2013) and expansion of the poultry industry

(COA, 2020a). These changes highlight the importance and urgency of the need for studies to: identify risk factors that are associated with poultry carcass contamination with "target" pathogens; identify the potential source and niche of these target pathogens; and evaluate control interventions to reduce or prevent product contamination with these pathogens.

The research reported in this thesis included a nationwide investigation of the prevalence of *Salmonella* spp. in Taiwanese broiler carcasses at slaughter and an analysis of the risk factors associated with the presence of *Salmonella* spp. in batches of broiler carcasses at processing. In addition, systematic analyses of the contamination with *Salmonella* spp. and *L. monocytogenes* of chickens processed at six abattoirs in Taiwan with different types of processing stages and slaughtering equipment were conducted to support and strengthen the existing processing procedures and to identify stages requiring improvement. Furthermore, distribution biomaps were developed and combined with the PFGE profiles of *Salmonella* isolates to identify cross-contamination points within an abattoir as well as to trace potential sources of contamination. The findings of the research reported in this thesis will, not only assist the poultry industry identify pathogen sources and their niches and evaluate control interventions, but also assist in reducing the risk of contamination of processed chickens with important pathogenic bacteria. In this final chapter, the overall findings of this study are reviewed, areas that need further research are highlighted and limitations of the current study are discussed.

7.1 Prevalence of contamination with important potentially pathogenic bacteria of processed chickens in Taiwan

Prior to the studies outlined in this thesis, there was little information available regarding *Salmonella* spp. contamination of broilers at slaughter in Taiwan and this

deficiency was the focus of the research presented in Chapter 3, where the nationwide prevalence of *Salmonella* spp. in Taiwanese broiler carcasses after chilling in 45 abattoirs were described. These findings provide the baseline prevalence of a major pathogen, *Salmonella*, in different types of broilers, i.e. commercial white broilers (BR) and Taiwan native chickens (TNC). The study found that at the individual bird level after chilling, 32.6% (589/1808; 95% CI: 30.4-34.8) of broiler carcasses were contaminated with *Salmonella* spp. with significantly more TNC (434/925; 46.9%, 95% CI: 43.7-50.1) contaminated than BR (155/883; 17.6%, 95% CI: 15.1-20.2). In addition, a detailed study of carcass and environmental contamination with *Salmonella* was undertaken in 6 of these abattoirs, and the results presented in Chapter 4. In that study the overall prevalence of *Salmonella* contaminated carcasses following chilling was 25% (30/120; 95% CI: 17.5-33.7); however again significant differences were detected between the different types of chickens processed: 0% (0/60; 95% CI: 0-6.0) for BRs and 50% (30/60; 95% CI: 36.8-63.2) for the TNCs. Another investigation involving sampling carcasses over a continuous 12-day-period in one selected BR abattoir that adopted a carcass air-chilling process revealed where in the processing line *Salmonella* contamination of the broiler carcasses occurred (Chapter 6). In this latter study, 21.9% (21/96; 95% CI: 14.1-31.5) of BR carcasses were found to be *Salmonella* positive at the final sampling site (post air-chilling).

Based on the aforementioned studies, the prevalence of *Salmonella* spp. on TNC carcasses after chilling in the sampled abattoirs was not significantly different between the studies (46.9% in Chapter 3 when 45 abattoirs were sampled vs. 50% in Chapter 4 when 6 abattoirs were sampled, $p = 0.6431$). In contrast, the prevalence in BR carcasses was significantly different between these two studies (17.6% in Chapter 3 vs. 0% in Chapter 4, $p = 0.0008$). The prevalence of *Salmonella* spp. in BR was also significantly

different between the 6 abattoirs that adopted water-chilling (Chapter 4) and the sole abattoir in Taiwan that uses air-chilling (Chapter 6) (0% vs. 21.9%, respectively; p = 0.0003), although there was no significant difference in the prevalence between BR chilled by water-chilling in Chapter 3 (45 abattoirs) and the abattoir that adopted air-chilling in Chapter 6 (17.6% vs. 21.9%, respectively; p = 0.295). These results highlight the higher and more constant prevalence of *Salmonella* contamination in TNC carcasses after chilling than in BR carcasses where the prevalence varied between studies and operations. In this thesis the possible factors that may result in the higher prevalence of *Salmonella* contaminated TNC carcasses compared to BR carcasses have been discussed, such as older processed birds (Arsenault et al., 2007) and the non-standardized size of processed birds within the same batch (Nunes, 2013). However, the higher contamination in TNC than BR may also be due to other factors, and future studies are required to identify why this difference occurs and what can be done by the industry to reduce this contamination. It is also suggested that a study involving sampling birds and the environment on-farm is required, similar to that reported in Chapter 6, to identify cross-contamination points within a farm, as well as to trace potential sources of contamination. Such a study could result in developing interventions that could reduce the supply of *Salmonella*-infected chickens into abattoirs.

While conducting the study reported in Chapter 4, the same samples were also used to culture and isolate *L. monocytogenes* and the results of this study are described in Chapter 5. The percentage of TNC carcasses contaminated with *L. monocytogenes* after chilling (5/60; 8.3%, 95% CI: 2.1-18.4) was found to be lower, although not significantly (p = 0.1675), than BR carcasses (10/60; 16.7%, 95% CI: 8.3-28.5), which was in contrast to that found for *Salmonella*. *Listeria monocytogenes* was not detected

in any cloacal (n = 120) or environmental (n = 256) samples collected before and during processing, whereas *Salmonella* was detected in 5.8% (7/120; 95% CI: 2.4-11.6) and 24.6% (63/256; 95% CI: 19.5-30.4) of those samples, respectively. These findings indicate that different target bacteria result in dissimilar contamination levels of carcasses, most likely as a result of their different growth characteristics. Therefore, if future studies are used to analyze the proportion of samples (carcasses) contaminated with indicator bacteria, it is recommended that the indicator bacteria used for such investigations are selected based on their growth in an environment with the temperature and humidity similar to the sampled abattoirs. Based on the results of the research presented in this thesis, *Salmonella* spp. would appear more suitable indicator bacteria than *L. monocytogenes*.

7.2 Distribution of the serotypes of *Salmonella* and *L. monocytogenes* in chickens processed at abattoirs in Taiwan

A total of 968 isolates of *Salmonella* were detected from all abattoirs in the three studies reported in this thesis (589 in Chapter 3, 156 in Chapter 4, and 223 in Chapter 6) with all isolates subsequently serotyped according to the Kauffmann–White scheme (Grimont and Weill, 2007). Thirty-three serotypes were identified (98.8%) with 12 isolates (1.2%) non-typeable. Overall, the most prevalent serotypes were *S.* Albany (30.9%; 95% CI: 28.0-33.9), *S.* Enteritidis (16.5%; 95% CI: 14.2-19.0), *S.* Schwarzengrund (9.7%; 95% CI: 7.9-11.8) and *S.* Typhimurium (6.7%; 95% CI: 5.2-8.5) (Table 7.1). These four serotypes were also responsible for 59.3% of human salmonellosis infections in Taiwan from 2013 to 2017 (Chiou et al., 2019). This finding demonstrates that the *Salmonella* serotypes affecting humans in Taiwan are consistent with those found at chicken abattoirs, supporting the belief that contaminated chicken meat is one source of human salmonellosis. However, the confirmation of the source(s)

of human salmonellosis should be informed by more detailed strain identification, such as through the use of whole-genome sequencing of isolates or elucidation of exposure factors (Koutsoumanis et al., 2019).

It is noteworthy that *S.* Anatum and *S.* Brancaster were infrequently detected in the surveys conducted (1.5 and 2.9% of all serotypes, respectively - Table 7.1), and were only detected in the study reported in Chapter 6 which was conducted in 2018. These two serotypes were not detected in the survey conducted in 45 Taiwanese chicken abattoirs during 2013 to 2014 (total of 589 *Salmonella* isolates cultured - Chapter 3) nor in the 2014 study where 6 abattoirs were sampled (total of 156 *Salmonella* isolates cultured - Chapter 4). Similarly, *S.* Anatum and *S.* Brancaster were not detected by Lin et al. (2008) who reported on 570 *Salmonella* isolates from 3 abattoirs and 2 retail stores sampled in 2006 and 2007. Chiou et al. (2019) found that *S.* Anatum was not a prevalent serovar among those collected from human cases of salmonellosis during 2004–2014 in Taiwan; however since then the number of *S.* Anatum linked human infections has increased quickly with 1.3%, 6.7% and 14.2% of all cases in humans in Taiwan being linked to this serovar in 2015, 2016 and 2017, respectively. In 2017 this serovar was the third most frequently identified serovar from humans in Taiwan (Chiou et al., 2019). Although, to date, there have been no reports of the involvement of *S.* Brancaster in human infections in Taiwan, this serovar has been associated with human salmonellosis through eating contaminated poultry meat in Senegal (Cardinale, Perrier Gros-Claude, et al., 2005). These results may indicate that *S.* Anatum and *S.* Brancaster could be emerging *Salmonella* enterica serovars in Taiwan and need further monitoring both in poultry meat and human infections.

Table 7.1 The serotypes of *Salmonella* isolates from all abattoirs in this thesis

Serotype	Chapter 3 n = 589	Chapter 4 n=156	Chapter 6 n=223	Total n=968	%	95% CI
Albany	187	65	47	299	30.9%	28.0-33.9
Enteritidis	80	2	78	160	16.5%	14.2-19.0
Schwarzengrund	44	32	18	94	9.7%	7.9-11.8
Typhimurium	57	2	6	65	6.7%	5.2-8.5
Tennessee	38	8	0	46	4.8%	3.5-6.3
Montevideo	38	2	0	40	4.1%	3.0-5.6
Hadar	35	1	3	39	4.0%	2.9-5.5
Kentucky	4	20	6	30	3.1%	2.1-4.4
Newport	16	5	8	29	3.0%	2.0-4.3
Brancaster	0	0	28	28	2.9%	1.9-4.2
Livingstone	24	2	0	26	2.7%	1.8-3.9
Anatum	0	0	15	15	1.5%	0.9-2.5
Livingstone var. O14+	8	5	0	13	1.3%	0.7-2.3
1,4,[5],12:i:-	11	0	0	11	1.1%	0.6-2.0
Muenster	1	5	3	9	0.9%	0.4-1.8
Agona	7	0	1	8	0.8%	0.4-1.6
Derby	6	0	0	6	0.6%	0.2-1.3
Cremieu	5	0	0	5	0.5%	0.2-1.2
Give	0	0	5	5	0.5%	0.2-1.2
Virchow	5	0	0	5	0.5%	0.2-1.2
Cerro	4	0	0	4	0.4%	0.1-1.1
Haardt	1	3	0	4	0.4%	0.1-1.1
Lindenberg	0	3	0	3	0.3%	0.1-0.9
Potsdam	2	0	0	2	0.2%	0.0-0.7
Stanley	2	0	0	2	0.2%	0.0-0.7
Alachua	0	0	1	1	0.1%	0.0-0.6
Bardo	1	0	0	1	0.1%	0.0-0.6
Chester	0	0	1	1	0.1%	0.0-0.6
Corvallis	0	0	1	1	0.1%	0.0-0.6
Havana	0	1	0	1	0.1%	0.0-0.6
Mbandaka	1	0	0	1	0.1%	0.0-0.6
Vejle	1	0	0	1	0.1%	0.0-0.6
Weltevreden	0	0	1	1	0.1%	0.0-0.6
Non-typeable	11	0	1	12	1.2%	0.6-2.2

In Chapter 5, 28 of 246 (11.4%; 95% CI: 7.7-16.0) carcass rinse samples collected from six Taiwanese chicken abattoirs during 2013 to 2014 were positive for *L.*

monocytogenes. Two serotypes were identified from those 28 isolates: 1/2a (82.1%; 95% CI: 63.1-93.9) and 1/2b (14.3%; 95% CI: 4.0-32.7) with one isolate (3.6%; 95% CI: 4.0-0.1-18.3) non-typeable. These findings are in agreement with other international studies (Jamshidi & Zeinali, 2019; Maung et al., 2019; Oliveira et al., 2018; Zeinali et al., 2016; Zhang et al., 2007) where serotypes 1/2a and 1/2b are commonly found in chickens and their products. In addition, these two serotypes were recognised as the predominant serotypes involved in human infections (34/46 isolates; 73.9%) at a Taiwanese hospital during 1996 to 2008 (Huang et al., 2011), as well as from sporadic cases of listeriosis in humans (189/295 isolates; 64.1%) in France in 1995 (Jacquet et al., 2002). This indicates that the serotypes affecting humans are consistent with those detected in chickens and their products. The results of this thesis show that the prevalence of *L. monocytogenes* in broiler carcasses in Taiwan is not as high as that of *Salmonella* spp.; however, the serotypes detected were the same as those causing the majority of human listeriosis cases in the country and other parts of the world. Taking into consideration the high mortality rate of *L. monocytogenes* infections, these findings highlight the potential risk from consumption of meat or manufactured product from contaminated broilers, and this contamination needs to be closely monitored to minimize the risk to public health.

In the four studies presented in this thesis (Chapters 3 - 6), colonies which had typical morphological characteristics of *Salmonella* spp. and *L. monocytogenes* on culture were selected for confirmation with biochemical assays. Samples with one or more colonies identified as *Salmonella* or *L. monocytogenes* were classified as positive; however, due to financial and personnel constraints only one colony from each positive sample was serotyped, even when multiple colonies with the characteristic appearance for these bacteria were present. Bouayad et al. (2015) reported that some *L. monocytogenes*

strains cultured from the same samples exhibited different PFGE profiles and belonged to different serogroups. Although the approach adopted in the studies described in this thesis would not have affected the overall proportion of positive samples, the percentage of specific serovars or PFGE profiles may have been affected by the sampling methodology. Nucera et al. (2010) suggested that selecting/isolating 3 colonies per sample, if available, was necessary to determine the variability of isolates within individual samples, particularly if the study was designed to characterize *L. monocytogenes* populations in food. Therefore, it is recommended that future studies should be expanded to follow the recommendation of Nucera et al. (2010) to enable a more accurate description of the serotypes and PFGE types present in samples.

7.3 Risk factors for *Salmonella* spp. contamination of slaughtered chickens in Taiwan

Contaminated poultry and poultry products have been identified as a major food source responsible for foodborne diseases (Finstad et al., 2012; Mead et al., 2010; Morar et al., 2014; Olsen et al., 2005), and these contaminations have been demonstrated to mainly occur during the slaughter and processing stages (Cardinale, Tall, et al., 2005; Chen et al., 2004; Cox et al., 1997; Rørvik et al., 2003; Rasschaert et al., 2007). However, the risk factors for *Salmonella* spp. contamination of chickens slaughtered/processed in Taiwan have rarely been investigated, and the effects of specific hygiene interventions conducted in abattoirs were unknown prior to the study reported in Chapter 3. This report is the first study to identify the risk factors associated with the presence of *Salmonella* in Taiwanese broilers and, in particular, emphasize the role of the abattoir's environmental conditions and bird type on contamination during processing. These findings can be used to implement targeted interventions to better control *Salmonella* cross-contamination between the environment and carcasses.

In the study described in Chapter 3, the characteristics/variables of each of the sampled batches were recorded for subsequent analyses, including; sampling year and season, bird type, processing conditions, as well as abattoir information. The results of the multivariable logistic regression analysis identified four risk factors associated with contamination of the chicken carcasses with *Salmonella*: season of sampling (warm season > cooler season); the location of the abattoir (the northern region > the southern region); duration of scalding (shorter scalding times > scalding times more than 90 seconds); and bird type (TNC > BR). Although the risk of *Salmonella* contamination of chickens slaughtered in northern Taiwan was greater than that of chickens slaughtered in the southern area of Taiwan, this may be affected by the duration of transport from the farm to the abattoir. As chicken farms in Taiwan are mainly located in the south (COA, 2014a), the transportation distance and hence duration of transport would be longer for those chickens sent from southern farms to northern abattoirs. This increase would result in greater stress for these birds and increased fecal excretion and ultimately higher levels of cross-contamination between birds prior to arrival at the abattoirs (FAO/WHO, 2002). However, no data on the transportation time from farm to the abattoir were collected in the current study and future research should be conducted to evaluate this factor on carcass contamination.

The higher percentage of contaminated TNC carcasses than BR carcasses may be influenced by the age of the chickens processed or the uniformity of the size/weight of the birds. As TNC are processed at an older age than BR and anecdotally there is a more variable body size/weight within a batch of TNC than BR, it is not surprising that more TNC carcasses are contaminated with *Salmonella* than BR carcasses. In future studies data needs to also be collected on the age and weight/size range of the processed birds within batches. Moreover, the results of the study presented in Chapter 6, along with

other studies conducted internationally (Corry et al., 2002; Nógrády et al., 2008; Olsen et al., 2003), found that *Salmonella*-infected/contaminated flocks are an important source of post-slaughter carcass contamination. However, in the study described in Chapter 3, data on whether the birds processed originated from flocks infected with *Salmonella* were not collected. Therefore, it is suggested that future research should ensure that more detailed data are collected and included in analyses for factors associated with carcass contamination to enable the development of preventive measures for carcass contamination.

Furthermore, although in this study background information of each sample was recorded, these "factors" were divided into only two categories for analyses to identify putative risk factors for *Salmonella* contamination. The effect of categorizing the data into more groups should be evaluated in future studies/analyses to potentially enrich the findings and lead to more informative and accurate results.

7.4 Use of PFGE and biomapping to investigate bacterial contamination during processing at one chicken abattoir

Pulsed-Field Gel Electrophoresis (PFGE) profiling is a DNA fingerprinting method which is dependent upon the restriction digestion of purified genomic DNA from bacteria and is considered the "gold standard" for typing bacteria (Neoh et al., 2019). The selective ability of rare-cutting restriction enzymes, primarily *Xbal* or *AvrII* (*BlnI*) for *Salmonella* and *Xbal*, *AscI*, or *ApaI* for *L. monocytogenes*, are used in PFGE to digest the bacterial genome into a limited number of restriction fragments (CDC, 2013b; Whittam & Bergholz, 2007). For two decades, PFGE was one of the most widely used methods for food safety surveillance, infection control and outbreak investigations (Neoh et al., 2019; Pietzka et al., 2019). Epidemiological studies and PFGE techniques are able to indicate the potential sources of contamination, trace the contamination in an

abattoir or processing plant and enhance knowledge about the environmental locations where potentially pathogenic bacteria can survive and develop in abattoirs (Chasseignaux et al., 2001). The studies reported in Chapters 4, 5, and 6 of this thesis used the PFGE profiles of *Salmonella* spp. and *L. monocytogenes* isolates collected from the abattoir environment, from carcasses and from the farm to investigate the potential source(s) of contamination, and to identify when and where carcass cross-contamination occurred. These studies resulted in the generation of valuable epidemiological information about the nature of the contamination encountered at the selected abattoirs.

In the study summarized in Chapter 4, all six abattoirs included in that study cleaned and disinfected the environment/equipment after the conclusion of each day's slaughtering/processing to remove contamination and to minimize contamination of birds slaughtered on the next processing day. However, *Salmonella* positive environmental samples were detected at these abattoirs after cleaning, indicating that the cleaning/disinfection processes were not sufficient to eliminate environmental contamination with *Salmonella*. In addition, characterization of the isolates by PFGE indicated that the same PFGE types of *Salmonella* that survived the cleaning process were subsequently detected on carcasses and in the environment during slaughtering. A similar situation was found in the study of Chapter 6, where the same PFGE types were detected from chicken carcasses sampled at the same site in the abattoir on different processing days, again supporting the belief that these strains had survived the cleaning and disinfection processes undertaken after the conclusion of each day's processing/slaughtering. These findings emphasize again the crucial importance of implementing effective cleaning and sanitation throughout the abattoir and should be the main focus for the control of such pathogens in the poultry meat processing line

(Iannetti et al., 2020). Of significant concern for public health are the findings of some studies of a correlation between antibiotic resistance and disinfectant resistance of certain bacteria (Khan et al., 2016; Mc Carlie et al., 2020). Therefore, in addition to using the recommended concentrations and application times of disinfectants before slaughter operations, more attention should be paid to the selection of disinfectants that are effective on residual bacteria.

In the study reported in Chapter 6, the same *Salmonella* spp. PFGE types were detected from birds sampled on the supply farms and on samples of carcasses from the same source batch at most processing steps in the abattoir. This finding was similar to that reported in Chapter 4, with two PFGE types detected from cloacal samples subsequently detected in environmental and carcass samples. These results highlight that *Salmonella*-infected flocks are important sources of *Salmonella* contamination within the abattoir leading to subsequent cross-contamination between carcasses (Corry et al., 2002; Nógrády et al., 2008; Olsen et al., 2003). One intervention that can be applied in abattoirs to reduce this cross-contamination is for batches from flocks which have tested negative for *Salmonella* on-farm to be processed before flocks that are infected with *Salmonella* (Evers, 2004; Zutter et al., 2005). However subsequent to the processing of the latter infected flocks and prior to processing batches on the following day it would be critical to undertake thorough cleaning of the abattoir environment as mentioned previously. The effectiveness of this end-of-day cleaning should be evaluated through collection of environmental samples at each of the processing stages/steps after cleaning/disinfecting has been conducted. The results of this sampling can help identify potential cross-contamination sites and the effectiveness of the cleaning/disinfection practices.

In the study presented in Chapter 5, *L. monocytogenes* was not detected in any cloacal

or environmental samples collected before and during processing, although it was subsequently detected from samples collected from carcasses after evisceration. Characterization by PFGE identified that *L. monocytogenes* isolates with the same PFGE types were detected from the carcass samples during evisceration, chilling, and post-chilling, indicating the potential for contamination through direct contact between carcasses during processing, or via contaminated water from the chilling tank (Chiarini et al., 2009; Reiter et al., 2005). These findings are similar to those outlined in Chapter 6, where certain PFGE types of *Salmonella* spp. were not isolated from carcasses immediately after exsanguination, but were detected from carcasses sampled at two or more subsequent processing sites. These findings also indicate this cross-contamination may have resulted from either direct contact between carcasses or have come from the abattoir environment after it had been contaminated by carcasses processed on previous days. The significant level of cross-contamination occurring during processing highlights the need for increased attention to determine and control both direct and indirect cross-contamination pathways during processing.

Through characterization by PFGE in the study of Chapter 5, *L. monocytogenes* isolates with the same PFGE profiles were detected in samples of chicken carcasses originating from different farms. Similarly, the study of Chapter 6 reported that isolates of *Salmonella* spp. with the same PFGE type(s) had been collected from carcasses of chickens originating from multiple source farms from different regions of Taiwan. These results imply that some PFGE types of *L. monocytogenes* and *Salmonella* spp. are widely distributed in the country's broiler industry and these findings are in agreement with other international studies (Boerlin et al., 1997; Bouayad et al., 2015; Chasseignaux et al., 2001). These researchers postulated that the widespread distribution of some clones was as a result of the movement of poultry via trade

channels. These results highlight that the cleaning and disinfection of poultry transportation vehicles and transport crates should be strengthened to prevent the spread of these pathogens between farms. Washing the live bird transport crates with water and disinfectant, and then leaving them to dry for 48 hours has been recommended to reduce the levels of residual *Salmonella* spp. found in and on transport cages (FAO/WHO, 2011; King et al., 2011).

A limitation of the study in Chapter 3 was that batches presented to each abattoir were only sampled on one day at one processing point in the abattoir and it is suggested that in the future birds presented to abattoirs should be sampled at multiple processing points to understand the impact processing stage has on the prevalence of *Salmonella* and on multiple days to determine variation in the prevalence with time. This suggestion was employed in the study of Chapter 6, and that study was also the first attempt to combine *Salmonella* biomapping and PFGE to reveal where in an abattoir processing line *Salmonella* contamination of broiler carcasses occurred, and to investigate and identify the potential sources of *Salmonella* contamination.

Two distribution biomaps were developed with the same data but were presented in a different format in the study of Chapter 6. The first biomap (Figure 6.2) demonstrated the presence of the same PFGE type at different processing stages on the same slaughtering day. These data can be used to identify cross-contamination points within an abattoir, as well as to identify when the *Salmonella* are introduced and assists in the tracing of potential sources of contamination on the slaughtering day. The second biomap (Figure 6.3) demonstrated the presence of the same PFGE type at the same processing stages on different slaughtering days. These data can be used to identify which processing stages result in on-going contamination and help confirm the need to implement or improve interventions to specific processing steps to minimize this

contamination. This information is invaluable for the poultry industry and can assist the industry to accurately identify the *Salmonella* contamination problems in abattoirs and help in the development of effective preventive measures to ensure the production of a more wholesome product for consumers.

In the studies outlined in Chapters 4, 5 and 6 isolates of *Salmonella* and *L. monocytogenes* with the same pattern from the same abattoir were considered to be the same strain/type. However, it is possible that the subtyping method was unable to detect differences in the genome of the strains or that the phenotypes of similar PFGE types were different (Lundén et al., 2003). If this is the case, it may lead to inadequate inferences for tracing the source(s) of contamination and pathways for cross-contamination within abattoirs processing broiler chickens. PFGE was developed in the 1980's and has been an excellent tool for the analytical analysis of isolates and was considered the "gold standard" for bacterial typing. However, in the 2000's, whole genome sequencing (WGS)-based new molecular epidemiological methods based on DNA sequencing, such as MLVA (multi-locus variable-number tandem-repeat analysis) and MLST (multi-locus sequence typing), emerged, and with the advent of next generation sequencers, whole genome MLST and core genome MLST have been adopted by many researchers in recent years (Ruppitsch et al., 2015; Siira et al., 2019; Singh et al., 2021; Yin et al., 2020). Compared to WGS, PFGE is lengthy and laborious and consequently WGS-based typing methods have gradually replaced PFGE as they have a higher accuracy and a superior discriminatory power (Pietzka et al., 2019). In addition, although PFGE has been used for source tracking to differentiate related and unrelated microbial strains to support epidemiological investigations of foodborne outbreaks, WGS is also replacing PFGE in these situations due to the higher resolution of the technique (Portmann et al., 2018) and now whole genomes of bacteria can be

sequenced in less than 24 hours (Neoh et al., 2019). Nevertheless, PFGE remains an affordable and relevant technique for small laboratories and hospitals, especially given the current high costs associated with the purchase of a next generation sequencer and the computational analyses required for WGS (Neoh et al., 2019). Currently the cost of running a WGS for a *Salmonella* strain is, on average, US$1,000 in Taiwan (personal communication with the head of the ATRI laboratory, July 10, 2019). It is suggested that, when the cost of operating and performing a WGS decreases, future studies investigating the bacterial contamination in abattoirs should be conducted by introducing new molecular epidemiological methods such as WGS or the next generation sequencers. This would potentially provide more accurate data and serve as a better reference for the poultry industry to help develop preventive and control interventions, resulting in enhanced meat hygiene and food safety for the general public.

7.5 Limitations and recommendations

As well as *Salmonella* and *L. monocytogenes*, other pathogens of animal origin, such as *Campylobacter* spp. or members of the *Enterobacteriaceae* family, also pose a serious health risk to humans in both developing and developed countries (Chlebicz & Śliżewska, 2018). It is recommended that the study design outlined in this thesis should be replicated for other pathogens. The studies reported in Chapters 3 to 6 of this thesis have established the models for determining the risk factors associated with *Salmonella* contamination of chicken carcasses at abattoirs, investigating the potential route of cross-contamination between poultry carcasses and the processing environment, and tracing the potential sources of carcass contamination in a poultry abattoir. It is suggested that future research can extend these survey models to livestock abattoirs to obtain similar information on different types of processed animals, such as pigs, cattle and sheep, as well as in other species of poultry, such as ducks and geese, in Taiwan.

Finally, as mentioned previously, limitations of the research described in this thesis include: the potential risk factors were divided into only two categories for analyses to identify putative risk factors for *Salmonella* contamination; only one colony from each positive sample was serotyped, even when multiple colonies with the characteristic appearance for these bacteria were present; and PFGE is lengthy, laborious and has a lower accuracy and discriminatory power compared to WGS. Because of these potential biases and limitations, it is also recommended that future studies:

- Should collect more detailed data to: allow the division of potential risk factors into multiple categories; and to study the effect on carcass contamination of chicken age, size/weight of processed birds, transportation time from farm to abattoir, and on-farm pathogen status of batches prior to processing.
- Be expanded through allocation of more personnel and financial support to: enable more sampling to be undertaken, including environmental sampling, to expand the results from the current biotyping study; and allow more colonies to be selected per plate to better understand the serotypes/phage types present in the Taiwanese chicken industry.
- Involve the use of WGS-based typing methods to investigate bacterial contamination problems in abattoirs and identify critical control points for contamination within the processing chain.

7.6 Conclusions

The studies outlined in this thesis provide the first research in Taiwan to: identify the risk factors associated with the presence of *Salmonella* in processed batches of broilers; detect and type *Salmonella* spp. and *L. monocytogenes* isolated from broiler chickens during processing and from the environment of six abattoirs; and develop distribution biomaps in combination with PFGE profiles of *Salmonella* isolates to demonstrate the

potential route of carcass cross-contamination in an abattoir.

This study involved collection of samples at the national level from 45 abattoirs and was followed by more detailed and intensive sampling at six abattoirs and finally intensive sampling of chicken carcasses from 12 farms processed at one abattoir on 12 consecutive processing days. Collectively the information obtained from this research can be used to assess control measures to minimize or prevent the contamination of chickens processed at abattoirs in Taiwan with potentially pathogenic bacteria (*Salmonella* spp. and *L. monocytogenes*). Implementation of such measures should result in fewer outbreaks of foodborne disease originating from contaminated poultry meat resulting in improved public health and greater confidence by the general public in the safety of consuming poultry meat.

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