

COMBINATION OF PHYSICAL AND CHEMICAL INTERVENTIONS FOR REDUCTION
OF LOOSELY AND TIGHTLY ASSOCIATED BACTERIA ON BROILER CARCASS SKIN

By

Pranjal Singh

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Food Science - Doctor of Philosophy

2016

ABSTRACT

COMBINATION OF PHYSICAL AND CHEMICAL INTERVENTIONS FOR REDUCTION OF LOOSELY AND TIGHTLY ASSOCIATED BACTERIA ON BROILER CARCASS SKIN

By

Pranjal Singh

The study was conducted to develop processing interventions to reduce pathogen load on broiler carcasses and to understand patterns of bacterial association to broiler skin with the following purposes: 1) assess the effect of hot water spray (HWS, 71°C, 1 min) on broiler carcasses for reduction of loosely, intermediately, and tightly associated bacteria, 2) quantify loosely and tightly associated bacteria on broiler skin using different sampling protocols, 3) assess the effect of trisodium phosphate dip, hot water dip, and their combinations with/without brushing on reduction of loosely- and tightly-associated bacteria on broiler carcasses and related structural changes in the skin, and 4) assess the characteristics of *Salmonella* association and penetration to broiler skin with and without stomaching.

In study 1, broiler skin was quantitatively assessed for loosely, intermediately, and tightly associated bacteria by rinsing, stomaching, and grinding the same skin. Hot water spraying (71°C, 1 min) of broiler carcasses followed by water immersion chilling reduced mesophilic aerobic bacteria (MAB) populations by 2.07, 1.84, and 2.04 log CFU/g and *Salmonella* prevalence by 65, 65, and 73% at loose, intermediate and tight association levels, respectively. Hot water spraying and chilling reduced loosely associated *Campylobacter* prevalence by 45% but no reduction was observed at intermediate and tight association levels. Broiler carcasses suffered from an undesirable partially cooked appearance after the hot water spray treatment.

In study 2, the left and right side skin (10 X 7 cm) of broiler carcasses were sampled by 10 swabings and 10 stomachings, and finally ground. Sixty five and 83 % of MAB populations

on broiler skin could not be recovered after 10 consecutive stomachings and swabbings, respectively, but were recovered after grinding. The first stomaching or swabbing recovered most of the loosely associated bacteria but not tightly associated bacteria, which were recovered by grinding. In general, stomaching was more effective than swabbing for bacteria recovery from broiler skin.

In study 3, microbial reductions and structural changes in broiler skin were evaluated after trisodium phosphate dip (TWD/TSP), hot water dip (TWD/HWD), and their combination (TSP/HWD) with (TSP/HWD/B)/without brushing. Microbial analysis, scanning electron microscopy and histological staining showed that TSP/HWD caused greater reduction in MAB, *E. coli*, total coliforms, and *Salmonella* with deeper penetration into the stratum compactum layer of the skin dermis than single application of TSP or hot water dip. TSP/HWD/B yielded lowest skin population of MAB whereas penetration into the dermis was similar to TSP/HWD but with sloughing off of part of stratum compactum in some areas. Both TSP/HWD and TSP/HWD/B changed skin color, with an increase in yellowness being the most prominent.

In study 4, loose and tight associated GFP tagged *Salmonella* were quantified and imaged after refrigerated incubation of inoculated broiler skins for up to 48 h. GFP tagged *Salmonella* were not able to associate tightly with broiler skin during exposure for up to 48 h at 4°C as application of two stomachings (1 min each) removed almost 88% of the *Salmonella* cells from the inoculated skin. Confocal imaging of broiler skins, either dipped (2 min) or stomached (2 min) in inoculated buffer, revealed that *Salmonellae* were present on the surface and inside crevices at average depths of 10 and 68 µm, respectively, after dipping, with some cells pushed to average depths of 62 and 132 µm, respectively, after stomaching. Trapped water seemed to hold *Salmonellae* deep inside the crevices.

ACKNOWLEDGMENTS

I would like to acknowledge all the people who contributed to this dissertation. First and foremost, I must express my sincere gratitude to Dr. Ike Kang. I thank him for giving me the opportunity to conduct this research. Dr. Kang helped in every way he could, and always encouraged me to think positively, even during the darkest and toughest moments. I will always be obliged that he provided a caring work atmosphere and made me feel at home. I look forward to future opportunities to continue working in collaboration with him in the area of meat safety. I sincerely thank Dr. Elliot Ryser, Dr. John Linz, and Dr. Darrin Karcher for their valuable time, and helpful discussions. Dr. Ryser has been a support and a guide in tough times. This dissertation was not possible without his consistent mentorship and well wishes. Dr. Ryser's lab is one of the best and most convenient places in the MSU Food Science Department to do applied microbiology. I have always looked up to Dr. Linz as a scientist, teacher and individual. It was a great learning experience for me to assist him in teaching Food Microbiology Lab course. Discussions and talks with him have enriched my dissertation and personal character. I wish to emulate some of his qualities and scientific acumen in my personal and professional life. Dr. Karcher has been a teacher and a friend to steer my dissertation in the right direction. He has always been positive about my potential and quality of the research towards this dissertation. I also thank my lab colleagues, technicians, and the MSU meat lab team for their willingness to help make this project possible. Most importantly, I deeply thank my family for their unconditional love, especially my father who was always there for me. I thank my wife, Pratibha, for her remarkable patience during the five years of my degree program.

TABLE OF CONTENTS

LIST OF TABLES	viii
LIST OF FIGURES	x
INTRODUCTION	1
CHAPTER 1 LITERATURE REVIEW	5
1.1 <i>Salmonella</i> , <i>Campylobacter</i> and <i>E. coli</i> association with poultry meat	6
1.2 Pathogen (<i>Salmonella</i> and <i>Campylobacter</i>) attachment and survival in poultry skin.....	8
1.3 Pathogen cross contamination during poultry processing	13
1.4 Current poultry carcass decontamination practices in the US	15
1.5 Trisodium phosphate (TSP/Na ₃ PO ₄) treatment	18
1.6 Hot water spray (HWS), and brushing.....	21
1.7 Combination of physical and chemical interventions	23
1.8 Poultry skin imaging (confocal and scanning electron microscopy)	24
CHAPTER 2 EFFECT OF HOT WATER SPRAY ON BROILER CARCASSES FOR REDUCTION OF LOOSELY-ASSOCIATED, INTERMEDIATELY-ASSOCIATED, AND TIGHTLY-ASSOCIATED PATHOGENIC (<i>SALMONELLA</i> AND <i>CAMPYLOBACTER</i>) AND MESOPHILIC AEROBIC BACTERIA.....	26
2.1 Abstract	27
2.2 Introduction.....	28
2.3 Materials and methods	30
2.3.1 Broiler processing	30
2.3.2 Sampling methods.....	31
2.3.3 Microbiological analysis	32
2.3.4 Statistical analysis	33
2.4 Results and discussion	38
CHAPTER 3 QUANTIFICATION OF LOOSELY- AND TIGHTLY-ASSOCIATED BACTERIA ON BROILER CARCASS SKIN USING SWABBING, STOMACHING, AND GRINDING METHODS	44
3.1 Abstract	45
3.2 Introduction.....	46
3.3 Materials and methods	48
3.3.1 Broiler carcasses for the quantification of loosely- and tightly-associated bacteria.....	48
3.3.2 Swabbing for 10 times	48
3.3.3 Stomaching for 10 times	49
3.3.4 Grinding of swabbed and stomached skin	49

3.3.5	Microbiological analysis	49
3.3.6	Total bacteria recovered after swabbing and grinding (TB-SWGR)	50
3.3.7	Total bacteria recovered after stomaching and grinding (TB-STGR)	50
3.3.8	Percentage of recovered bacteria	50
3.3.9	Statistical analysis	51
3.4	Results and discussion	52
CHAPTER 4	EFFECT OF TRISODIUM PHOSPHATE DIP, HOT WATER DIP, AND THEIR COMBINATION WITH/WITHOUT BRUSHING ON BROILER CARCASSES FOR REDUCTION OF LOOSELY- AND TIGHTLY-ASSOCIATED BACTERIA AND SKIN STRUCTURAL CHANGES	59
4.1	Abstract	60
4.2	Introduction	61
4.3	Materials and methods	63
4.3.1	Broiler carcasses	63
4.3.2	Experiment I: Control (CTR), trisodium phosphate dip (TSP), hot water dip (HWD), and combination of TSP and HWD (COM)	64
4.3.3	Experiment II: Control (CTR), CTR with brushing (CTR-B), combination of TSP and HWD (COM), and COM with brushing (COM-B)	65
4.3.4	Skin sampling methods	66
4.3.5	Microbiological analysis	66
4.3.6	Muscle and skin color measurements	67
4.3.7	Scanning electron microscopy (SEM) imaging of skin	68
4.3.8	Histological staining and imaging	68
4.3.9	Statistical analysis	69
4.4	Results and discussion	70
4.4.1	Experiment I: Control (CTR), trisodium phosphate dip (TSP), hot water dip (HWD), and combination of TSP and HWD (COM)	70
4.4.2	Experiment II: Control (CTR), CTR with brushing (CTR-B), combination of TSP and HWD (COM), and COM with brushing (COM-B)	74
4.4.3	Scanning electron microscopy (SEM) and histological imaging of skin...79	
CHAPTER 5	CHARACTERISTICS OF <i>SALMONELLA</i> ASSOCIATION AND PENETRATION TO BROILER SKIN DURING EXPOSURE WITH/WITHOUT STOMACHING	91
5.1	Abstract	92
5.2	Introduction	93
5.3	Materials and methods	94
5.3.1	<i>Salmonella</i> strains	94
5.3.2	Experiment I: Time required for <i>Salmonella</i> to associate tightly to broiler skin	94
5.3.2.1	Microtiter plate attachment assay	94
5.3.2.2	Broiler skin model	95
5.3.2.3	Storage of broiler skin after <i>Salmonella</i> inoculation	96
5.3.2.4	Skin sampling for microbiological analysis	96
5.3.2.5	Skin sampling for confocal imaging	97

5.3.2.6	Microbiological analysis	97
5.3.2.7	Confocal microscopy	97
5.3.3	Experiment II: Effect of stomaching on <i>Salmonella</i> penetration in broiler skin.....	98
5.3.3.1	Skin treatment	98
5.3.3.2	Confocal microscopy	98
5.3.4	Statistical analysis	98
5.4	Results and discussion	99
5.4.1	Experiment I: Time required for <i>Salmonella</i> to associate tightly to broiler skin.....	99
5.4.2	Experiment II: Effect of stomaching on <i>Salmonella</i> penetration in broiler skin.....	104
CONCLUSIONS AND FUTURE RECOMMENDATIONS.....		107
BIBLIOGRAPHY		112

LIST OF TABLES

Table 2.1	Populations (log CFU/g) of mesophilic aerobic bacteria (MAB) and <i>Campylobacter</i> that were loosely, intermediate, and tightly associated on chicken skin at different sampling sites	36
Table 2.2	Prevalence of <i>Salmonella</i> and <i>Campylobacter</i> that were loosely, intermediately, and tightly associated with chicken skin at different sites.....	37
Table 3.1	Mean populations (log cfu/cm ²) of mesophilic aerobic bacteria (MAB) with (SD) on broiler skin from 10 consecutive stomaching or swabbing repetitions and subsequent grinding	55
Table 3.2	Mean <i>E. coli</i> populations (log cfu/cm ²) on broiler skin obtained from 1 st , 2 nd and 10 th consecutive swabbing or stomaching repetitions and subsequent grinding ...	57
Table 3.3	Mean total coliforms populations (log cfu/cm ²) (SD) on broiler skin obtained from 1 st , 2 nd and 10 th consecutive swabbing or stomaching repetitions and subsequent grinding	57
Table 4.1	Mean populations (log cfu/g) (SD) of mesophilic aerobic bacteria (MAB), <i>E. coli</i> and total coliforms and prevalence (%) of <i>Salmonella</i> and <i>Campylobacter</i> on broiler skin after treatments in Exp. I	73
Table 4.2	Mean color values ¹ (L*, a*, b*) (SD) of broiler breast muscle after treatments in Exp. I.....	73
Table 4.3	Pooled mean population (log cfu/g) ± SEM of MAB, <i>E. coli</i> , and total coliforms and pooled mean prevalence (%) of <i>Salmonella</i> on broiler skin obtained after the four treatments using two sampling methods in Exp. II	77
Table 4.4	Mean color values (L*, a*, b*) (SD) of broiler breast muscle after treatments in Exp. II	78
Table 4.5	Mean color values (L*, a*, b*) (SD) of broiler carcass scapula area skin after treatments in Exp. II.....	78
Table 4.6	Mean color values (L*, a*, b*) (SD) of broiler carcass breast area skin after treatments in Exp. II.....	79
Table 4.7	Observations on skin structure before and after treatments based on light microscopy of histologically stained skin sections and percentages of representative microscopic fields with appearances corresponding to the observations described	88

Table 5.1	<i>Salmonella</i> population ¹ (SD)/percent ² recovery from two stomachings and one grinding of broiler skin after refrigerated exposure to a <i>Salmonella</i> cocktail (1 x 10 ⁹ CFU/ml)	101
Table 5.2	Average (SD) and range of depth of <i>Salmonella</i> cells on broiler skin before and after two 1 min stomachings in a <i>Salmonella</i> cocktail (1 x 10 ⁹ CFU/ml)	101
Table 5.3	Average (SD) and range of depth of <i>Salmonella</i> cells on flat surfaces and inside crevices of broiler skin after a 2 min immersion (no stomaching) and 2 min of stomaching in a <i>Salmonella</i> cocktail (1 x 10 ⁸ CFU/ml)	105

LIST OF FIGURES

Figure 2.1	Sampling sites during broiler processing with or without hot water spray (HWS)	35
Figure 2.2	Appearance of chicken carcass breast muscle after spraying with a) cold water and b) hot water	43
Figure 3.1	Recovered (%) ^c mesophilic aerobic bacteria (MAB) after the 1 st swabbing, 2 nd to 10 th consecutive swabbings, and grinding of the swabbed skin.....	56
Figure 3.2	Recovered (%) ^c mesophilic aerobic bacteria (MAB) after the 1 st stomaching, 2 nd to 10 th consecutive stomachings, and grinding of the stomached skin	56
Figure 4.1	Experiment I design and sampling plan.....	64
Figure 4.2	Experiment II design and sampling plan	65
Figure 4.3	Scanning electron microscope (A1 - G1, 10000X magnification) and light microscope (A2 - G2 and G3, 20X magnification) images of skin surface and histologically stained skin sections, respectively, for reference and treated skins.	83
Figure 4.4	Scanning electron microscope images (10000X magnification) of skin crevices for reference and treated skins	86
Figure 5.1	Confocal image of uninoculated control skin (A) and images taken before (B, C, D and E) and after (F, G, H and I) two 1 min stomachings of broiler skin inoculated with a <i>Salmonella</i> cocktail (1×10^9 CFU/ml) and incubated at 4°C for 48 h.....	102
Figure 5.2	Representative confocal images of skin surface without <i>Salmonella</i> exposure (A) ¹ , flat skin surfaces after <i>Salmonella</i> exposure (B, C and D) ¹ , and skin crevices after <i>Salmonella</i> exposure (E, F, and G) ¹	106

INTRODUCTION

Salmonella, *Campylobacter* and pathogenic *Escherichia coli* (*E. coli*) account for more than 99% of total bacterial foodborne illnesses in the United States (US) (USDA ERS 2000). Generic *E. coli* is indicator of fecal contamination on food products and processing surfaces. Poultry, the most consumed meat in the U.S., is an excellent vehicle for pathogens especially *Salmonella* and *Campylobacter* (Gast, 1997; Corry and Atabay, 2001; Zhao et al., 2001; Capita et al., 2004). Birds are contaminated from naturally existing bacteria at the poultry farm and gastrointestinal/fecal contents at the processing plant (Dincer and Baysal, 2004). Once contaminated, the bacteria can lodge deeply in hair follicles and crevices of the skin that can provide physical protection from chemicals such as chlorine and organic acids (Yang et al., 2001; Chantarapanont et al., 2004). Poultry meat is usually purchased raw and cooked at home leading to additional risk of foodborne illness due to cross contamination and product mishandling.

To minimize the risk, good agricultural practices and preventive measures are required from farm to table. In spite of years of research, however, not much progress has been made in this direction. The US poultry industry still relies heavily on chlorine for poultry carcass microbiological safety (James et al., 2006), especially for controlling cross contamination during processing, although chlorine has several limitations as follows: 1) Inactivation with heat, light and organic material, 2) pH monitoring, and 3) corrosive to stainless steel at acidic pH (Mariott, 1999; Mckee, 2004). US regulations allow chlorine up to 50 ppm chlorine for carcass washing and chilling (USDA FSIS, 2003). A study conducted at the Western Regional Research Station of Agricultural Research Service indicated that more than 400 ppm of total chlorine needs to be added to meet the chlorine demand which is negatively impacted by the organic load of the chill water in industrial carcass chilling tanks (Tsai et al., 1992). Only after meeting the chlorine

demand is further added chlorine freely available for bactericidal action. This amount of 400 ppm is more than 8 times the standard 50 ppm set by USDA. It promotes some processing plants to use chlorine at concentrations much higher than the USDA specifications (Tsai et al., 1992; Scott, 2012).

Chlorine at high concentration is an irritant and has the potential to form carcinogens such as haloacetic acids and trihalomethanes with organic substances in water (Dunnick and Melnick, 1993; Li et al., 1996; CDC, 1997). Furthermore, chlorine typically does not reduce more than 1 log total bacteria population on broiler carcasses during water chilling (James et al., 1992; Bautista et al., 1997).

While chlorine is the most popular antimicrobial agent used in the US broiler industry, other chemicals such as organic acids, cetylpyridinium chloride (CPC), and trisodium phosphate (TSP) are also often used to reduce pathogen load on carcasses (Li et al., 1997; Lillard, 1990; Sakhare et al., 1999; Whyte et al., 2001). However, most of these chemicals are not GRAS (such as chlorine and CPC), impart undesirable organoleptic properties to meat (such as organic acids), or present waste management issues (TSP). Scientific literature is available on the effects of other antimicrobial treatments such as electrolyzed water, ozonated water, irradiation, and ultrasound (Boysen and Rosenquist, 2009; Fabrizio et al., 2002; Nassar et al., 1997). However, such treatments have not been fully adopted by the industry.

During poultry processing, loosely associated bacteria on broiler skin are likely to cross contaminate equipment and other carcasses while tightly associated bacteria can survive antibacterial treatments by hiding in protective niches, such as skin crevices and cracks. Industry needs effective GRAS intervention strategies to reduce both loosely and tightly associated

bacteria on carcasses during processing with minimum meat quality changes.

Since multiple hurdles are often more successful against bacteria than individual treatment, this study was designed to evaluate the efficacy of TSP dip, hot water dip, and brushing alone and in combination for improving the microbiological quality of broiler carcasses. This study also tried to understand loosely and tightly associated bacteria and assessed multiple hurdles against bacteria at both the association levels on broiler skin. Since data are more relevant and useful for efficacy evaluations or risk assessment when decontamination studies are done in industrial settings (Boysen et al., 2013), the processing interventions in this study were tested against naturally occurring bacteria in commercial plants.

CHAPTER 1

LITERATURE REVIEW

1.1 *Salmonella*, *Campylobacter* and *E. coli* association with poultry meat

Sofos (2008) indicated several limitations of pathogen control on broiler meat such as increased antibiotic resistance among pathogens, intensive bird rearing practices, inadequate processing interventions, increased international trade, and consumer preference for minimally processed meat. Compared to 2006-2008, *Salmonella* incidence remained unchanged and *Campylobacter* incidence went up by 16% in 2012 in the US (CDC FoodNet report 2012). The prevalence values for *Salmonella*, *E. coli*, and *Campylobacter* in retail poultry were 13.2, 82.9, and 46.6%, respectively, in 2002 – 2009, which were similar to 13%, 73%, and 38% values for the same bacteria in 2013 (NARMS Retail Meat Report, 2011; NARMS Integrated Report, 2012-2013).

Raw broiler meat is one of the leading causes of bacterial foodborne illness, especially campylobacteriosis and salmonellosis (Shane, 1997; Altekruze et al., 1999; ACMSF, 2005; EFSA panel of biological hazards, 2010; Chaine et al. 2013). Consumption of contaminated chicken is responsible for 20% of human salmonellosis and 20-40% of campylobacteriosis (Vellinga and Van Loock, 2002). Poultry meats associated foodborne illnesses are under-reported because of their sporadic and isolated nature (Mead, 2004). Although health agencies attempt to track the outbreaks, direct linkage to poultry and other products remains challenging (Mead, 2004). Reduction of contamination levels on broiler carcass is linked to the reduced incidence of foodborne campylobacteriosis in humans (Rosenquist et al., 2003).

Kotula and Pandya (1995) reported that the incidences of *Salmonella*, *E. coli* and *Campylobacter* on broiler carcass before scalding were 100-60%, 100%, and 100-80% respectively. In a year-round study conducted in 20 of 127 large USDA-inspected chicken

slaughter establishments, 21% of post chill chicken carcasses were positive for *Salmonella* and almost 90% were positive for *E. coli* (Altekruse et al. 2009). Zhang et al. (2013) reported that 65% and 50% of post chlorine chill poultry carcasses were positive for *Salmonella* and *Campylobacter*, respectively.

Zhao et al. (2001) screened 212 retail broiler meat samples from 59 stores in greater Washington DC and reported that the prevalence of *Salmonella*, generic *E. coli* and *Campylobacter* were 4.2, 38.7, and 70.7%, respectively. In a similar study in metropolitan Fargo, North Dakota, Kegode et al. (2008) reported that out of 123 retail chickens, 4.1, 68, and 9% were positive for *Salmonella*, generic *E.coli* and *Campylobacter*, respectively. A recent study in Canada reported comparatively higher *Salmonella* prevalence (31-33%) in retail poultry meat samples than most of the studies conducted in the US (Cook et al., 2012). Mazengia et al. (2014) surveyed poultry retail establishments in Seattle, Washington and found that 11% of the samples were positive for *Salmonella*. Drumsticks, gizzards and ground chicken were the retail items with the highest *Salmonella* prevalence, ranging from 12 to 15.6%. Out of all the establishments tested, the highest *Salmonella* prevalence was 29.6%. Broiler carcass pathogen prevalence on processed birds is generally above acceptable levels, which can negatively impact public health in the US and around the world.

Small scale, pasture-raised poultry is growing in the local food sector. Broilers produced without antibiotics for the organic brand had a higher *Salmonella* prevalence than those using antibiotics. One study conducted for pathogen prevalence on post-chill carcasses of pasture-raised broilers processed on-farm, in a small USDA inspected slaughter facility, and in a mobile processing unit (MPU) found that *Salmonella* incidence was 89, 43, and 0% and *Campylobacter* incidence was 72, 82, and 100%, respectively (Trimble et al., 2013). While these figures are

concerning, small scale processing plants are likely to vary in *Salmonella* and *Campylobacter* prevalence from region to region and a large scale study is required to have a refined picture.

Bone-in and boneless broiler parts along with neck skin are used to prepare ground meat products. Ground broiler meat commonly has a higher *Salmonella* prevalence than intact cuts (Mazengia et al., 2014). Wu et al. (2014) reported that *Salmonella* presence was 0.8% in drumstick bone marrow and 21.4% in neck skin. These data indicated that there is a significantly higher pathogen risk associated with ground chicken containing skin. The study also found that *Salmonella* prevalence in skin sampled by stomaching was higher (20.7%) than in skin sampled by rinsing (2.3%), which suggested different attachment affinities of *Salmonella* to broiler skin.

A recent study conducted with retail poultry meat interestingly showed that only 31% of *Salmonella* isolates from poultry meat were positive for hydrogen sulfide (H₂S), an important indicator of *Salmonella* presence in classical isolation and identification approach (Lin et al., 2014). The results indicate that *Salmonella* selective enrichment should be plated on more than one selective agar medium, including a medium not having H₂S dependence for *Salmonella* identification.

1.2 Pathogen (*Salmonella* and *Campylobacter*) attachment and survival in poultry skin

Bacteria on broiler carcass skin originate from either the farm or processing plant where cross contamination can readily occur via other birds, equipment, and employees (Jang et al., 2007). Upon introduction, the bacteria can attach to broiler skin at various locations from easily accessible flat surfaces to protected niche areas such as hair follicles, ridges, and crevices (Kim

et al., 1996a; National Advisory Committee on Microbiological Criteria for Foods, 1997).

Bacterial cells may need only a few minutes to attach tightly to chicken skin (Arritt et al., 2002).

Using confocal scanning laser microscopy, Chantarapanont et al. (2003) reported that *Campylobacter* was initially retained within the thin water layer on carcass skin and entered hair follicles or skin crevices during processing or storage. The skin epidermal layer, colonized predominantly by gram- positive bacteria, is damaged or removed during scalding and picking operations, exposing underlying fresh dermal skin that is vulnerable to colonization by gram-negative pathogens. The dermal skin has channels and crevices, which can protect bacteria from antimicrobial treatments (Thomas and McMeekin, 1980). Broiler carcass skin and muscle fibers swell during water immersion, which exposes crevices and provides more surface area for bacteria entrapment and attachment (Chantarapanont et al., 2003). Opening of feather follicles during scalding facilitates bacterial attachment and closing of the follicles during chilling entraps the bacteria within the follicles (Bryan and Doyle, 1995). One study using scanning electron microscopy reported that some bacteria on chicken skin were deeply lodged in crevices. Such bacteria are not recovered by simple rinsing or stomaching but by shredding the skin (Nayak et al., 2001). Other researchers have also reported that gentle rinsing was not sufficient to remove tightly attached microbes from broiler skin (Notermans and Kampelmacher, 1974; McMeekin et al., 1984; Lillard, 1986; Hinton and Cason, 2008). Lillard (1988) reported that even 5 consecutive stomachings of broiler skin yielded similar numbers of bacteria, indicating that a single stomaching cannot remove tightly attached bacteria from chicken skin. Bacteria can penetrate skin and potentially muscle when the tissue absorbs moisture (Jang et al., 2007). Using confocal microscopy, Morild et al. (2011) showed that bacteria trapped in liquid suspension were drawn into hair follicles, clefts, and crevices by capillary-like forces when pigskin was washed

with water for cleaning or sanitization purposes.

Inoculated bacteria tended to attach firmly to pig skin in larger numbers when the skin was previously exposed to hot water (80°C) and lactic acid solution (1%) compared to the unexposed control skin (Morild et al., 2011). Hence, the decontamination treatment may affect the strength of attachment of bacteria that cross contaminate carcasses down the processing line. Morild et al. (2011) also found that structural and environmental conditions of the skin surface played a more important role than did bacterial species in the attachment process.

In addition to physical entrapment in skin microstructures, bacteria can attach to skin using flagellae, fimbriae, pili, ionic forces, dipole interaction, covalent bonds and/or hydrophobic interactions (Jang et al., 2007). Liquid pH and surface charge can affect bacterial attachment to the surface. Generally, as pH of the suspension liquid increases above the isoelectric point of the bacterial cell (about pH 3 for most bacteria), the bacterial surface charge becomes more negative due to ionization of surface carboxyl and amino groups. As a result, oppositely charged ions in the suspension fluid are attracted to the bacterial surface charge forming an electrical double layer of opposite and attractive charges between the bacteria and suspension fluid (van der Wall et al., 1997). The attractive force of the electrical double layer can be interfered by the charge on the surfaces of attachment (Sheng et al., 2008), such as broiler skin. In the presence of water, hydrophobic forces develop between two hydrophobic particles where they tend to stick together to reduce the association with water to go into a state requiring less energy (Magnusson, 1982). Sinde and Carballo (2000) studied the role of bacteria and contact surface hydrophobicity in the attachment process. They found that bacteria attached to more hydrophobic surface in higher numbers with greater binding force. However, bacteria with lower hydrophobicity (*Listeria monocytogenes*) attached to hydrophobic surfaces in greater number than bacteria with higher

hydrophobicity (*Salmonella*). This discrepancy suggests that apart from hydrophobicity, other forces are also involved in the bacterial attachment to surfaces. Sinde and Carballo (2000) reported that sanitizers altered surface properties, changing the degree of bacterial attachment.

While physical and chemical forces can play an important role in bacterial attachment, it is difficult to predict the specific role of each force because of the complexity of the skin surface composition and different ways of interaction between the skin surface and bacterial surface. Bacterial surface composition and properties change according to bacterial species, bacterial surface structures, types of available nutrients, and suspension fluid pH and ionic strength (Araujo et al., 2010).

Surface roughness and presence of organic matter may also influence the attachment affinity of bacteria. Moore et al. (2007) studied attachment affinities of *Salmonella* Typhimurium for up to 6 h after inoculation on different surfaces using stainless steel (S), Formica laminate (F), polypropylene (P), and wood (W), in the presence or absence of protein rich debris (tryptic soy broth with 5% horse serum). Regardless of surface type, *Salmonella* recovery fell sharply in the first hour after inoculation, followed by a slower decrease in recovery over the next 5 hours. Recovery of *Salmonella* was higher ($P < 0.05$) from smooth S and F surfaces than W and P surfaces, having peaks, crevices, and pits. When the surfaces contained protein debris, the recovery was greater ($P < 0.05$) than for surface without debris, possibly due to clogging of surface pores and crevices preventing *Salmonella* transfer to deeper locations. Other studies also showed that increased roughness promotes attachment of bacteria to the surfaces (Arnold et al., 2000 and 2004; Medilanski et al. 2002). While surface roughness is generally considered to facilitate bacterial attachment, Boyd et al. (2002) showed that a certain degree of roughness, in stainless steel, was optimum for bacterial attachment strength with an increase in roughness

beyond the threshold resulting in less corresponding increase in attachment strength.

Attachment to broiler skin can also increase bacterial resistance/tolerance towards antimicrobials. Yang et al. (2001) concluded that almost 30 ppm free chlorine reduced *Salmonella* Typhimurium and *Campylobacter jejuni* populations in chilling water by 6 logs CFU/ml or more compared to only 1 log CFU/cm² or less when the pathogens were tightly attached to chicken skin pieces. The paper concluded that a possible reason for the lower reduction might be the presence of an oil layer on the skin surface, preventing chlorine from accessing to bacteria in crevices and follicles. Similarly, Lee et al. (2014) showed that bacteria associated loosely with broiler skin were less resistant to chemical (sodium hypochlorite and thiamine dilauryl sulfate) and physical (ultrasound) treatments than tightly associated bacteria.

Strength of bacterial attachment to broiler skin is not well defined. Morild et al. (2011) described loosely and tightly attached bacteria as those recovered by rinsing, and by single swabbing or stomaching of the rinsed skin, respectively. Similarly, Arritt et al. (2002) collected loosely attached bacteria by shaking broiler skin with buffer and tightly attached bacteria by stomaching the same skin with fresh buffer. Nayak et al. (2001) classified loosely and tightly attached bacteria on poultry skin when the bacteria were collected by the first and the second stomaching, respectively. Tamblyn et al. (1997) classified loosely and tightly attached bacteria on poultry skin when bacteria were collected by shaking of the skin with buffer and blending of the same skin with fresh buffer, respectively. Since most of the studies arbitrarily quantify loosely and tightly associated bacteria on broiler skin, more refined sampling methods based on classification are required.

1.3 Pathogen cross contamination during poultry processing

During processing, cross contamination of broiler carcasses within a lot or from previous lots is a major risk to public health (Venkata et al. 2007). Processing of broiler chickens involves a range of events such as catching, hauling, transporting, shackling, stunning, bleeding, scalding, plucking/de-feathering, evisceration, washing, and finally chilling. Using Pulse Field Gel Electrophoresis (PFGE), investigators have found that even after cleaning the facility, pathogens from contaminated carcasses persist on equipment especially in scalding, de-feathering, and evisceration areas (Posch et al., 2006; Rasschaert et al., 2007; Venkata et al. 2007; Ellerbroek et al., 2010). Similar results for cross contamination of equipment were observed using multilocus sequence typing (Allen et al., 2007), restriction fragment length polymorphism (Peyrat et al. 2008), and *Salmonella* serotyping (Olsen et al. 2003).

Scalding can minimize bacteria load and pathogen prevalence if the scald water temperature is 58°C or above, whereas, scalding at 55°C or below is not effective in reducing the bacterial load and can result in cross contamination of other carcasses (Notermans et al., 1977; Oosterom et al., 1983; Wempe et al., 1983). However, scalding at high temperatures results in sloughing of the skin epidermal layer exposing a fresh dermal layer suitable for gram-negative pathogens to colonize. Some processing techniques such as pre-scald brushing, frequent addition of fresh water to the scalding tank, regulating pH of the scalding water, and multiple tank countercurrent scalding can reduce pathogen load, cross-contamination, and organic material on carcasses and scalding water (Cason et al., 2000; FAO/WHO, 2009). Clouser et al. (1995) showed that steam spray non-immersion scalding (62°C) prevented *Salmonella* cross contamination unlike conventional water immersion scalding (58°C) which resulted in a significant ($P<0.05$) increase in *Salmonella* prevalence.

De-feathering and evisceration also lead to carcass cross contamination from the gastrointestinal content (Berrang et al., 2001; Miwa et al., 2003; Alter et al., 2005; Rasschaert et al., 2006, Zhang et al., 2013). Allen et al. (2003) showed that when an inoculated carcass was introduced at the de-feathering step, the inoculated bacteria could be found in up to 200 carcasses thereafter and up to 30 carcasses ahead of the artificially inoculated carcass. Rubber fingers of the de-featherer were suggested to play a major role in the cross contamination in this study. Guerin et al. (2010) systematically reviewed 32 published studies to conclude that prevalence of *Campylobacter* increases after de-feathering (10 - 72%) and evisceration (15%) due to cross contamination at these steps. Sarlin et al. (1998) suggested that crop removal is the critical step that causes *Salmonella* cross contamination during evisceration. High *Campylobacter* populations of 2-3 log CFU/ml with a prevalence rate of 90% immediately after evisceration are not uncommon for carcasses in commercial processing plants (Oyarzabal et al., 2004). Appropriate fasting of broilers before slaughter, prevention of feather build-up on equipment, continuous rinsing of equipment, and regular replacement of plucker fingers can reduce chances of cross contamination during de-feathering (FAO/WHO, 2009). Similarly, minimizing rupture of viscera and spread of feces by minimizing bird size variation among batches and careful adjustment of automatic machines can avoid cross-contamination during evisceration (FAO/WHO, 2009).

Another important source of cross contamination is aerosol formation in the processing environment (Allen et al., 2003; Allen et al., 2007). Aerosol formation primarily takes place during de-feathering (Allen et al., 2003; Allen et al., 2007) and evisceration (Allen et al., 2007) due to vigorous physical motion of the carcass associated with these operations. These aerosols can persist in the processing environment and can contaminate carcasses and personnel.

Some studies reported a decrease in *Salmonella* prevalence at the end of processing (Morrison et al., 1985; Stopforth et al., 2007), whereas other studies indicated no change or an increase in pathogen prevalence after processing (Kramer et al., 2000; Dickins et al., 2002; Goksoy et al., 2004; Oyarzabal et al., 2004; Rosenquist et al., 2006; Rasschaert et al., 2008; Zhang et al., 2011). Using a simulation model, Hayama et al. (2011) reported that although processing was successful in reducing *Campylobacter* in a heavily contaminated flock, distribution or prevalence of *Campylobacter* in such a flock tends to increase after processing due to cross contamination.

1.4 Current poultry carcass decontamination practices in the US

The most commonly used agent for controlling cross contamination during poultry processing is chlorine (Russell, 2009). Elemental chlorine (Cl_2), also known as chlorine gas, is the most effective form of chlorine against microbes but processors widely use milder sodium hypochlorite (NaOCl) to avoid the high toxicity and corrosiveness of elemental chlorine (Russell, 2009). Sodium hypochlorite reacts with water to form hypochlorous acid (HOCl), which further ionizes into hypochlorite ions (OCl^-). Both hypochlorous acid and hypochlorite ions lead to amino acid degradation and hydrolysis in bacterial cell. Amino acids further react with HOCl to form chloramine, which interferes in cell metabolism (Estrela et al., 2002).

During poultry chilling, a thin layer of liquid film, containing organic matter, is generated on carcasses, which may render chlorine ineffective against bacteria on skin (Thomas and McMeekin, 1980). Buhr et al. (2005) reported that addition of 20 ppm chlorine to poultry chilling water significantly reduced total aerobes, *E. coli*, coliforms and *Campylobacter* on

carcasses but the reduction was less than 0.5 log for each category with no significant reduction in *Salmonella*. In a similar study, 50 ppm chlorine significantly reduced *Campylobacter* and *Salmonella* numbers by 4 to 5.5 log in chilling water but no reduction was observed on chicken skin (Yang et al., 2001). Their findings show that chlorine can prevent cross contamination through chilling water but not through direct contact between carcasses. Even chilling water may act as a medium of pathogen transfer from one carcass to other, if the chlorine effectiveness is compromised due to excessive organic load (Sanchez et al., 2002). Most studies have shown that chlorine in chilling water cannot reduce more than 1 log total aerobic bacteria on chicken skin (James et al., 1992; Bautista et al., 1997; Zhang et al. 2011). High concentrations of chlorine (>50 ppm as used in some processing plants) in chilling water are a health hazard for workers, induce strong chlorine odor, induce off flavor, and cause stainless steel corrosion (Teotia and Miller, 1975; Izat et al., 1989; Mckee, 2004; Davis et al., 2010). Excess chlorination may result in formation of three kinds of airborne irritants in processing plants: 1) detectable chlorine in the atmosphere, 2) organochloramines via free chlorine and organic compound interaction, and 3) nitrogen trichloride resulting from an increase in the chlorine to ammonia ratio (Upsher and Fletcher, 1996).

Cetylpyridinium chloride (CPC) is one of the most commonly used chemicals to disinfect poultry carcasses in the US. It is non-volatile, water soluble, and a stable quaternary ammonium compound with a neutral pH, and renders no adverse effect on product quality. Being a cationic surfactant, CPC kills bacteria through interaction of basic cetylpyridinium ions with the acid groups of bacteria to form weakly ionized compounds which inhibit bacterial metabolism (Kim and Slavik, 1996). Spraying or dipping in a 0.1% CPC solution reduced *Salmonella* on artificially inoculated broiler skin by 0.9 to 1.7 log (Kim and Slavik, 1996). Spray temperature of

15 or 50°C and dipping time of 1 or 3 min did not influence CPC activity against *Salmonella* in the study. Spraying CPC (0.5%) inside and outside carcasses at 35°C and 413 kPa reduced artificially inoculated *Salmonella* by almost 2 log CFU/carcass and naturally occurring mesophilic aerobic bacteria (MAB) by 2.16 log CFU/carcass (Yang et al., 1998). Similarly, Xiong et al. (1998) showed that spraying broiler skin with 0.1 and 0.5% CPC at 20°C reduced artificially inoculated *Salmonella* by 1.5 and 1.9 log CFU/mL, respectively, and naturally occurring MAB by 1.6 and 2 log CFU/mL, respectively. Wang et al. (1997) showed that spraying pressure played no role at 10°C but significantly influenced CPC (0.1%) activity against *Salmonella* at 60°C on broiler skin. While CPC is effective against poultry associated pathogens, non-GRAS status is its major drawback leading to serious export impediments, especially for the European market.

Organic acids are GRAS chemicals that can effectively reduce the bacterial load on broiler carcass (Mountney and O'Malley, 1965; Lillard et al., 1987; Bolder, 1997; Tamblyn and Conner, 1997; Capita et al., 2002b; Fabrizio et al., 2002). USDA-FSIS has approved the use of 1.5-2.5% organic acids for poultry carcass decontamination (USDA FSIS, 2006). Organic acids, like lactic acid and acetic acid (AA), which can be directly added to chilling water are less sensitive to organic substances than chlorine (Dickens et al., 1994; Dickens and Whittemore, 1994,1997; Bolder, 1997; Cherrington et al., 1992). Among all organic acids, acetic acid and lactic acid are most accepted by the poultry industry and researchers (Dincer and Baysal, 2004). Pre-chilling in 0.6% acetic acid for 10 min significantly reduced enterobacteriaceae on broiler carcasses (Dickens et al., 1994). Dickens and Whittemore (1995) showed that chilling broiler carcasses for 1 h in static ice slush with 0.6% AA and in a paddle type chiller with 0.6% AA reduced MAB by 0.34 and 1.16 log CFU/ml, Enterobacteriaceae by 0.5 and 1.4 log CFU, and

artificially inoculated *Salmonella* by 7% and 80.3%, respectively. These results indicate the importance of agitation of the chilling solution for improving antimicrobial activity. Using a commercial broiler processing line, Meredith et al. (2013) demonstrated that post-bleeding cloacal washing with 5 and 10% lactic acid reduced population of mesophiles and psychrophiles on eviscerated broiler carcasses. The results suggest that cloacal leaking may be one of the sources of bacterial contamination on carcasses during processing. Mountney and O'Malley (1965) reported that adjusting the chilling water pH to 2.5 with acetic acid was the most effective antimicrobial treatment over 5 other acids (adipic, succinic, citric, fumaric and lactic acids), but resulted in hard and leathery poultry skin. Discoloration and a leathery appearance of carcasses with a sharp smell are major drawbacks associated with organic acids (Dincer and Baysal, 2004).

1.5 Trisodium phosphate (TSP/Na₃PO₄) treatment

The US Food and Drug Administration granted GRAS status to TSP for use as a processing aid with no label declaration requirement in 1992. The same year, TSP was approved for use in poultry slaughter operation as an antimicrobial solution at levels of 8-12% (Capita et al., 2002b). TSP has a high pH (around 12) and ionic strength, which result in bacterial cell lysis (Capita et al., 2002b; USDA FSIS, 2002). TSP also acts as a detergent, dissolving the thin layer of fat, which aids in the release of bacteria during washing (SCVPH, 1998). The hydroxyl ions remaining on the treated product also help to prevent further growth of bacteria during storage (Ramirez et al., 2001).

TSP is more active against gram-negative bacteria such as *Salmonella* and *Campylobacter* compared to gram-positive bacteria such as *Listeria* and *Staphylococcus* due to

differences in their cell membrane structure (Colin and Salvat, 1996; Liao and Cooke, 2001).

Colin and Salvat (1996) reported a 100 fold reduction in gram negative bacteria compared to 10 fold reduction in total aerobic mesophiles, including both gram positive and negative, after application of 10% TSP.

Stopforth et al. (2007) showed that spraying with 8 to 12% TSP reduced naturally occurring MAB, *E. coli*, and total coliforms by 0.5, 0.7, and 0.8 log CFU/mL, respectively, reducing the *Salmonella* incidence from 10% to 3%. Compared to a pure water spray, mesophilic aerobic bacteria and artificially inoculated *Salmonella* were reduced by 0.71 and 1.36 log CFU/carcass after a 10% TSP spray (Yang et al., 1998). Compared to distilled water, use of a 10% TSP spray on eviscerated broiler carcasses, inoculated with poultry fecal material and *Salmonella* Typhimurium, reduced MAB, *E. coli*, total coliforms and *Salmonella* Typhimurium by 0, 0.29, -0.12, and 0.03 log CFU/mL, respectively (Fabrizio et al., 2002). In the same study, compared to immersion chilling of carcasses to an internal temperature of 4°C in distilled water, chilling of carcasses in 10% TSP solution reduced the populations of MAB, *E. coli*, total coliforms, and *Salmonella* Typhimurium by 1.45, 3.33, 3.7, and 1.9 log CFU/mL, respectively (Fabrizio et al., 2002). Whyte et al. (2001) reported that compared to plain water dipping, 10% TSP dipping of eviscerated broiler carcasses for 15 s reduced MAB, *E. coli*, *Enterobacteriaceae*, *Salmonella* (Most Probable Number), and *Campylobacter* (Most Probable Number) by 0.83, 1.58, 1.86, >0.56, and 1.16 log CFU/g, respectively.

Thirty seconds of spraying with 12% TSP and 1000 ppm acidified sodium chlorite (ASC) were similar effective in reducing naturally occurring *Campylobacter*, *Enterobacteriaceae* and *Pseudomonas* on broiler carcass while they were more effective than 6 ppm chlorine dioxide or a peroxyacetic acid solution, containing 400 ppm of peracetic acid, 1600 ppm of hydrogen

peroxide, and 800 ppm of acetic acid (Purnell et al., 2014). When the treatment time was increased from 15 to 30 s, the increase in efficacy was larger for TSP than the other antimicrobial agents (Purnell et al., 2014). Bolton et al. (2014) showed that TSP (10 and 14%) and citric acid (1 and 5%) were similar in reducing total viable bacteria, *Enterobacteriaceae*, *Pseudomonas*, lactic acid bacteria and yeasts/molds on broiler skin, while they were more effective than lactic acid (1 and 5%), peroxyacids (100 and 200 ppm), and ASC (500 and 1200 ppm).

Since TSP is more effective against gram negatives than gram positives, it was a concern that refrigerated storage of TSP treated carcasses could result in domination of gram-positive bacteria such as *Listeria*. However, Salvat et al. (1997) showed that when *Pseudomonas*, gram-negative and dominant spoilage bacteria, population decreased after TSP treatment on broiler carcass, *Brochothrix thermosphacta*, a gram positive spoilage bacteria became dominant rather than *Listeria* during refrigerated storage because the growth rate of *B. thermosphacta* was greater than that of *Listeria*. Shelf life of TSP treated broiler meat increased under refrigeration as *B. thermosphacta* developed slower than *Pseudomonas*. Alonso-Hernando et al. (2015) inoculated broiler legs with same concentrations of *L. monocytogenes* and *B. thermosphacta* and showed that after 72 h of refrigerated storage under mild temperature abuse, 12% TSP-dipped (15 min, 20°C) broiler legs had almost 1 log CFU/cm² of artificially inoculated *L. monocytogenes* and almost 3.5 log CFU/cm² of artificially inoculated *B. thermosphacta*. After 120 h of storage, *L. monocytogenes* and *B. thermosphacta* populations were almost 4 and 5 log CFU/cm², respectively. On untreated legs, the differences between the two bacteria populations were 1 log CFU/cm² or less after 72 and 120 h of storage. Results again show that *B. thermosphacta* outcompetes *Listeria* in terms of growth during refrigerated storage of TSP treated broiler meat.

A study on beef carcass showed that TSP yielded a greater reduction (3.04 logs) of *E. coli*

when applied immediately after inoculation than after 2 h (1.76 log reduction) and 4 h (1.26 log reduction). These results suggest that the earlier the TSP application, the better the bacterial reduction. Antimicrobial activity of TSP is greater at higher temperatures (Xiong et al., 1998). More *Salmonella* cells were reduced after TSP treatment at 50°C compared to 10°C (Kim et al., 1994), and after 90 s as compared to 30 s of exposure (Korber et al., 1997).

1.6 Hot water spray (HWS), and brushing

Hot water spray is one of the more promising decontamination methods to improve microbiological quality of poultry carcasses (Tompkins et al., 2008). Several studies have reported that pathogenic and spoilage bacteria were reduced on poultry carcass by hot water spraying, immersion or steam exposure (Cox et al., 1974b; James et al., 2000; Purnell et al., 2004; James et al., 2007). Hot water spraying for 1 min at 65.6°C or 71°C decreased total aerobic bacteria on chicken carcasses by 1 to 2 logs, but with cooked visual appearance of the carcass (Cox et al., 1974b; Thomson et al., 1974). Hot water spraying at 55 and 60°C (12 s, 80 psi) reduced inoculated *C. jejuni* by more than 0.78 log CFU/carcass with no damage to skin color at temperatures below 60°C, although some changes in b* value were observed on skin at 60°C (Li et al., 2002). Corry et al. (2007) also showed that hot water dipping at 70°C/ 40 s, 75°C/ 30 s, and 80°C/ 20 s reduced inoculated *Escherichia coli* K12 by 1.16, 1.3, and 1.31 log CFU/ cm², respectively, and inoculated *Campylobacter jejuni* by 0.98, 1.66, and 1.27 log CFU/cm², respectively (Corry et al., 2007). Dipping carcasses in 70°C water caused no visual damage but some damage was observed when dipping at 75 and 80°C (Corry et al., 2007). Berrang et al. (2000) showed that hot water dipping (60°C, 28 s) or spraying (71°C, 20 s) reduced naturally

occurring aerobic mesophiles but not *Campylobacter*, *E. coli*, and coliforms on broiler carcasses. Zhang et al. (2013) reported that hot water spraying (HWS) (71°C for 1 min) after evisceration reduced naturally present mesophilic aerobic bacteria on post-evisceration carcasses, and prevalence of loosely, intermediately and tightly attached *Salmonella* but not intermediately or tightly attached *Campylobacter* on post-chill carcasses. However, carcasses exposed to HWS suffered from a partially cooked appearance (Zhang et al., 2013). Hot water dipping (70°C, 1 min) was more effective than hot water spraying (70°C, 1 min) against presumptive coliforms on naturally contaminated carcasses (Sinhamahapatra et al., 2004).

Based on previous work, it appears that hot water dipping or spraying is effective against inoculated *Campylobacter* and *E. coli* (Corry et al., 2007; Li et al., 2002) but not against naturally occurring *Campylobacter* and *E. coli* (Berrang et al., 2000, Zhang et al., 2013) on broiler carcasses. From the carcass appearance perspective, hot water spraying at 60°C or higher, even for a few seconds, seems to produce visual damage on carcass (Li et al., 2002; Thomson et al., 1974; Zhang et al., 2013). Similarly, hot water dipping at more than 70°C for 30s seems to adversely affect carcass appearance (Corry et al., 2007).

Not much research has been conducted to study the effect of brushing with hot water dipping or spraying on microbiological quality of broiler carcasses. Berrang and Belly (2009) reported that carcass brush washers helped control *E. coli* (0.5 log reduction) and coliforms (0.5 log reduction) but not *Campylobacter* or *Salmonella* prevalence. Since brushing can reach bacteria in deeper hidden areas, the combination of brushing and antimicrobial additives may act synergistically to remove difficult to access bacteria cells that are more tightly associated.

1.7 Combination of physical and chemical interventions

Dipping of artificially inoculated chicken wings in TSP (10%, 10°C, 15 min) or/and hot water (95°C, 5 s) showed that TSP dipping alone reduced *Salmonella* Typhimurium by 93.45% and 62.42% after 24 h of storage at 10°C and 4°C, respectively, and hot water dipping alone reduced *S. Typhimurium* by 83.5% and 47.44%, while their combination led to reduction of 94.76% and 99.67% at the two storage temperatures (De Ledesma et al., 1996). Li et al. (1994) showed that *Salmonella* Typhimurium inoculated on chicken skin was reduced 34 to 76% when treated with 1% NaCl, Na₂CO₃, or TSP alone but more than 90% when these treatments were combined with an electrical treatment (4mA/cm², 1kHz frequency). Among the combination treatments, TSP and electrical treatments resulted in maximum reduction in *Salmonella* prevalence. Mild lactic acid (5%) and 70°C steam treatments synergistically reduced *Listeria innocua* on chicken skins while preserving the skin's raw appearance, although the most effective antibacterial treatment proved to be a combination of 10% lactic acid and 98°C steam (Lecompte et al., 2008). Koolman et al. (2014) showed that ultrasound treatments (from 40 to 80 kHz) reduced natural total viable bacteria (TVC) and *Enterobacteriaceae* counts as well as inoculated *C. jejuni* on broiler drumsticks by almost 1 log CFU/cm². The combination of ultrasound and 12% TSP caused an additional 1 log CFU/cm² reduction in natural TVC and *Enterobacteriaceae* populations and an additional 1 to 2 log CFU/cm² reduction in inoculated *C. jejuni* (Koolman et al., 2014).

Combined use of physical and chemical interventions seems to generate synergistic or additive effects against pathogens on broiler carcasses, with the potential for preserving appearance and organoleptic properties of the carcasses at milder intensities.

1.8 Poultry skin imaging (confocal and scanning electron microscope)

Thomas and McMeekin (1980) used scanning electron microscopy (SEM) and transmission electron microscopy (TEM) to visualize broiler skin before processing, after plucking, and after immersion chilling. The study indicated that scalding and de-feathering removed the skin epidermis exposing the underlying dermis, which had capillary size channels and crevices entrapping bacteria. The study emphasized that structural changes in the broiler skin surface during processing induced entrapment of water along with bacteria (primarily gram-negative), leading to a tight association of bacteria and cross-contamination of carcasses. Using scanning electron microscopy, Lee et al. (2014) showed that inoculated *Salmonella* Typhimurium became entrapped inside crevices and follicles. Such entrapped *Salmonella* were difficult to remove by physical (ultrasound) or chemical (sodium hypochlorite) treatment alone, although their combination showed better results. A study using confocal microscopy showed that crevices (0.15, 0.3, and 0.5 mm wide), unlike flat surfaces created on glass coverslips, were able to protect *Salmonella enteritidis* from TSP treatment (Korber et al., 1997).

Using electron microscopy, Kim et al. (1994) showed that a 10% TSP rinse could remove surface debris and *Salmonella* from broiler skin surfaces. The TSP treated skin looked cleaner due to removal of dirt and debris that were not removed by a plain water rinse. Chantarapanont et al. (2003) used confocal microscopy to show that *C. jejuni* were removed from broiler skin surfaces after rinsing, while those cells located in crevices and feather follicles remained. *Campylobacter* populations on the surface decreased by 1 log after storage at 25°C for 24 h whereas cells located 20 to 30 µm beneath the surface maintained their viability. *Campylobacter* cells were initially trapped in a thin layer of water on the skin and then penetrated into follicles and channels during storage (Chantarapanont et al., 2003). Similarly, Nayak et al. (2001) used

electron microscopy to observe deep channels and crevices in broiler skin that may trap bacteria that may not be recovered unless the skin is shredded. However, Chantarapanont et al. (2004) used confocal microscopy to report that there was no evidence that deeper located *Campylobacter* cells were better protected from chemical sanitizers (sodium hypochlorite, peracetic acid, and acidified sodium chlorite). The study also reported that inoculated *C. jejuni* cells were mostly found at a depth of 0-10 μm with very few viable cells found in deeper locations (21 to 30 μm) of the skin (Chantarapanont et al., 2004).

While there are some discrepancies among past studies regarding the role of skin structures in attachment/protection of bacteria, skin microstructures seem to provide some protection to bacteria against rinsing and chemical sanitizers in most cases. It would be of interest to understand the effect of sanitizers on loosely and tightly associated bacteria on broiler carcasses along with the effect on skin microstructures.

CHAPTER 2

EFFECT OF HOT WATER SPRAY ON BROILER CARCASSES FOR REDUCTION OF LOOSELY-ASSOCIATED, INTERMEDIATELY-ASSOCIATED, AND TIGHTLY- ASSOCIATED PATHOGENIC (*SALMONELLA* AND *CAMPYLOBACTER*) AND MESOPHILIC AEROBIC BACTERIA

2.1 Abstract

Chickens are known to harbor many bacteria, including pathogenic microorganisms such as *Salmonella* and *Campylobacter*. The objective of this study was to evaluate the efficacy of hot water spray (HWS, 71°C for 1 min) in reducing bacterial contamination of pre-chilled broiler carcasses. For each of 4 replications, skin samples from 5 broilers were collected at 3 processing stages: after bleeding (feathers removed manually), after evisceration (with/without HWS), and after water chilling. Broiler skin was quantitatively assessed for loosely associated (by rinsing the skin), intermediately associated (by stomaching the rinsed skin), and tightly associated (by grinding the rinsed and stomached skin) mesophilic aerobic bacteria (MAB) and *Campylobacter* as well as for the prevalence of *Salmonella* and *Campylobacter*. Broiler skins possessed 6.4 to 6.6 log cfu/g, 3.8 to 4.1 log cfu/g, and 2.8 to 3.5 log cfu/g of MAB populations after bleeding, evisceration, and chilling, respectively. The HWS resulted in more than 1 log unit of reduction in MAB immediately after evisceration and immediately after chilling regardless of microbial sampling method. Compared with MAB, the contamination of *Campylobacter* was low (1.7 to 2.6 log cfu/g) after bleeding, but the level was not reduced throughout the processing steps regardless of HWS. The application of HWS reduced the prevalence of *Salmonella* after chilling, but not of *Campylobacter* except for loosely associated cells. After hot water exposure, a partially cooked appearance was seen on both broiler skin and skinless breast surface. More research is required to effectively reduce pathogenic organisms, regardless of association type, during processing.

2.2 Introduction

Chickens, carrying millions of bacteria internally and externally, are an excellent vehicle for foodborne pathogens particularly *Campylobacter* and *Salmonella* (Izat et al., 1988; Corry and Atabay, 2001; Capita et al., 2004). At each step of processing, there is always possibility that some of those bacteria can contaminate equipment, employees and carcasses. Therefore, intervention strategies that reduce the microbial contamination on poultry meat are necessary to lower the potential of foodborne illness. Both *Salmonella* and *Campylobacter* have been known to persist on chicken skin during poultry processing because of their ability to attach to skin and become entrapped in deeper skin layers, crevices or feather follicles (Notermans and Kampelmacher, 1975; McMeekin et al., 1984; Lillard, 1986). These sites may provide a suitable microenvironment for bacteria to lodge (McMeekin et al., 1979; Chantarapanont et al., 2003) and physical protection from antimicrobial agents such as chlorine, acidified sodium chlorite and peracetic acid (Yang et al., 2001; Chantarapanont et al., 2004).

Lillard (1989a) indicated that a high number of bacteria were recovered after 40 consecutive rinses of a single carcass due to a firm attachment to poultry skin, some of which were extremely difficult to eliminate using a conventional washing method. Zhang et al. (2011) reported that the incidence of *Salmonella* (12%) and *Campylobacter* (92%) on eviscerated broiler carcasses decreased to 0% and 4%, respectively, after in-line spraying of disinfectant. However, the incidence rate was increased to 13% for *Salmonella* and 52% for *Campylobacter* after carcass-chilling, indicating that the chemical spray did not reduce pathogens sufficiently on the broiler surface.

Hot water treatments have been used for many years to improve the safety of food products. A number of researchers have had some success in decontaminating poultry meat using

either hot water sprays/immersion or steam exposure (Cox et al., 1974a; James et al, 2000; Purnell et al., 2004; James et al., 2007). It appears that these treatments are more effective at higher temperatures and longer exposures. Morrison and Fleet (1985) found that by immersing a pre-chill carcass in 60°C water for 10 min, the number of *Salmonella* was reduced 100-fold without adversely affecting carcass appearance. Cox et al. (1974b) reported that by immersion in water at 71°C for 1 min, total aerobic bacterial count on carcass was lowered by almost 2 log unit, but a partially cooked appearance was produced. Thomson et al. (1974) found that microbial counts on skins were reduced by about 1 log unit, and shelf life was subsequently extended for 1 to 2 days with the spray of hot water at 65.6 or 71.1°C. Again, an inferior appearance was observed on broiler carcass after washing with hot water at 71.1°C. Li et al. (2002) found that the inside-outside spraying of carcasses at 55 and 60°C significantly reduced the inoculated *Campylobacter jejuni* by more than 0.78 log CFU/carcass compared with a water spray at 20°C.

It is desirable to use appropriate microbial enumeration method to precisely assess the bacterial populations on carcasses after implementing intervention technology. Carcass rinsing is one of many sampling techniques (Capita et al., 2004; Cox et al., 2010). However, the gentle force of rinsing may not be sufficient to remove microorganisms on carcasses or from selected samples (Notermans and *Kampelmacher*, 1974; McMeekin et al., 1984; Lillard, 1986; Hinton and Cason, 2008). Subjecting samples to stomaching likely increases the number of bacteria that are attached tightly to deeper layers of skin (Avens and Miller, 1970; Notermans et al., 1975) although a quite number of bacteria still remain in the skin (Hannah et al., 2011). Lillard (1988) indicated that five consecutive stomaching of broiler skin showed similar results. These types of cells attached tightly or internally may be recovered when the whole sample is ground. Therefore,

the objective of this study was to assess the efficacy of hot water spray on pre-chilled (or eviscerated) carcasses for the reduction of so-called loosely-associated (by rinsing skin), intermediately-associated (by stomaching the rinsed skin), and tightly-associated (by grinding the rinsed/stomached skin) mesophilic aerobic bacteria, as well as *Salmonella* and *Campylobacter*.

2.3 Materials and methods

2.3.1 Broiler processing

A total of 100 approximately 46-day-old live birds (HubbardM99/ross 708) were used for this study. For each of 4 replications, 25 broilers were taken from different flocks on 4 different days at a local commercial plant. After a 12 h feed withdrawal period, the birds were cooped in plastic cages and transported to the Michigan State University poultry-processing laboratory. Upon arrival, broilers were electrically stunned for 3 s (40 mA, 60 Hz, 110 V) and bled for 90 s by severing both carotid artery and jugular vein on one side of the neck. Following bleeding, 5 out of 25 birds were randomly picked and the feathers on breast and neck areas were manually removed. The remaining 20 birds were subjected to conventional processing for scalding at 56.7°C for 120 s, de-feathering in a rotary drum picker (SP38SS automatic pickers, Brower Equipment, Houghton, IA) for 25 s, and eviscerating manually. After washing, half of the eviscerated carcasses received hot water spray (HWS at 71°C for 1 min, outside only) while the remaining half had a tap water spray from the same water source.

These two groups of birds were then separately chilled by immersing to one of two ice slurry mixtures (0.5°C; 50 ppm chlorine; 7.6 L/bird). The chlorine level (50 ppm) in the chilling

water was measured using chlorine testing kits 321 (Ecolab Inc., St. Paul, MN) and the water was mechanically agitated (0400-025GV1S potable agitator, Grovhac Inc., Brookfield WI) during the entire chill. For microbial evaluation, breast and neck skins (25g / bird) were taken from 5 carcasses at each of 3 processing steps – after bleeding, after evisceration/washing (regular or hot water), and after chilling (Figure 2.1).

2.3.2 Sampling methods

Skin samples (25g) from breast and neck area were aseptically taken, placed in WhirlPak[®] bags (Nasco, Modesto, CA), and stored on ice in a cold room (4°C) for less than 2 h before analysis. Two hundred and twenty five milliliter of buffered peptone water (BPW) was added to each of skin-contained-WhirlPak[®] bags, manually shaken for 1 min, and used to analyze so-called loosely-associated microorganisms. The rinsed skin was then aseptically transferred to a new WhirlPak[®] bag having the same amount of BPW, stomached for 1 min (Stomacher[®] 400 Circulator, Seward, Worthing, UK) and used to analyze so-called intermediately-associated cells. Lastly, the rinsed and stomached skin was transferred to a stile bottle and physically ground with the same amount of BPW using Brinkmann Polytron Homogenizers (Plytron[®] PT10/35, Brinkmann Instruments, Co. Westbury, NY) to analyze so-called tightly-associated cells. From this point, those 3 groups of bacteria i.e. “loosely-associated, intermediately-associated, and tightly-associated” mean the bacteria that were analyzed from the rinsed, stomached, and ground skins, respectively. These experiments were repeated 4 times for 4 replications, except that tightly associated cells were not analyzed in replication 1.

2.3.3 Microbiological analysis

A serial 10-fold dilutions of the rinsed-, stomached- or ground-samples were surface-plated (0.1 ml) in duplicate on standard method agar (Acumedia, Lansing, MI) to enumerate mesophilic aerobic bacteria (MAB). In case of *Campylobacter*, both quantitative and qualitative assessments were carried out. For *Campylobacter* enumeration, the sample solution (0.1 ml) from rinsing, stomaching or grinding was spread-plated on each of two Campy-Cefex agar plates (CCA, Acumedia, Lansing, MI). These plates were then placed in 1 gallon re-sealable Whirl-Pak bags (Nasco, Modesto, CA) that were flushed with a mixture of 5% O₂, 10% CO₂, and 85% N₂ (Airgas Great Lakes, Lansing, MI), and incubated at 42°C for 48 h. In addition to direct plating, 30 ml aliquots of the rinsed, stomached or ground solutions were added to 4 oz. Whirl-Pak[®] bags, containing 30 ml of 2x Blood-Free Bolton's enrichment broth (Oxoid LTD., Basingstoke, UK). These bags were flushed with the same gas mixture, incubated at 42°C for 48 h for enrichment, streaked onto plates of CCA, and incubated again as previously stated for *Campylobacter* prevalence. Five to 10 presumptive *Campylobacter* colonies from each treatment were examined microscopically for typical cellular morphology and mobility, and then confirmed using the Campy detection system (Remel Inc., Lenexa, KS).

The presence of *Salmonella* was assessed by adding 30 ml of the rinsed-, stomached- or ground-sample to 30 ml of BPW (Acumedia, Lansing, MI) followed by 20 h of incubation at 37°C. After incubation, 100 µL of the pre-enriched solution was transferred to 10 ml of Rappaport-Vassiliadis broth (RV broth, Acumedia) and incubated again at 42°C for 20 h. An aliquote (120 µl) of this enrichment was then examined for *Salmonella* using Reveal[®] *Salmonella* test kits (Neogen Corp., Lansing, MI). All positive samples were streaked onto brilliant green sulfur and xylose lysine tergitol-4 agar (Acumedia, Lansing, MI), incubated at

37°C for 24 h and then inspected for typical *Salmonella* colonies to confirm the Reveal® *Salmonella* results.

2.3.4 Statistical analysis

Bacterial counts were transformed to log units. The GLM and Duncan's multiple range test (SAS Institute Inc., 2002) were used to assess the differences in bacterial counts among the sampling sites for statistically significant differences at $P < 0.05$.

$$y_{ij} = \mu + \alpha_i + \varepsilon_{ij},$$

in which y_{ij} is the j th replicate observation of bacterial number (log CFU/g) from the i th sampling site ($i = 5$), $j=1$ to n ($n=3$, for tightly associated cells; $n=4$, for loosely and intermediately associated cells). The μ is the overall population mean bacterial number (log CFU/g) from the 5 sampling sites. The α_i is the effect of the sampling site on bacterial number. The ε_{ij} is random error associated with each replicate observation within any of the 5 sampling sites.

Prevalence differences for *Salmonella* and *Campylobacter* were determined using the chi-squared test (FREQ procedure).

$$\chi^2 = \sum_{i=1}^k (O_i - E_i)^2 / E_i,$$

in which O_i is the observed positive/negative number for *Salmonella* or *Campylobacter* at each sampling site i (data were consolidated: 3 replications for tightly associated cells, 4

replications for loosely and intermediately associated cells), and E_i is the expected positive/ negative number for *Salmonella* or *Campylobacter* at each sampling site i ($i = 5$, represents sampling sites; data were consolidated: 3 replications for tightly associated cells, 4 replications for loosely and intermediately associated cells). A P -value of <0.05 was considered significant.

Figure 2.1 Sampling sites during broiler processing with or without hot water spray (HWS)

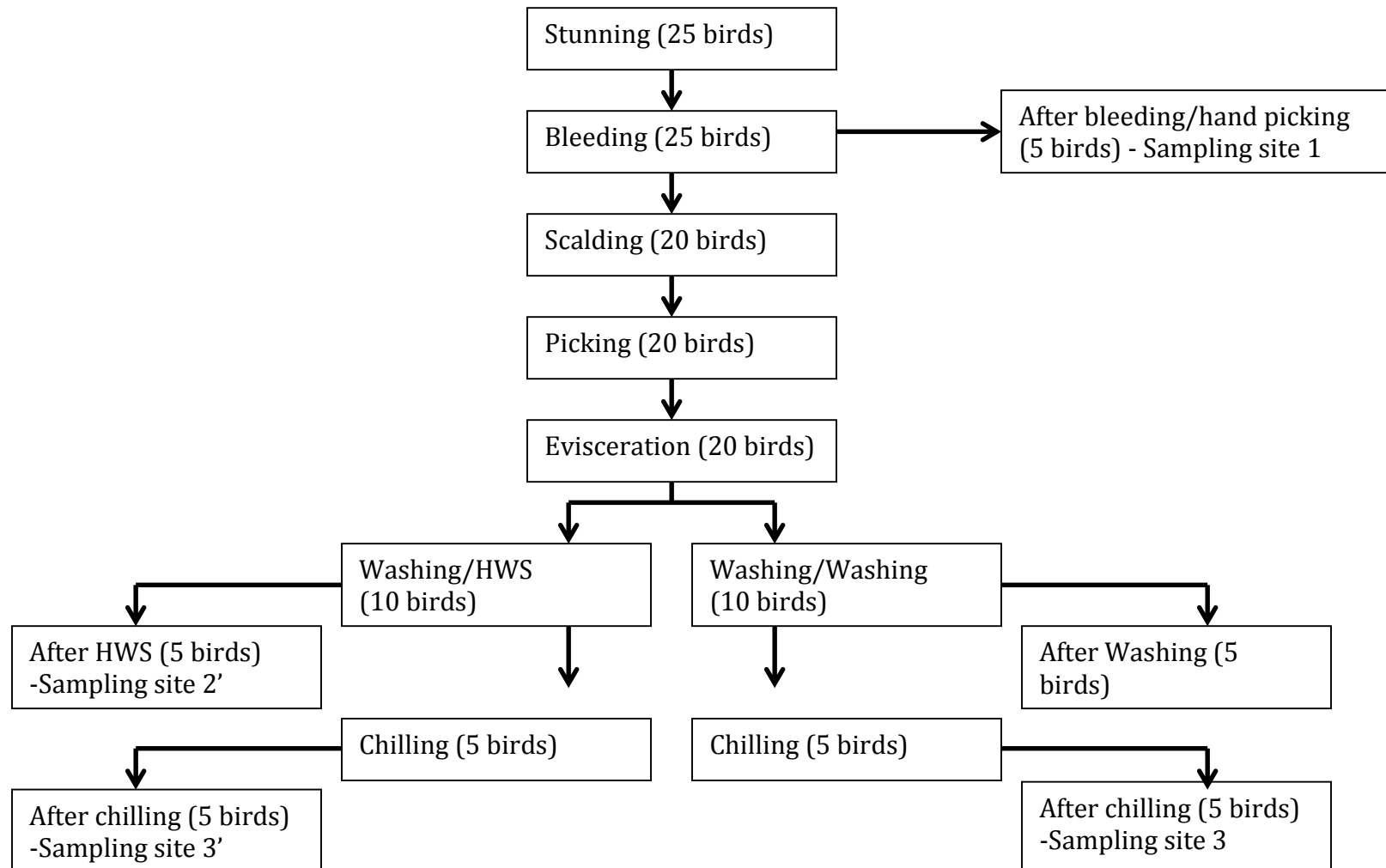


Table 2.1 Populations (log CFU/g) of mesophilic aerobic bacteria (MAB) and *Campylobacter* that were loosely, intermediately, and tightly associated on chicken skin at different sampling sites

Item	MAB ¹			<i>Campylobacter</i> ¹		
	Loosely	Intermediately	Tightly	Loosely	Intermediately	Tightly
Postbleeding	6.5 ^{a,x}	6.4 ^{a,x}	6.6 ^{a,x}	1.7 (1/20, 5%)	2.6 (4/20, 20%)	2.2 (6/15, 40%)
Postevisceration	3.8 ^{b,x}	3.8 ^{b,x}	4.1 ^{b,x}	3.4 (3/20, 15%)	2.2 (7/20, 35%)	2.5 (2/15, 13%)
Postevisceration/ HWS²	2.7 ^{c,x}	2.8 ^{c,x}	2.8 ^{c,x}	2.4 (1/20, 5%)	2.4 (2/20, 10%)	1.7 (1/15, 7%)
Postchilling	2.8 ^{c,x}	3.2 ^{bc,x}	3.5 ^{b,x}	3.3 (4/20, 20%)	3.2 (3/20, 15%)	3.3 (2/15, 13%)
Postchilling/ HWS²	1.7 ^{d,x}	2.0 ^{d,x}	2.1 ^{c,x}	2.3 (3/20, 15%)	1.7 (1/20, 5%)	1.9 (4/15, 27%)

^{a-c}Means within a column with no common superscripts are different ($P < 0.05$).

^xMeans within a row with common superscripts are not different ($P > 0.05$).

¹Mean values (direct plating) for loosely and intermediately associated ($n = 20$), and for tightly associated ($n = 15$); numbers in parentheses are the number of positive samples out of 15 or 20 samples and percentages for loosely, intermediately, and tightly associated *Campylobacter* cells without enrichment.

²HWS = hot water applied after evisceration.

Table 2.2 Prevalence of *Salmonella* and *Campylobacter* that were loosely, intermediately, and tightly associated with chicken skin at different sampling sites

Item	<i>Salmonella</i> positive % ¹			<i>Campylobacter</i> positive % ¹		
	Loosely	Intermediately	Tightly	Loosely	Intermediately	Tightly
Postbleeding	45 ^{ab,x} (9/20)	40 ^{ab,x} (8/20)	67 ^{ab,x} (10/15)	60 ^{ab,y} (12/20)	60 ^{a,y} (12/20)	60 ^{a,y} (10/15)
Postevisceration	85 ^{a,x} (17/20)	80 ^{a,x} (16/20)	93 ^{a,x} (14/15)	85 ^{a,y} (17/20)	90 ^{a,y} (18/20)	33 ^{a,y} (5/15)
Postevisceration/HWS²	45 ^{ab,x} (9/20)	40 ^{ab,x} (8/20)	53 ^{ab,x} (8/15)	65 ^{ab,y} (13/20)	65 ^{a,y} (13//20)	60 ^{a,y} (10/15)
Postchilling	65 ^{a,x} (13/20)	50 ^{ab,x} (10/20)	60 ^{ab,x} (9/15)	45 ^{ab,y} (9/20)	50 ^{a,y} (10/20)	47 ^{a,y} (7/15)
Post chilling/HWS	20 ^{b,x} (4/20)	15 ^{b,x} (3/20)	20 ^{b,x} (3/15)	40 ^{b,y} (8/20)	55 ^{a,y} (11/20)	40 ^{a,y} (6/15)

^{a,b}Values (%) within a column with no common superscripts are different ($P < 0.05$).

^xMeans within a row (*Salmonella*) with common superscripts are not different ($P > 0.05$).

^yMeans within a row (*Campylobacter*) with common superscripts are not different ($P > 0.05$).

¹Numbers in parenthesis are the numbers of positive out of 20 samples for loosely and intermediately (n=20), and tightly associated (n=15) cells.

²HWS = hot water applied after evisceration.

2.4 Results and discussion

The mean populations of mesophilic aerobic bacteria (MAB) and *Campylobacter*, and prevalence of *Salmonella* and *Campylobacter* on broiler skins were evaluated for their association profiles during broiler processing. After bleeding, the populations of loosely-associated, intermediately-associated and tightly-associated MAB on broiler skins were 6.5, 6.4, and 6.6 log CFU/g, respectively, with no significant differences among each other ($P > 0.05$, Table 2.1). These populations were significantly reduced ($P < 0.05$) to 3.8, 3.8, and 4.1 log CFU/g after evisceration, and to 2.7, 2.8, and 2.8 log CFU/g after hot water spray (HWS) at 71°C for 1 min in the same association order. The 1 log unit reduction in HWS samples over no HWS was also seen after chilling, resulting in the lowest MAB populations for loose (1.7 log CFU/g), intermediate (2.0 log CFU/g), and tight (2.1 log CFU/g) association levels (Table 2.1).

The total microbial loads (over 10^6 CFU / g) on broiler skin prior-to scalding were agreed with the report ($\sim 10^6$ microorganisms per 16 cm² of skin before processing) of Thomas and McMeekin (1980). According to their research, the majority of these bacteria was *Micrococcus* spp. and located within clumps on the skin surface. During processing, numbers of viable and aerobic bacteria on the broiler skin were significantly reduced, whereas a heterogeneous population (mainly gram-negative bacteria) replaced the predominant gram-positive microflora (McMeekin and Thomas, 1978; Thomas and McMeekin, 1980).

When samples were not enriched, *Campylobacter* were seldom detected (5 – 40%) with the levels < 2.6 log CFU/g after bleeding, but the cells were not significantly reduced throughout processing, regardless of HWS application and association types (Table 2.1). When these samples were enriched, 60% of broiler skins were positive for *Campylobacter* after bleeding for the 3 association levels (Table 2.2). After evisceration and chilling, the percentage was increased

to 85 – 90% (except for tightly associated) and decreased to 45 – 50%, respectively, with no significant difference ($P > 0.05$) except loosely associated *Campylobacter* on HWS/post-chilled samples. The prevalence (40 – 67%) of *Salmonella* after bleeding was also increased to 80 – 93% following evisceration with a significant reduction ($P < 0.05$) to 15 – 20% after HWS and chilling (Table 2.2). As compared to post bleeding, number of MAB decreased but prevalence of *Salmonella* tends to increase post evisceration (Table 2.1, 2.2). The difference in MAB and *Salmonella* population/prevalence pattern between the two stages confirms findings of Chantarapanont et al. (2003) and Thomas and McMeekin (1980) that scalding and picking operations remove skin epidermal layer, inhabited predominantly by gram positives, to expose fresh dermal skin to gram negatives, such as *Salmonella*, originating primarily from chicken gut material and processing environment.

Several studies have shown that live birds are contaminated with bacterial flora internally and externally, both of which can come in and spread over other birds during processing (Notermans and Kampelmacher, 1974; Thomas and McMeekin, 1980; Firstenberg-Eden, 1981; Lillard, 1989b). A certain portion of these bacteria (inherent and/or newly introduced) can be recovered in an initial rinsing while repeated rinsing could capture the remaining cells. Lillard (1988) reported that only a small percentage of the total bacteria are removed in the first rinse while the rest of majority remains in the carcasses. Other studies also showed that *Salmonella* is not always isolated from the initial rinse but from consecutive rinses of the same carcass (Lillard, 1989b; Izat et al., 1991).

In scanning electron microscopy studies, bacteria were found to attach preferentially to loose-connective tissues (fascia) on chicken skin layers (McMeekin et al., 1979; Benedict et al., 1991; Kim et al., 1996c). Observing closely, Chantarapanont et al. (2003) reported that a large

number of *Campylobacter* cells were found at 0 to 10 µm depths in folds or follicles of chicken skin after chemical treatments. Similar to *Campylobacter*, *Salmonella* were mostly located in the crevices and feather follicles (Kim et al., 1996c). It is suggested that bacteria located in deeper layers or in feather follicles are less accessible and more protected by the microenvironment of skin (McMeekin et al., 1979; Thomas and McMeekin, 1980). Notermans and Kampelmacher (1975) also noted that bacteria became firmly attached during plucking and the resulting cells were not only difficult to remove but also gained more heat resistance.

The efficacy of several sampling techniques (e.g., whole carcass rinsing, excised skin sampling, and skin swabbing) for bacterial recovery from broiler carcasses has been evaluated by numerous researchers (Cox et al., 1976; Cox et al., 1978; D'aoust et al., 1982; Lillard, 1988; Capita et al., 2004). Results indicated that destructive methods (e.g., excision) generally recovered more microorganisms than non-destructive ones (e.g., rinsing, Avens and Miller, 1970; Notermans and Kampelmacher, 1975). Hannah et al. (2008) found that bacterial levels recovered from rinsates with sand were significantly higher than the levels without sand. However, other studies showed no difference in bacterial recovery between rinsing and excision methods, whereas, swabbing detected significant fewer cells than the other two (Sarlin et al., 1998; Gill et al., 2005; Cox et al., 2010; Zhang et al., 2012).

In our study, a skin sample was first rinsed to detect loosely-associated microorganisms, stomached for intermediately-associated bacteria, and finally ground for tightly-associated cells. No significant differences ($P > 0.05$) were found for loosely-, intermediately-, and internally associated MAB (colony forming number), *Salmonella* (prevalence) and *Campylobacter* (prevalence) regardless of processing stage except for MAB at post-chilling stage (Table 2.1, 2.2). Based on these findings, microorganisms appear to be already spread over surface and

potentially lodged at a certain depth of broiler skin prior to processing. While initial population of loose and tight MAB was same, more number of tight MAB seem to survive processing with higher population post-chilling than loose MAB (Table 2.1). The disinfectants during carcass washing might not be sufficient to eliminate entrapped bacteria, nor penetrate feather follicles effectively (Yang et al., 2001; Chantarapanont et al., 2004). Another possibility is that surface bacteria were freshly introduced and lodged deeper in skin throughout the processing when the carcass skin layers were physically damaged during scalding and feather follicles opened after picking. As a result, post-chilling MAB population was higher at tight association level than loose association level (Table 2.1).

A number of researchers have had some success in decontaminating poultry meat using either hot water spray or immersion (De Ledesma et al., 1996; Göksoy et al., 2001; Avans et al., 2002; Purnell et al., 2004). Bacteria and pathogens can be easily killed by direct contact with hot water at or above 70°C (Corry et al., 2007), whereas skin discoloration or cooked appearance can occur. Sinhamahapatra et al. (2004) compared the effect of different surface decontaminants on chicken quality with hot water at 70°C for 1 min, 2% lactic acid for 30 s, 1200 ppm acidified sodium chlorite solution for 5 s, or 50 ppm chlorine solution for 5 min. Results indicated that hot water treatment was the most effective in reducing the surface microbial load without affecting the appearance, smell, tenderness and overall acceptability (Sinhamahapatra, 2004).

Several processing steps, such as scalding, picking, evisceration and washing have been linked to increase in prevalence or populations of pathogens on carcasses (Mulder et al., 1978; Wempe et al., 1983; Rosenquist et al., 2006; Rasschaert et al., 2008). In this study, the prevalence of *Salmonella* decreased significantly ($P < 0.05$) at the end of chilling when the HWS was applied regardless of sampling methods. Considering no significant reduction with tap water

washing, the hot water treatment immediately after evisceration was effective for *Salmonella* reduction. Regarding *Campylobacter*, however, the hot water showed mixed results after chilling: significantly lowered prevalence ($P < 0.05$) of loosely-associated cells but not of intermediately- and tightly-associated cells.

Whyte et al. (2003) reported that hot water immersion for 10 s at 80 and 85°C resulted in a significant reduction of total viable bacteria in naturally and artificially contaminated broiler samples. However, the same immersion was not effective against naturally contaminated *Campylobacter* over controls, although, hot water spray was more effective than chlorinated cool water spray for *Campylobacter* reduction (Li et al., 2002). When the hot water immersion time was extended to 20 s, both naturally and artificially contaminated *Campylobacter* on broiler skin were significantly reduced.

Regarding appearance of broiler carcasses, it appears to be sensitive to both water temperature and exposure time. Sinhamahapatra et al. (2004) indicated that hot water treatment (70°C for 1 min, dip or spray) resulted in no effect on appearance, smell, tenderness and overall acceptability of carcasses. However, Cox et al. (1974b) showed that immersion of broiler carcasses in 71°C water for 1 min caused a partially cooked appearance. The present study supported the results of Cox et al. (1974b) of cooked appearance on carcass surfaces after spray of hot water at 71°C for 1 min (Figure 2.2).

Figure 2.2 Appearance of chicken carcass breast muscle after spraying with a) cold water and b) hot water



In summary, the results of present study demonstrated the potential of hot water spray in reducing total microbial load and *Salmonella* prevalence on the broiler carcasses. In general, the hot water spray was effective for reducing MAB population and *Salmonella* prevalence, but less effective for reducing *Campylobacter*, which are relatively thermo-tolerant cells. Further research is required to evaluate the efficacy of hot water, potentially with other intervention strategies, for pathogen reduction during processing. Furthermore, fundamental research is required to evaluate the survival mechanisms of cells attached differently, and their functional roles during processing and storage.

CHAPTER 3

QUANTIFICATION OF LOOSELY- AND TIGHTLY-ASSOCIATED BACTERIA ON BROILER CARCASS SKIN USING SWABBING, STOMACHING, AND GRINDING METHODS

3.1 Abstract

This research was conducted to quantify bacterial populations after swabbing or stomaching, followed by grinding the swabbed- or stomached-broiler skins. For each of three replications, three eviscerated broilers were randomly taken from a processing line in a local broiler processing plant. Ten swabs and ten stomachs per bird were conducted on the left- and the right-side skins (10 x 7 cm), respectively, which were then finally ground. Results indicated that mesophilic aerobic bacteria (MAB) in the 1st-swabbed sample were significantly lower than those in the 1st-stomached sample ($P < 0.05$), with no difference in most of the rest of sampling times ($P > 0.05$). During 10 swabbings followed by final grinding, 8, 9, and 83% of MAB were enumerated after 1st swabbing, after 2nd – 10th swabbings, and after final grinding of the skin, respectively. During 10 stomachings followed by final grinding, 17, 18, and 65% of MAB were enumerated after 1st stomaching, after 2nd – 10th stomachings, and after final grinding of the skin, respectively. *Escherichia coli* (*E. coli*) and total coliforms were significantly lower in the 1st swabbing than those in the 1st stomaching ($P < 0.05$), with no difference in most of the rest of sampling times. Populations of *E. coli* and total coliforms decreased step-wisely from the highest after grinding to the intermediate after 1st and 2nd sampling, and to the least after 10th sampling ($P < 0.05$), regardless of swabbing or stomaching. In this study, less than 35% of MAB seemed to be loosely-associated to the skin of eviscerated broiler, whereas more than 65% of MAB seemed tightly-associated, which were not recovered by stomaching or swabbing for even 10 consecutive times but were recovered by grinding the skin.

3.2 Introduction

During poultry processing, bacteria can migrate and find shielding niches on poultry skin such as hair follicles, ridges, and crevices (Kim et al., 1996c; National Advisory Committee on Microbiological Criteria of Foods, 1997; Chantarapanont et al., 2003). These bacteria can then locate themselves deeper in skin for better protection from physical and chemical disinfectants (McMeekin et al., 1979; Thomas and McMeekin, 1980). The epidermis, previously colonized by various bacteria, is usually damaged or removed during scalding and picking. The freshly exposed dermis is now vulnerable to be colonized by Gram-negative pathogens from boiler gut materials and other birds. The dermis has many channels and crevices, which can provide physical protection and tight attachment (Thomas and McMeekin, 1980). During water immersion, broiler carcass skin and muscle fibers swell, providing more surface area for bacteria to attach (Chantarapanont et al., 2003).

Tightly associated or less accessible bacteria are difficult to recover by common sampling methods such as, rinsing, swabbing and stomaching (Lillard, 1988; Gill et al., 2005; Hinton and Cason, 2008). Studies have shown that a single rinse was less effective in recovering *Salmonella* from a carcass compared to multiple rinses in a consecutive manner (Lillard, 1989b; Izat et al., 1991). Hannah et al. (2008) reported that rinsing with sand recovered more bacteria than rinsing without sand. A scanning electron microscope study reported that the bacteria lodged deeper in broiler skin could not be recovered by rinsing or stomaching but by shredding (Nayak et al., 2001).

For many reasons, raw broiler meat has been attributed as one of the major sources for bacterial foodborne illness (Shane, 1997), especially, salmonellosis and campylobacteriosis (ACMSF, 2005; EFSA panel of biological hazards, 2010; Chaine et al., 2013). The infection rate

of *Campylobacter* in 2014 was increased by 13% when compared with the rate in 2006 – 2008, whereas *Salmonella* infection rate remained unchanged in the U.S. (CDC, 2014). Two potential reasons for increased or persistent infection rates could be: 1) ineffective bacterial control strategies on farms and at processing plants, and 2) inaccurate sampling methods leading to false negative results when pathogens are physically present on the carcasses.

Studying broiler carcass chilling, Northcutt et al. (2006) reported that increasing chilling water volume from 2.1 to 16.8 L/kg did not change bacterial concentration in the water, suggesting that bacteria cannot be recovered in the rinsate beyond a certain equilibrium concentration. Similarly, extension of rinsing time from 1 to 4 min resulted in a similar bacterial concentration, indicating that the equilibrium concentration was reached within the first minute (Hannah et al., 2008). While mechanisms involving structural and chemical factors for bacterial attachment and detachment need to be elucidated to address the limitations of bacterial detection, quantification of bacterial populations based on three sampling methods (swabbing, stomaching and grinding) may serve as a framework to address the knowledge gap between degree of bacteria association affinity with skin and ease of recovery. Loosely-associated bacteria can likely contribute to cross contamination from bird to bird while tightly-associated bacteria can survive during process and cause problems, thereafter.

The goal of this study was to evaluate the strength of bacterial association to broiler carcass using the three sampling methods; swabbing, stomaching and grinding. Most of the bacterial cross-contamination on carcasses from gut material and other birds occurs during processing, especially during evisceration and de-feathering steps (Berrang et al., 2001; Alter et al., 2005). Once a carcass is contaminated, bacteria may attach tightly to the skin within a few minutes (Arritt et al., 2002). Therefore, in order to understand the association pattern during

actual processing conditions and improve the relevance of the study to processors and inspectors, the present investigation was conducted on site during processing at a large size commercial poultry processing plant in Indiana, US.

3.3 Materials and methods

3.3.1 Broiler carcasses for the quantification of loosely- and tightly-associated bacteria

In each of three visits, three broiler birds (about 46-day-old, HubgardM99/ross 708) were randomly picked from the post-evisceration line at a local broiler processing plant. For the quantification of bacterial populations, each of the carcasses was subjected to swabbing on the left side skin for 10 times, consecutively. The swabbed skin was then excised and ground. The right side skin of same bird was excised and subjected to stomaching for 10 consecutive times followed by grinding as before. The cells, detected from the 10 times swabbing or stomaching, were named loosely-associated bacteria, while the cells detected from the subsequent grinding were named tightly-associated bacteria.

3.3.2 Swabbing for 10 times

A stable plastic mold (13 x 10 x 0.25 cm) having a hole (10 x 7 x 0.25 cm) was used to swab on the left side skin of carcass in a same area. Each carcass was placed laterally on sterile surface, and the plastic mold was firmly attached to the surface between left wing and left thigh of the carcass. Swabbing was conducted for 10 consecutive times on the exposed skin, using the plastic mold, which was sterilized with 75% ethanol, and 10 different Kimwipes® (one-ply

composite tissue) which were moisturized with 1 mL sterile phosphate buffer saline (Vorst et al., 2004) each time.

The swabbed Kimwipes® were then individually placed in clean WhirlPak® bags, diluted with 9 mL sterile Phosphate Buffer Saline (PBS), and stomached (Stomacher 400, Seward, Worthing, UK) for one minute for microbial analysis.

3.3.3 Stomaching for 10 times

For stomaching, a new plastic mold of the same size as used in swabbing was placed on the surface area between right wing and right thigh of a carcass as before, and the exposed skin was aseptically excised using a sterile scalpel and a forceps. The excised skin (10 x 7cm, approximately 10 g) was then transferred to a clean WhirlPak® bag, filled with 90 mL sterile PBS, and stomached for 1 min. The stomaching was repeated consecutively using the same skin for 10 times with fresh PBS each time. Skin was rinsed with sterile PBS between stomachings.

3.3.4 Grinding of swabbed or stomached skin

Broiler skin that was swabbed or stomached 10 times was ground using a sterile homogenizer (Polytron, Model PT10/35, Brinkmann Instruments Inc., Westbury, NY), having two 1 cm blades, to quantify any undetected bacteria previously.

3.3.5 Microbiological analysis

Mesophilic aerobic bacteria (MAB) were assessed after each swabbing, stomaching, and grinding of broiler skin. For enumeration of *Escherichia coli* (*E. coli*) and total coliforms, samples after 1st, 2nd, and 10th swabbing or stomaching and final grinding of the swabbed- or

stomached-skin were used. After swabbing, stomaching or grinding, serial 10-fold dilutions were made and surface-plated (1 mL) in duplicate on Petrifilm™ Aerobic Count Plates (3 M Microbiology Products, St. Paul, MN) for mesophilic aerobic bacteria (MAB) enumeration and Petrifilm™ *E. coli*/coliform count plates (3 M Microbiology Products, St. Paul, MN) for *E. coli* and total coliforms enumeration. All samples were incubated at 37°C for 24 h prior to enumeration.

3.3.6 Total bacteria recovered after swabbing and grinding (TB-SWGR)

Total mesophilic aerobic bacteria populations from swabbed and ground skin (TB-SWGR) for each replication were obtained by adding the mean counts (CFU/cm²), which were mean of MAB counts from 3 birds in each replication, obtained from 1st – 10th swabbings and from the final grinding of the swabbed-skins (10 x 7 cm).

3.3.7 Total bacteria recovered after stomaching and grinding (TB-STGR)

Total mesophilic aerobic bacteria populations from stomached and ground skin (TB-STGR) were similarly attained for each replication by adding the mean counts (CFU/cm²), which were mean of MAB counts from 3 birds in each replication, from 1st – 10th stomaching and from the final grinding of the stomached-skins (10 x 7 cm).

3.3.8 Percentage of recovered bacteria

The percentages of bacteria recovered from three detection steps (1st swabbing, 2nd – 10th swabbed, and finally ground skin) for each replication were calculated. The calculation is based on non log-transformed bacterial counts/cm² using the three equations as follow: 1) the mean

count recovered from the 1st swabbed skins/TB-SWGR x 100, 2) the mean cumulative count recovered from the 2nd – 10th swabbed skin/TB-SWGR x 100, and 3) the mean count recovered from the ground skin/TB-SWGR x 100. Similarly, the percentages of bacteria recovered from three detection steps (1st stomached, 2nd – 10th stomached, and finally ground skin) were calculated using the three equations as follow: 1) the mean count recovered from the 1st stomached skin/TB-STGR x 100, 2) the mean cumulative count recovered from the 2nd – 10th stomached skin/TB-STGR x 100, and 3) the mean count recovered from the ground skin/TB-STGR x 100.

3.3.9 Statistical analysis

Mesophilic aerobic bacteria from triplicate experiments were converted to log CFU/cm². General linear model (GLM) and Duncan's multiple range test were used to compare difference in MAB counts among 10 repetitions of stomaching or swabbing and final grinding, in addition to difference between each stomaching and swabbing repetition at $P < 0.05$. For *E. coli* and total coliforms, Duncan's multiple range test was similarly used to evaluate any difference among 1st, 2nd, and 10th repetitions (swabbing or stomaching) and final grinding, in addition to any difference between each swabbing and stomaching repetition at $P < 0.05$. To compare the efficacies (%) of bacteria detection from grinding, swabbing, and stomaching, non-log transformed bacterial counts were used using GLM and Duncan multiple range test at $P < 0.05$ (SAS, 2013).

3.4 Results and discussion

In comparison of swab and stomach methods on broiler carcasses, the first swabbing resulted in lower MAB, *Escherichia coli* (*E. coli*), and total coliform bacteria recovery than the first stomaching ($P < 0.05$), with no difference seen for the most of the rest of sampling times ($P > 0.05$) (Tables 3.1, 3.2, and 3.3). After 10 swabbings and final grinding, the populations of MAB decreased step-wisely from the highest (4.3 log CFU/cm²) for the grinding to the intermediate (3.3 log CFU/cm²) for the 1st – 2nd swabbings, and to the least (< 2.8 log CFU/cm²) for the 3rd– 10th swabbings ($P < 0.05$). After 10 stomachings and final grinding, the highest population (4.2 log CFU/cm²) of MAB was seen at the final grinding, followed by the 1st (3.7 log CFU/cm²) and the 2nd – 10th (< 2.9 log CFU/cm²) stomachings ($P < 0.05$) (Table 3.1). Similarly, *E. coli* and total coliforms showed the highest populations after the grinding, followed by the 1st, the 2nd, and the 10th samplings ($P < 0.05$), regardless of swabbing or stomaching (Tables 3.2, and 3.3).

Previously, our laboratory observed similar results that the mean populations of mesophilic aerobic bacteria on post-eviscerated broiler skin were 3.78, 3.84, and 4.13 log CFU/g, respectively, after rinsing, stomaching, and grinding the same broiler skin (Zhang et al., 2013). In accordance with our results, Zhang et al. (2012) also reported that stomaching of excised broiler skin yielded 0.5 and 0.46 log CFU/cm² higher aerobic bacteria and *Enterobacteriaceae*, respectively, than did swabbing ($P < 0.05$). Applying repeated stomachings on poultry skin, Lillard (1988) recovered more *Enterobacteriaceae* in the 1st stomaching than the 3rd through 5th stomachings, with intermediate populations seen in the 2nd stomaching. A similar trend of higher aerobic bacteria recovery (0.43 – 0.49 log CFU/cm²) was reported after stomaching than after swabbing of poultry skin (Berrang et al., 2014; McEvoy et al., 2005). Berrang et al. (2014)

showed that the first blending of excised broiler skin in a stomacher recovered 0.1 to 0.2 log more inoculated *Salmonella* and *Campylobacter*, respectively, than 1st swabbing. Stomaching recovered 0.43 log more total aerobic bacteria and 0.56 log more Enterobacteriaceae per cm² from hot pig carcasses than swabbing (Snijders et al., 1984). Similarly, in pig carcasses, Ghafir and Daube (2008) showed that excision and stomaching yielded almost 1 log higher total aerobic bacteria and 0.9 log higher *E. coli* per square cm of tissue than swabbing. In a study on red meat species, Martinez et al. (2010) concluded that compared to swabbing, stomaching of excised tissue can recover significantly higher total aerobic bacteria in carcasses having high microbial load (> 4.5 log CFU/ cm²) but the difference was not as pronounced in carcasses with low microbial load (<4 log CFU/cm²). Sharpe et al. (1996) indicated that in the case of red meat carcasses, swabbing yields only a fraction of the bacteria present compared to stomaching.

In general, destructive methods such as grinding or stomaching appear to be more effective for pathogen detection than non-destructive methods such as swabbing or rinsing, especially for the poultry skin having rough surface with feather follicles. Sarlin et al. (1998) found that both rinse of whole carcass and stomach of excised skin (2 cm x 6 cm) identified more *Salmonella* incidences than swab on pre-chill broiler carcasses (2 cm x 6 cm) at a processing facility. In case of *Salmonella* prevalence, however, swabbing of entire carcass surface area resulted in higher prevalence than stomaching of neck skin possibly due to the heterogeneous nature of *Salmonella* contamination (McEvoy et al., 2005).

It is commonly known that difference in bacterial counts between excision and swabbing methods are influenced by swab material, animal species, sampling person, and bacterial contamination levels (Martinez et al., 2010), in addition to sampling area (lean or fatty) and sampling time (immediately after processing or after storage) (Pepperell et al., 2005). One

limitation of swabbing is that large volume of the dilution fluid is not in direct contact with the sample during sampling. As a result, a bacteria concentration gradient is not created for the bacteria movement from higher to lower concentration. Another limitation for swabbing, as a sampling method, is under-representation of sample bacteria population due to imperfect transfer efficacy during two transfers, from sample to swab and from swab to dilution fluid (Pepperell et al., 2005).

When the MAB populations were combined after 10 swabbings and final grinding of the swabbed-skin, only 8 and 9% of MAB were recovered from the 1st and the 2nd – 10th swabbings, respectively, while the remaining (83%) were recovered from grinding the swabbed-skin (Figure 3.1). In a similar manner, 17, 18, and 65% of MAB were recovered from the 1st and the 2nd – 10th stomachings, and the final grinding of the stomached-skin, respectively (Figure 3.2). These results indicate that the 10 repeats of swabbing and stomaching on broiler skin can release only 17 and 35% of MAB populations (most likely loosely-associated), respectively, whereas grinding of the swabbed- and stomached skins can release 83 and 65% MAB populations (most likely tightly-associated), respectively, in the dilution fluid. These results support the previous finding that bacteria were recovered in high numbers (4.62 log CFU/carcass) even after 40 consecutive rinses of a single broiler carcass, presumably due to different attachment levels to the poultry skin (Lillard, 1988).

Avens and Miller (1970) reported that a swab method enumerated 7 and 22% of the aerobic bacteria that were counted by a skin blending method at low and high contamination levels, respectively. Zhang et al. (2013) indicated that 2.1 log CFU/g of tightly-attached bacteria were enumerated on broiler skin after hot water spray and carcass chilling, whereas, only 1.7 and 2.0 log CFU/g were enumerated as loosely- and intermediately-attached bacteria, respectively.

Table 3.1 Mean populations (log cfu/cm²) of mesophilic aerobic bacteria (MAB) with (SD) on broiler skin from 10 consecutive swabbing or stomaching repetitions and subsequent grinding

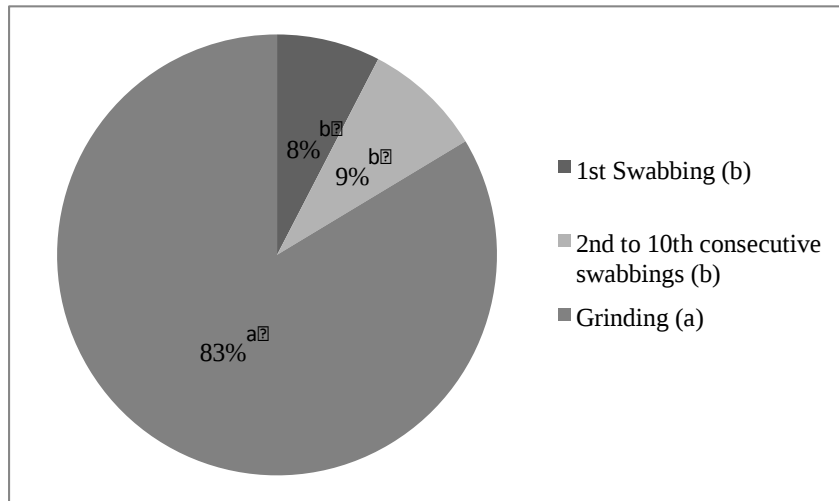
Repetition	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	Grind
Swab	3.30 ^{B,y} (0.23)	2.82 ^{BC} (0.22)	2.45 ^{CD} (0.26)	2.39 ^{CD} (0.18)	2.34 ^{CD} (0.34)	2.18 ^D (0.3)	2.27 ^D (0.28)	2.06 ^D (0.29)	2.14 ^D (0.56)	1.95 ^D (0.19)	4.34 ^A (0.18)
Stomach	3.69 ^{B,x} (0.21)	2.92 ^C (0.14)	2.92 ^C (0.11)	2.57 ^C (0.12)	2.76 ^C (0.29)	2.48 ^C (0.17)	2.48 ^C (0.53)	2.65 ^C (0.30)	2.71 ^C (0.61)	2.69 ^C (0.31)	4.27 ^A (0.17)

^{A-D}Means within a row with no common superscripts are different (P<0.05)

^{x-y}Means within the first column with no common superscripts are different (P<0.05)

Number of observations = 3

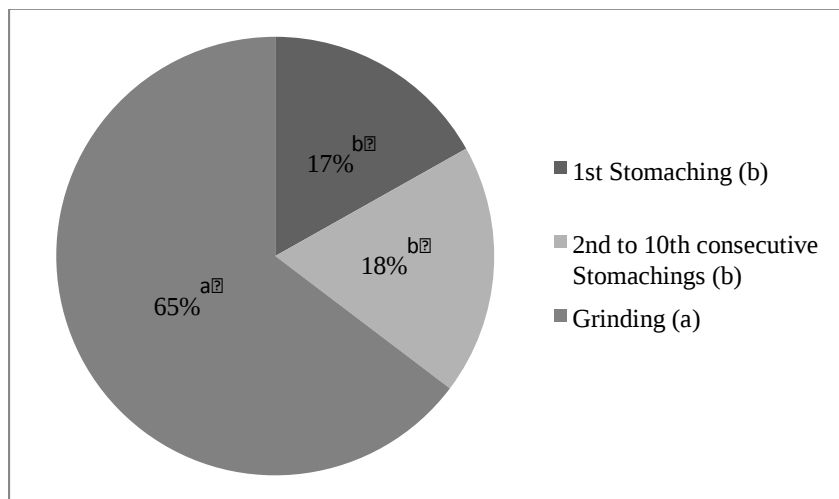
Figure 3.1 Recovered (%)^c mesophilic aerobic bacteria (MAB) after the 1st swabbing, 2nd to 10th consecutive swabbings, and grinding of the swabbed skin



^{a-b}Means with different letters are different ($P < 0.05$)

^cPercentages are based on total MAB population recovered from 10 consecutive swabbings + grinding post swabbings (TB-SWGR)

Figure 3.2 Recovered (%)^c mesophilic aerobic bacteria (MAB) after the 1st stomaching, 2nd to 10th consecutive stomachings, and grinding of the stomached skin



^{a-b}Means with different letters are different ($P < 0.05$)

^cPercentages are based on total MAB population recovered from 10 consecutive stomachings + grinding post stomachings (TB-STGR)

Table 3.2 Mean *E. coli* populations (log cfu/cm²) on broiler skin obtained from 1st, 2nd and 10th consecutive swabbing or stomaching repetitions and subsequent grinding

Repetition	1 st	2 nd	3 rd	4 th	5 th	6 th	7 th	8 th	9 th	10 th	Grind
Swab	1.35 ^{B,y} (0.05)	0.86 ^C (0.30)	←—————→							0.29 ^D (0.33)	2.37 ^A (0.08)
Stomach	2.10 ^{B,x} (0.07)	1.43 ^C (0.09)	←—————→							0.53 ^D (0.13)	2.80 ^A (0.27)

^{A-D}Means within a row with no common superscripts are different (P<0.05)

^{x-y}Means within the first column with no common superscripts are different (P<0.05)

Number of observations, n = 3

¹N/C data not collected from third to ninth repetition

Table 3.3 Mean total coliforms populations (log cfu/cm²) (SD) on broiler skin obtained from 1st, 2nd and 10th consecutive swabbing or stomaching repetitions and subsequent grinding

Repetition	1 st	2 nd	3 rd	4 th	5 th	6 th	7 th	8 th	9 th	10 th	Grind
Swab	1.48 ^{B,y} (0.17)	0.94 ^C (0.35)	←—————→							0.41 ^D (0.34)	2.52 ^A (0.09)
Stomach	2.18 ^{B,x} (0.08)	1.59 ^C (0.14)	←—————→							0.74 ^D (0.23)	3.02 ^A (0.23)

^{A-D}Means within a row with no common superscripts are different (P < 0.05)

^{x-y}Means within the first column with no common superscripts are different (P < 0.05)

Number of observation, n = 3

¹N/C data not collected from third to ninth repetition

Since the number of MAB, *E. coli*, and total coliforms recovered and MAB recovery efficacy (%) fall steeply after first or first two sampling repetitions (Table 3.1, 3.2, and 3.3; Figure 3.1, and 3.2), subsequent swabbings or stomachings appear to be futile in recovering remaining bacteria on skin as major proportions of remaining bacteria appear to be tightly associated with skin. A large portion of presumably loosely associated bacteria can be collected by one or two consecutive sampling repetitions while a large portion of remaining bacteria (presumably tightly associated) cannot be collected by further repetitions of swabbing or stomaching, due to fall in recovery efficacy, unless the skin was completely shredded by grinding.

In conclusion, swabbing, stomaching, and grinding methods were compared for their efficacies in recovering bacteria from broiler skin. The 1st stomaching of excised broiler skin released more numbers of MAB, *E. coli* and total coliform than the 1st swabbing of skin, both of which were outnumbered by the grinding of the resulting skins ($P < 0.05$). Comparing the recovery efficacy of MAB, no significant difference between the 1st sampling (8 – 17%) and the 2nd – 10th sampling (9 – 18%) was found for loosely-associated MAB, regardless of swabbing or stomaching, whereas significantly higher recovery (65 – 83%) of tightly-associated MAB were observed by grinding the swabbed- or stomached-skin ($P < 0.05$). In a similar manner, the major portions of tightly-associated *E. coli* and total coliforms were not recovered after 10 swabbings or 10 stomachings but recovered after grinding the skin. Although a single rinse of whole carcass is one of the recommended methods, more reliable detection methods, involving sampling of entire carcass surface area, need to be considered to detect both loosely- and tightly-associated bacteria on poultry carcasses to prevent any false negative results when pathogens are physically present.

CHAPTER 4

EFFECT OF TRISODIUM PHOSPHATE DIP, HOT WATER DIP, AND THEIR COMBINATION WITH/WITHOUT BRUSHING ON BROILER CARCASSES FOR REDUCTION OF LOOSELY- AND TIGHTLY-ASSOCIATED BACTERIA AND SKIN STRUCTURAL CHANGES

4.1 Abstract

The purpose of this research was to evaluate the effects of trisodium phosphate (TSP) dip at 8%/25°C, hot water dip (HWD) at 71°C, and their combination (TSP/HWD) with (TSP/HWD/B)/without brushing on broiler breast skin for bacterial reduction and structural changes. Eviscerated broiler carcasses were randomly obtained from a processing line in a local slaughter plant and immediately subjected to two control treatments of tap water dip at 25°C with (TWD/TWD/B) and without (TWD/TWD) brushing in addition to four other treatments (TWD/TSP, TWD/HWD, TSP/HWD, and TSP/HWD/B). Each carcass dip was for 45 s and brushing was applied intermittently for 45 s with 5 s on and 5 s off. Compared to TWD/TWD, TSP/HWD significantly reduced populations of MAB, *Escherichia coli* (*E. coli*), and total coliforms by 1.1, 0.9 and 1.0 log CFU/g, respectively, and *Salmonella* prevalence by 53.3% ($P < 0.05$), whereas TWD/TSP and TWD/HWD showed intermediate reductions ($P < 0.05$). Upon brushing, TSP/HWD/B more effectively reduced populations of MAB, *E. coli*, and total coliforms, and the prevalence of *Salmonella* than TWD/TWD/B ($P < 0.05$). Comparing the two sampling methods, stomaching and grinding, initial stomaching released fewer MAB and total coliforms (named loosely-associated cells) than subsequent grinding of the stomached skin (named tightly-associated cells), with no difference observed in *E. coli* numbers or *Salmonella* prevalence. Compared to TWD/TWD and TWD/TWD/B, both TSP/HWD and TSP/HWD/B generally resulted in darker, less reddish, and more yellowish breast skin. Scanning electron microscope and histological images indicated that both TSP/HWD and TSP/HWD/B had deeper skin penetration than controls or TWD/HWD and TWD/TSP, supporting the greater antimicrobial activities of TSP/HWD and TSP/HWD/B. However, TSP/HWD and TSP/HWD/B treatments resulted in skin color changes on broiler carcasses.

4.2 Introduction

Broiler chicken is the most favorite meat in the United States and many other countries. According to OECD-FAO (2014), poultry is expected to become the world's most consumed meat over the next 5 years. Poultry meat, however, is a leading cause of foodborne illnesses, particularly salmonellosis and campylobacteriosis, in the United States and the European Union (Hoffman et al., 2007; European Food Safety Authority [EFSA], 2008; CDC FoodNet report, 2012; and Chain et al., 2013). While low infectious dose of *Salmonella* and *Campylobacter* is fundamentally responsible, two other potential reasons for these poultry-related illnesses could be ineffective pathogen control strategies and inaccurate pathogen detection methods that are currently being used at farms and/or at processing plants.

Over the last two decades, the broiler industry has adopted various intervention strategies against pathogens, which include the use of chlorine, organic acids, cetylpyridinium chloride (CPC), trisodium phosphate (TSP), etc. (Lillard, 1990; Li et al., 1997; Sakhare et al., 1999; Whyte et al., 2001; and Zhang et al., 2011). While these microbial reduction strategies have been partly successful, many chemicals such as chlorine and CPC are not generally recognized as safe (GRAS), and others such as organic acids and TSP have negative organoleptic effects and waste management issues, respectively (Ricke et al., 2005).

Hot water sprays or dips have been shown to reduce the bacterial load on broiler carcasses with their effectiveness dependent on water temperature and exposure time (Cox et al., 1974; and Purnell et al., 2004). In our previous study, hot water spraying (71°C, 1 min) reduced total bacteria counts and the incidence of *Salmonella* on broiler carcasses but caused a partially cooked appearance (Zhang et al., 2013).

On-line brushing with a water spray has been primarily used to remove fecal material from the surface of broiler carcasses before scalding. In 2009, Berrang and Bailey assessed on-line washing with/without brushing of broiler carcasses between bleed-out and chilling in a commercial processing plant. Overall, their multiple-sequential washing steps decreased *Campylobacter* and *E. coli* populations as well as *Salmonella* prevalence, although no single step caused a significant difference.

Trisodium phosphate received GRAS status and was approved for use in broiler processing in 1992 (Capita et al., 2002b). However, TSP has not been fully utilized in the broiler industry due to issues related to wastewater management and equipment cleaning.

In processing plants, most bird-to-bird contamination occurs during de-feathering and evisceration (Allen et al., 2003; Sarlin et al., 1998; Rasschaert et al., 2008; and Guerin et al., 2010). Loosely associated bacteria are likely to contribute to cross-contamination of carcasses during processing, while tightly associated bacteria are likely to survive during chemical and physical antimicrobial treatments. Therefore, immediate intervention strategies for contaminated carcasses are needed to prevent bacteria from becoming tightly associated as processing continues. During antimicrobial application, physical brushing is expected to expose hidden bacteria, located in crevices and ridges, to chemical and other physical treatments. Our recent study indicated that 10 swabbings and 10 stomachings recovered only 17 and 45% of mesophilic aerobic bacteria present on the broiler skin, respectively, whereas the remaining bacteria were recovered after grinding the swabbed or stomached skin (Singh et al., 2015). Previously, several studies also used grinding or blending of broiler skin to recover tightly associated bacteria from chicken skin (Tamblyn et al., 1997; Zhang et al., 2013; Lee et al., 2014).

Many studies have shown that a combination of physical and chemical treatments, such as TSP with electricity, sodium carbonate with hot water, sodium carbonate with electricity, lactic acid with steam, and sodium hypochlorite with acidic electrolyzed water, was more effective against pathogenic and spoilage bacteria on broiler carcasses than individual treatments (Li, et al., 1994; Rodriguez de Ledesma et al., 1996; Northcutt et al., 2007; and Lecompte et al., 2008). Until today, no research has been conducted to evaluate the effect of trisodium phosphate dip (TSP), hot water dip (HWD), and brushing (B) against bacteria on broiler carcasses. Therefore, the objective of this study was to evaluate the effects of TSP, HWD, and their combination with and without brushing on broiler carcasses for the reduction of loosely-associated and tightly-associated bacteria. As tightly associated bacteria are likely to be located deeper in the dermal layer of broiler skin (Thomas & McMeekin, 1980), visual changes in skin structure resulting from the treatments were also evaluated using scanning electron microscopy and histological staining.

4.3 Materials and methods

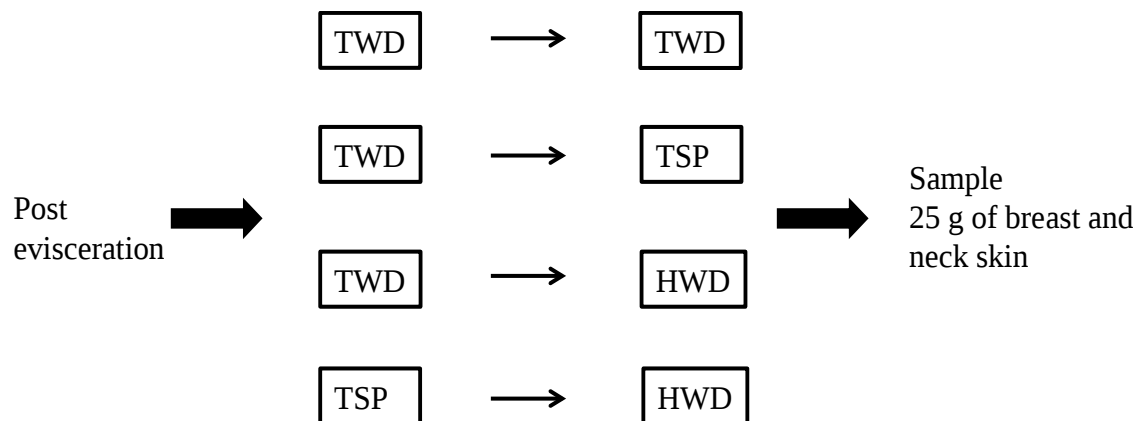
4.3.1 Broiler carcasses

A total of nine visits were made to a local broiler processing plant on nine different days to assess the effects of chemical and/or physical treatments on microbiological quality and structure of broiler carcass skin (~ 46-day-old, HubgbardM99/ross 708), using microbiological analysis and visual imaging (scanning electron microscope and histological staining), respectively.

4.3.2 Experiment I: Control (TWD/TWD), trisodium phosphate dip (TWD/TSP), hot water dip (TWD/HWD), and combination of TSP and HWD (TSP/HWD)

In each of three visits, 20 broiler carcasses were randomly selected from a broiler processing line after evisceration. The carcasses were immediately subjected to one of four treatments (5 carcasses/treatment, Figure 4.1) as follows: 1) Tap water dipping (TWD) at 25°C for two times – TWD/TWD, 2) TWD followed by 8% trisodium phosphate dipping at 25°C – TWD/TSP, 3) TWD followed by hot water dipping at 71°C – TWD/HWD, and 4) 8% trisodium phosphate dipping followed by hot water dipping at 71°C - TSP/HWD. Each dip was done for 45 sec.

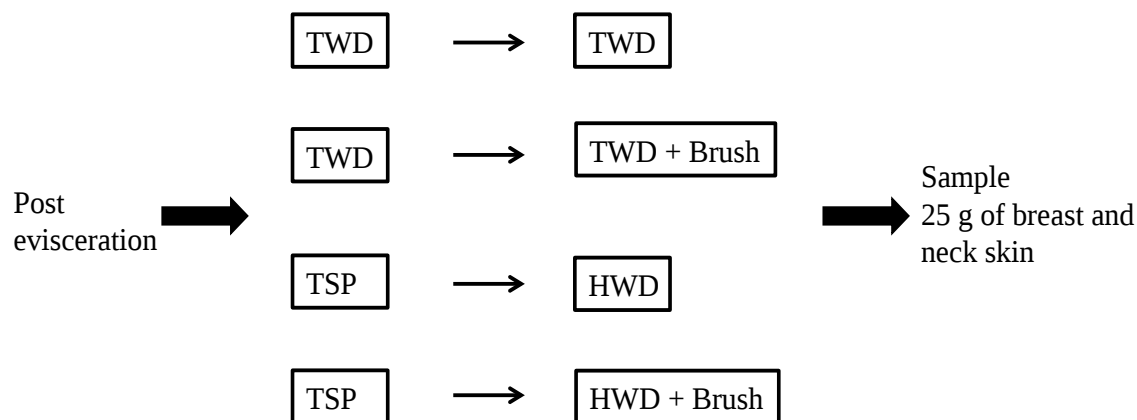
Figure 4.1 Experiment I design and sampling plan



4.3.3 Experiment II: Control (TWD/TWD), control with brushing (TWD/TWD/B), combination of TSP and HWD (TSP/HWD), and TSP/HWD with brushing (TSP/HWD/B)

During each of three additional visits, 20 broiler carcasses were similarly selected and subjected to one of four treatments (5 carcasses/treatment, Figure 4.2) as follows: 1) control TWD at 25°C for two times - TWD/TWD, 2) TWD at 25°C followed by a second TWD with brushing – TWD/TWD/B, 3) 8% trisodium phosphate dipping at 25°C followed by hot water dipping at 71°C – TSP/HWD, and 4) 8% trisodium phosphate dipping at 25°C followed by hot water dipping at 71°C with brushing - TSP/HWD/B. Each dip was done for 45 s and each brushing was applied at 5 s on/off intervals for 45 s on carcass breast and neck area. For brushing, polyester brushes (Sparta® Spectrum® All Purpose Utility Scrub Brushes), having a bristle density of 38/cm², bristle diameter of 1 mm, and bristle length of 4.5 cm, were used after purchasing from Carlisle Foodservice Products (Batavia, IL).

Figure 4.2 Experiment II design and sampling plan



4.3.4 Skin sampling methods

After each treatment, 25 g of skin was aseptically taken from the breast and neck area of the bird, placed in sterile WhirlPak bag (Nasco, Modesto, CA), and transported on ice to Michigan State University campus. The sample bags were poured with 225 ml of sterile phosphate buffer saline (PBS) and stomached for 1 min (Stomacher 400 Circulator, Seward, Worthing, UK) to assess loosely-associated bacteria in Exp. I and II. For tightly-associated bacteria in Exp. II, the stomached-skin was transferred to a new WhirlPak bag containing 225 ml of fresh PBS and ground using a Brinkmann Polytron Homogenizer (Plytron PT10/35, Brinkmann Instruments Co., Westbury, NY). Between stomaching and grinding, the skin was washed with sterile water to remove any free bacteria remaining on the surface.

Last three visits were made to prepare breast skin samples for SEM imaging (5 samples/treatment) and histological staining (3 samples/treatment) after all the treatments mentioned above in Exp I and II. Three additional skin samples were collected from broiler carcasses after bleeding and before scalding to serve as a reference for the SEM and histological images.

4.3.5 Microbiological analysis

A serial 10-fold dilution after stomaching of the skin sample or grinding of the stomached skin was made in phosphate buffer saline, plated in duplicates on aerobic and *E. coli*/coliform count plates (3M Company, St. Paul, MN), and incubated at 37°C for 24 to 48 h for bacterial enumeration in Exp. I and II. *Campylobacter* presence in Exp. I and *Salmonella* presence in Exp. I and II were identified. For *Campylobacter* presence, 30 ml aliquots of the stomached solutions were added to 4 oz. Whirl-Pak® bags, containing 30 ml of 2x Blood-Free

Bolton's enrichment broth (Oxoid LTD., Basingstoke, UK). These bags were flushed with a mixture of 5% O₂, 10% CO₂, and 85% N₂ (Airgas Great Lakes, Lansing, MI), and incubated at 42°C for 48 h for enrichment, streaked onto plates of CCA, and incubated again as previously stated for *Campylobacter* prevalence. Five to 10 presumptive *Campylobacter* colonies from each treatment were examined microscopically for typical cellular morphology and mobility, and then confirmed using the Campy detection system (Remel Inc., Lenexa, KS). The presence of *Salmonella* was assessed by adding 30 ml of the stomached or ground solution to 30 ml of BPW (Acumedia, Lansing, MI) followed by 20 h of incubation at 37°C. After incubation, 100 µL of the pre-enriched solution was transferred to 10 ml of Rappaport-Vassiliadis broth (RV broth, Acumedia) and incubated again at 42°C for 20 h. An aliquote (120 µl) of this enrichment was then examined for *Salmonella* using Reveal[®] *Salmonella* test kits (Neogen Corp., Lansing, MI). All positive samples were streaked onto brilliant green sulfur and xylose lysine tergitol-4 agar (Acumedia, Lansing, MI), incubated at 37°C for 24 h and then inspected for typical *Salmonella* colonies to confirm the Reveal[®] *Salmonella* results.

4.3.6 Muscle and skin color measurements

Commission Internationale de l'Éclairage (CIE) color (lightness-L*, redness-a*, and yellowness-b*) was measured in duplicate on the surface of breast muscle (section 4.3.2 and 4.3.3) and breast and scapular skin (section 4.3.3), using a colorimeter (8-mm aperture, illuminant C; CR-400, Konica Minolta Sensing Inc., Japan) after calibration with a white plate (L*, 97.28; a*, -0.23; b*, 2.43).

4.3.7 Scanning electron microscopy (SEM) imaging of skin

After the dip treatments (section 4.3.2 and 4.3.3), skin samples (3 mm x 3 mm) were removed from breast area and fixed at 4°C for 12 h in 4% glutaraldehyde buffered at pH 7.4 with 0.1 M sodium phosphate. The samples were then rinsed for 4 h in the buffer followed by post-fixation for 12 h in 1% osmium tetroxide buffered with 0.1 M sodium phosphate. After fixation, the samples were rinsed again in the buffer for 4 h followed by dehydration by exchanging with graded ethanol series (25%, 50%, 75%, 95%) for 2 h at each gradation followed by three 2 h changes in 100% ethanol. The samples were critically point dried in a Leica Microsystems model EM CPD300 critical point dryer (Leica Microsystems, Vienna, Austria) using liquid carbon dioxide as the transitional fluid. The samples were then mounted on aluminum stubs using carbon suspension cement (SPI Supplies, West Chester, PA) and coated with gold (~20 nm thick) in an Emscope Sputter Coater model SC 500 (Ashford, Kent, England) purged with argon gas. Samples, mounted on the stubs, were examined at 10,000X magnification in a JEOL 6610LV SEM (tungsten hairpin emitter) scanning electron microscope (JEOL Ltd., Tokyo, Japan). Total of 30 microscopic fields (6 fields/sample) were observed for each treatment.

4.3.8 Histological staining and imaging

After the dip treatments (section 4.3.2 and 4.3.3), skin samples (2 cm by 2 cm) were similarly obtained from breast area and mounted on wax squares followed by fixation in 10% Neutral Buffered Formalin for 48 h. The fixed skins were processed and vacuum infiltrated with paraffin using a Sakura VIP 2000 tissue processor, followed by embedding with a ThermoFisher HistoCentre III embedding station. Once the sample-mounted blocks were cooled, excess paraffin was removed from the edges. The blocks were then placed on a Reichert Jung 2030

rotary microtome and faced to expose the tissue sample. The blocks were then finely sectioned at 4 - 5 µm and the sections were dried for 2 - 24 h at 56°C in a slide incubator to ensure adherence to the slides.

The slides were removed from the incubator and stained using a standard Hematoxylin and Eosin method which included two changes of Xylene for 5 minutes each, two changes of absolute ethanol for 2 minutes each, and two changes of 95% ethanol for 2 minutes each. Thereafter, samples were rinsed in running tap water for 2 minutes and Endure Hematoxylin (Cancer Diagnostics Inc., Morrisville, NC) for 1½ minute followed by a 10 - 15 s differentiation in 1% aqueous glacial acetic acid and running tap water for 2 minutes to enhance nuclear detail. After the tap water rinse, the slides were placed in one change of 95% ethanol for 2 minutes, 1% Alcoholic Eosin-Phloxine B for 2 minutes to stain cytoplasm, one change of 95% ethanol for 2 minutes, four changes of 100% ethanol for 2 minutes each, and four changes of Xylene for 2 minutes each followed by coverslipping with synthetic mounting media for permanent retention and visualization by light microscopy at 20X magnification. The anatomical terminology of Weir and Lunam (2004) was used to classify the skin dermal layers. Total of 48 microscopic fields (16 fields/sample) were observed for each treatment.

4.3.9 Statistical analysis

Mesophilic aerobic bacteria, *E. coli* and total coliforms counts per gram of skin from triplicate experiments were converted to log units for statistical analysis. In experiment I (section 4.3.2), bacterial counts and muscle color values (L^* , a^* , b^*) were compared among treatments, using a General linear model (GLM) and Duncan's multiple range test at $P < 0.05$ (SAS 9.4, 2013, SAS Institute Inc.). To compare prevalence of *Salmonella* among treatments, binary

distribution in GLIMMIX was used with Tukey's adjustment at $P < 0.05$ (SAS 9.4, 2013, SAS Institute Inc.).

In experiment II (section 4.3.3), a two-factor (Treatment x Sampling method) analysis was conducted to compare bacterial counts and *Salmonella* presence. Since there was no interaction between the two factors, data were pooled together. For bacterial counts, comparisons among 4 treatments and between 2 sampling methods were conducted using GLM and Duncan's multiple range tests at $P < 0.05$. For *Salmonella* presence, binary distribution in GLIMMIX with Tukey's adjustment at $P < 0.05$ was used as before. Skin color values on breast and scapula area were also compared among treatments, using GML and Duncan's multiple range test at $P < 0.05$.

4.4 Results and discussion

4.4.1 Experiment I: Control (TWD/TWD), trisodium phosphate dip (TWD/TSP), hot water dip (TWD/HWD), and combination of TSP and HWD (TSP/HWD)

Compared to the control carcasses dipped in tap water (TWD/TWD), the populations of MAB, *E. coli* and total coliforms on carcasses after trisodium phosphate dip (TWD/TSP) were reduced by 0.5, 0.7, and 0.8 log CFU/g, respectively ($P < 0.05$) (Table 4.1). These results were identical with that of Stopforth et al. (2007), who observed reductions in MAB, *E. coli* and coliforms of 0.5, 0.7, and 0.8 log CFU/ml, respectively, after spraying TSP (8-12%) on poultry carcasses in commercial processing plants. Spraying 10% TSP on poultry carcasses, previously inoculated with fecal bacteria, resulted in reduction of 0.5, 0.5, and 0.2 log CFU/ml in carcass rinsate for MAB, *E. coli* and coliforms, respectively (Fabrizio et al., 2002). del Rio et al. (2007)

reported that 15 min dip of chicken legs in 12% TSP resulted in 1.7 and 0.9 log reductions in MAB and coliforms, respectively.

Compared to control carcasses (TWD/TWD), hot water dipping (TWD/HWD) reduced MAB, *E. coli* and total coliforms by 1.2, 0.7, and 0.7 log CFU/g, respectively, ($P < 0.05$) (Table 4.1). In 2013, Zhang et al. reported that hot water spraying at 71°C for 1 min lowered loosely- and tightly-attached MAB on broiler carcasses by 1.1 log and 1.3 log CFU/g, respectively. Hot water dipping of broiler carcasses at 70°C for 40 s and 75°C for 30 s resulted in 1.1 and 1.0 log CFU/ml reductions in MAB populations, and 1.0 and 0.91 log CFU/ml reductions in Enterobacteriaceae populations, respectively (Purnell et al., 2004). After comparing different surface decontamination strategies, Sinhamahapatra et al. (2004) indicated that hot water treatment is the cheapest, most convenient and simplest approach.

The combination of trisodium phosphate and hot water dips (TSP/HWD) yielded greater reduction of *E. coli* and total coliforms than hot water dip alone (TWD/HWD) and a greater reduction in MAB than trisodium phosphate dip alone (TWD/TSP) ($P < 0.05$) (Table 4.1). The combination (TSP/HWD) lowered the prevalence of *Salmonella* from 80 to 26.7% ($P < 0.05$), with intermediate reductions seen for TWD/TSP and TWD/HWD (Table 4.1). Considering the 40 and 20% reductions after TWD/TSP and TWD/HWD, respectively, the reduction (53.5%) by TSP/HWD appeared to be an additive effect. Trisodium phosphate acts as a detergent that can dissolve the thin layer of skin fat and expose hidden bacteria to hot water (Scientific Committee on Veterinary Measures Relating to Public Health, 1998). Similar reductions in *Salmonella* and other pathogenic bacteria were observed when 10% TSP at 10°C/15 s and HWD at 95°C/5 s were combined (Rodriguez De Ledesma et al., 1996). In terms of treatment order, TSP followed by hot water was more effective than the reverse (Gorman et al., 1995). The microbial reductions

seen in the present study are with respect to the control dip (45 s + 45 s) rather than the initial carcass load. Therefore, the reductions by treatments reported in the present study are conservative.

With regard to color change (L^* , a^* , b^*), no significant difference in broiler breast muscle was observed regardless of treatment, except for more yellowness after TWD/TSP than TWD/HWD (Table 4.2). In the case of skin color, however, Purnell et al. (2004) reported that 60% and 5 - 9% of the carcasses were down-graded after HWD at 75°C for 30 s and 70°C for 40 s, respectively, due to epidermal damage or skin tears during the trussing process. Immersion or spraying of broiler carcasses at 71°C for 1 min resulted in lower appearance scores with the surface muscle having a partially cooked appearance (Cox et al., 1974; Zhang et al., 2013).

Table 4.1 Mean populations (log cfu/g) (SD) of mesophilic aerobic bacteria (MAB), *E. coli* and total coliforms and prevalence (%) of *Salmonella* and *Campylobacter* on broiler skin after treatments in Exp. I

	n ¹	Treatments ²			
		TWD/TWD	TWD/TSP	TWD/HWD	TSP/HWD
MAB	15	3.5 ^a (0.46)	3.0 ^b (0.29)	2.3 ^c (0.23)	2.3 ^c (0.36)
<i>E. coli</i>	15	1.6 ^a (0.58)	0.9 ^{bc} (0.45)	0.9 ^b (0.29)	0.7 ^c (0.05)
Total coliforms	15	1.7 ^a (0.62)	0.9 ^{bc} (0.46)	1.0 ^b (0.32)	0.7 ^c (0.05)
<i>Salmonella</i>	15	80 ^a (12/15)	40 ^{ab} (6/15)	60 ^{ab} (9/15)	26.7 ^b (4/15)
<i>Campylobacter</i>	10	20 (2/10)	40 (4/10)	30 (3/10)	30 (3/10)

^{a-c}Means within a row with no common superscripts are different ($P < 0.05$).

¹n = Number of observations

²TWD/TWD: tap water dipping (TWD) (25°C, 45 s) followed by TWD (25°C, 45 s)

²TWD/TSP: TWD (25°C, 45 s) followed by Trisodium phosphate (TSP) (8%) dipping (25°C, 45 s)

²TWD/HWD: PWD (25°C, 45 s) followed by hot water dip (HWD) (71°C, 45 s)

²TSP/HWD: TSP (8%) dipping (25°C, 45 s) followed by HWD (71°C, 45 s)

Table 4.2 Mean color values¹ (L*, a*, b*) (SD) of broiler breast muscle after treatments in Exp. I

	Treatment ¹			
	TWD/TWD	TWD/TSP	TWD/HWD	TSP/HWD
L*	44.20 ^a (2.63)	43.40 ^a (2.70)	45.59 ^a (3.90)	42.55 ^a (4.41)
a*	0.99 ^a (0.66)	1.21 ^a (0.84)	0.77 ^a (1.43)	0.81 ^a (0.92)
b*	-0.36 ^{ab} (1.72)	0.73 ^a (1.50)	-0.92 ^b (1.22)	0.27 ^{ab} (1.72)

^{a-b}Means within a row with no common superscripts are different ($P < 0.05$).

¹Number of observations for each treatment, n = 10.

¹TWD/TWD: tap water dipping (TWD) (25°C, 45 s) followed by TWD (25°C, 45 s)

¹TWD/TSP: TWD (25°C, 45 s) followed by Trisodium phosphate (TSP) (8%) dipping (25°C, 45 s)

¹TWD/HWD: PWD (25°C, 45 s) followed by hot water dip (HWD) (71°C, 45 s)

¹TSP/HWD: TSP (8%) dipping (25°C, 45 s) followed by HWD (71°C, 45 s)

4.4.2 Experiment II: Control (TWD/TWD), control with brushing (TWD/TWD/B), combination of TSP and HWD (TSP/HWD), and TSP/HWD with brushing (TSP/HWD/B)

In this test, the best antibacterial treatment (TSP/HWD) found previously (4.4.1) was used with and without brushing to evaluate antimicrobial efficacy on broiler carcasses. For populations of loosely- and tightly-associated bacteria, stomaching and stomaching followed grinding of skin samples were both used, respectively, as described by Singh et al. (2015). Data were pooled together because no significant interaction ($P > 0.05$) was found between treatment and sampling method. Compared to tap water dipping (TWD/TWD), no bacterial reduction ($P > 0.05$) was observed after brushing with tap water dipping (TWD/TWD/B), regardless of species (Table 4.3). However, brushing with TSP and HWD (TSP/HWD/B) further reduced MAB populations in addition to the reduction by TSP/HWD compared to TWD/TWD or TWD/TWD/B ($P < 0.05$) (Table 4.3). The populations of *E. coli* and total coliform bacteria were also significantly reduced after TSP/HWD, although no additional reduction was found after TSP/HWD/B, potentially due to the low initial populations (1.2 – 1.5 log CFU/g) compared to MAB (3.6 log CFU/g). Similarly, no effect of brushing was seen on *Salmonella* prevalence (Table 4.3).

In accordance with our results for controls with (TWD/TWD/B) and without brushing (TWD/TWD), Berrang and Bailey (2009) found no difference before or after pre-scald brushing of broiler carcasses in populations of *E. coli*, and coliforms or prevalence of *Salmonella*. However, post-evisceration brushing significantly reduced *E. coli* and coliforms by ~ 0.5 log CFU/ml in carcass rinsate. Shackelford et al. (1992) showed that brushing with thicker bristles removed more total solids from broiler carcasses than thinner bristles. Overall, in this study,

brushing of carcasses in combination with TSP and HWD (TSP/HWD/B) effectively reduced MAB, present at 2.4 log CFU/g after TSP/HWD, but not *E. coli* and coliforms, present at 0.7 log CFU/g after TSP/HWD, presumably due to the low initial populations.

For the comparison of loosely- and tightly-associated bacteria collected using stomaching of a skin sample and grinding of the stomached-skin sample, respectively, fewer loose MAB and total coliforms were enumerated on broiler skin than tightly associated cells ($P < 0.05$), whereas no difference was found between loosely and tightly associated *E. coli* populations or *Salmonella* prevalence (Table 4.3). It is generally known that bacteria introduced on poultry skin migrate and find protected niches such as hair follicles, ridges, and crevices (Kim et al., 1996; and National Advisory Committee on Microbiological Criteria of Foods, 1997). As a result, those microorganisms are more difficult to eliminate. Lee et al. (2014) found that shaking of broiler skin in a buffer solution recovered fewer MAB and coliforms than grinding of the previously shaken skins. While study using electron microscopy indicated that rinsing or stomaching of broiler skin did not recover bacteria located in deep skin crevices, the same organisms were recovered after shredding of the skin (Nayak et al., 2001). Using confocal microscopy, Chantarapanont et al. (2003) indicated that a large number of bacteria were still on chicken skin after physical rinsing, primarily inside crevices and feather follicles. Such bacteria are better protected by the microenvironment and are less accessible to antimicrobial treatments (McMeekin et al., 1979; and Thomas and McMeekin, 1980).

During scalding and picking, the epidermis can be easily removed along with colonizing bacteria. The freshly exposed dermal layer is vulnerable to colonization, primarily by gram-negative bacteria. Many channels and crevices in the dermal layer allow bacteria associate tightly (Thomas and McMeekin, 1980), especially when the skin is swollen during water chilling

(Chantarapanont et al., 2003; and Jeong et al., 2011). In addition, mutational changes responsible for conformational or antigenic modifications in bacterial surface structures can help bacteria attach and colonize more effectively (Wilson et al., 2010).

Carcass muscle color was not affected by any of the treatments except TWD/TWD/B, which increased yellowness (higher b^* value) (Table 4.4). Both scapula and breast skin darkened (lower L^* value) and became more yellowish (higher b^* value) after TSP/HWD and TSP/HWD/B ($P < 0.05$) compared to TWD/TWD and TWD/TWD/B (Table 4.5 and 4.6). A decrease in breast skin redness (lower a^* value) was also seen after TSP/HWD/B compared to TWD/TWD and TWD/TWD/B ($P < 0.05$) (Table 4.6). When pig carcasses were dipped in 8% TSP at 35°C, Morris et al. (1997) also reported less redness and an increase in yellowness compared to the control. However, dipping chicken in TSP at 12% did not affect sensory properties of raw and fried meat as well as consumer purchase intent (Hathcox et al., 1995).

Table 4.3 Pooled mean population (log cfu/g) $\pm$ SEM of MAB, *E. coli*, and total coliforms and pooled mean prevalence (%) of *Salmonella* on broiler skin obtained after the four treatments using two sampling methods in Exp. II

	Treatments ¹				Sampling method ²	
	TWD/TWD	TWD/TWD/B	TSP/HWD	TSP/HWD/B	Stomach	Grind
MAB $\pm 0.07^x, \pm 0.05^y$	3.6 ^a	3.5 ^a	2.4 ^b	2.1 ^c	2.5 ^b	3.3 ^a
<i>E. coli</i> $\pm 0.05^x, \pm 0.04^y$	1.2 ^a	1.1 ^a	0.7 ^b	0.7 ^b	0.9 ^a	1.0 ^a
Coliforms $\pm 0.08^x, \pm 0.05^y$	1.5 ^a	1.6 ^a	0.7 ^b	0.8 ^b	1.1 ^b	1.2 ^a
<i>Salmonella</i>	77 ^a (23/30)	50 ^{ab} (15/30)	23 ^{bc} (7/30)	20 ^c (6/30)	38.3 ^a (23/60)	46.7 ^a (28/60)

^{a-c} and ^{a,b} Means within a row on each side of the divider with no common superscripts are different ($P < 0.05$)

^xStandard error of mean (SEM) for treatments (TWD/TWD, TWD/TWD/B, TSP/HWD, and TSP/HWD/B)

^yStandard error of mean (SEM) for sampling methods (stomach and grind)

¹Number of observations for each treatment, n = 30.

²Number of observations for each sampling method, n = 60.

¹TWD/TWD: tap water dipping (TWD) (25°C, 45 s) followed by TWD (25°C, 45 s)

¹TWD/TWD/B: TWD (25°C, 45 s) followed by TWD (25°C, 45 s) with intermittent manual brushing (5 s on/ 5 s off)

¹TSP/HWD: TSP (8%) dipping (25°C, 45 s) followed by HWD (71°C, 45 s)

¹TSP/HWD/B: TSP dipping (25°C, 45 s) followed by HWD (71°C, 45 s) with intermittent manual brushing (5 s on/ 5 s off)

Table 4.4 Mean color values (L*, a*, b*) (SD) of broiler breast muscle after treatments in Exp. II

	Treatments ¹			
	TWD/TWD	TWD/TWD/B	TSP/HWD	TSP/HWD/B
L*	43.51 ^a (2.98)	41.10 ^a (1.90)	41.10 ^a (4.00)	41.57 ^a (2.27)
a*	0.86 ^a (0.55)	1.03 ^a (0.55)	1.10 ^a (0.68)	1.21 ^a (0.50)
b*	0.01 ^b (1.45)	1.28 ^a (1.39)	0.85 ^{ab} (1.01)	0.93 ^{ab} (1.18)

^{a-b}Means within a row with no common superscripts are different ($P < 0.05$)

¹Number of observations for each treatment, n = 10

¹TWD/TWD: tap water dipping (TWD) (25°C, 45 s) followed by TWD (25°C, 45 s)

¹TWD/TWD/B: TWD (25°C, 45 s) followed by TWD (25°C, 45 s) with intermittent manual brushing (5 s on/ 5 s off)

¹TSP/HWD: TSP (8%) dipping (25°C, 45 s) followed by HWD (71°C, 45 s)

¹TSP/HWD/B: TSP dipping (25°C, 45 s) followed by HWD (71°C, 45 s) with intermittent manual brushing (5 s on/ 5 s off)

Table 4.5 Mean color values (L*, a*, b*) (SD) of broiler carcass scapula area skin after treatments in Exp. II

	Treatments ¹			
	TWD/TWD	TWD/TWD/B	TSP/HWD	TSP/HWD/B
L*	67.73 ^a (1.56)	66.93 ^a (0.71)	61.92 ^b (2.08)	61.98 ^b (2.15)
a*	3.51 ^{ab} (1.51)	4.6 ^a (1.36)	2.64 ^b (1.33)	2.83 ^b (0.76)
b*	4.32 ^b (1.57)	0.72 ^c (1.03)	8.35 ^a (0.97)	7.07 ^a (1.37)

^{a-c}Means within a row with no common superscripts are different ($P < 0.05$).

¹Number of observations for each treatment, n = 6

¹TWD/TWD: tap water dipping (TWD) (25°C, 45 s) followed by TWD (25°C, 45 s)

¹TWD/TWD/B: TWD (25°C, 45 s) followed by TWD (25°C, 45 s) with intermittent manual brushing (5 s on/ 5 s off)

¹TSP/HWD: TSP (8%) dipping (25°C, 45 s) followed by HWD (71°C, 45 s)

¹TSP/HWD/B: TSP dipping (25°C, 45 s) followed by HWD (71°C, 45 s) with intermittent manual brushing (5 s on/ 5 s off)

Table 4.6 Mean color values (L*, a*, b*) (SD) of broiler carcass breast area skin after treatments in Exp. II

	Treatments ¹			
	TWD/TWD	TWD/TWD/B	TSP/HWD	TSP/HWD/B
L*	62.70 ^a (0.71)	60.18 ^{ab} (1.88)	58.47 ^{bc} (1.80)	56 ^c (1.16)
a*	1.5 ^a (1.64)	1.47 ^a (1.13)	-0.35 ^{ab} (0.95)	-1.13 ^b (0.82)
b*	1.94 ^{bc} (1.33)	0.04 ^c (1.65)	6.15 ^a (2.23)	4.81 ^{ab} (1.60)

^{a-c}Means within a row with no common superscripts are different ($P < 0.05$)

¹Number of observations for each treatment, n = 3

¹TWD/TWD: tap water dipping (TWD) (25°C, 45 s) followed by TWD (25°C, 45 s)

¹TWD/TWD/B: TWD (25°C, 45 s) followed by TWD (25°C, 45 s) with intermittent manual brushing (5 s on/ 5 s off)

¹TSP/HWD: TSP (8%) dipping (25°C, 45 s) followed by HWD (71°C, 45 s)

¹TSP/HWD/B: TSP dipping (25°C, 45 s) followed by HWD (71°C, 45 s) with intermittent manual brushing (5 s on/ 5 s off)

4.4.3 Scanning electron microscopy (SEM) and histological imaging of skin

Scanning electron microscope (SEM) images showed that the surface of reference skin samples, obtained from carcasses after bleeding, had an intact epidermal layer with few naturally occurring bacteria (Figure 4.3-A1). Light microscope images of histologically stained reference skin samples also showed no apparent damage to the epidermis (Figure 4.3-A2). Control (TWD/TWD) skin samples, obtained from carcasses after scalding and evisceration, had oil/fat particles surrounded by various dense debris materials (Figure 4.3-B1). The source of debris on the control carcasses might be organic material from the scald tank. The skin surface was hardly visible due to debris accumulation. These results are in accordance with the SEM images of Kim et al. (1994), showing extensive debris on chicken skin after evisceration and water rinsing. The histological images of the control (TWD/TWD) carcasses showed no epidermis but intact layers of dermis including stratum superficiale and stratum compactum, with no apparent damage or

erosion (Figure 4.3-B2). Based on the observations, the epidermis appears to have been washed off during scalding.

TSP treatment (TWD/TSP) removed most of the debris and exposed clean fat particles and connective tissues (Figure 4.3-C1), which were expected due to erosion of the dermal stratum superficiale layer and exposure of the dermal stratum compactum layer, which is rich in connective tissue bundles. The exposed connective tissues appeared swollen, presumably due to water retention at the high pH of TSP (Christensen et al., 1994; Capita et al., 2002a; and Capita et al., 2002b). Erosion of the dermis was supported by the histological images as most of the stratum superficiale layer was eroded or damaged after TSP treatment (Figure 4.3-C2). Kasschau et al. (1995) showed that a pH of 7.5 caused lysis of red blood cells. Compared to the control treatment (TWD/TWD) (Figure 4.3-B2), lysis of red blood cells was seen (white arrows) in the histological images for TWD/TSP (Figure 4.3-C2), TSP/HWD (Figure 4.3-E2), and TSP/HWD/B (Figure 4.3-G3) treated samples, possibly due to the high pH of TSP.

Hot water dipping (TWD/HWD) completely removed surface debris, resulting in a smooth surface with few fat particles (Figure 4.3-D1). This smooth surface is expected from the precipitation of major dermal components such as glycoproteins, glycosaminoglycans, and proteoglycans (Haake et al., 2001). In SEM images, no connective tissue was visible after TWD/HWD, presumably because surface collagen was dissolved or gelatinized at 50 to 71°C (Figure 4.3-D1) (Vaclavik and Christian, 2013). Histological images indicated that TWD/HWD caused tissue discoloration in the stratum compactum and partial erosion of stratum superficiale (Figure 4.3-D2), although not as much as the TSP treatment (TWD/TSP) (Figure 4.3-C2).

After TSP/HWD, SEM images showed a smooth skin surface that resembled that after TWD/HWD, but with some connective tissue embedded in the dermal layer, possibly due to the

trisodium phosphate effect (Figure 4.3-E1). Histological images showed tissue discoloration, lysis of red blood cells (indicated by arrows), and deeper textural damage in the stratum compactum layer (Figure 4.3-E2). These visual appearances helped to explain greater reduction in bacterial populations using TSP/HWD treatment than individual treatments (TWD/HWD or TWD/TSP): TSP/HWD penetrated deeper to reach hidden bacteria in the dermis.

When brushing was applied to control carcasses (TWD/TWD/B), surface debris was eliminated exposing some connective tissue, but unlike the TSP treatment (TWD/TSP) (Figure 4.3-C1), no erosion of the dermal layer was observed as most of the connective tissue was still embedded in the dermal layer (Figure 4.3-F1). Histological images showed deformation of the physical structure due to the physical force by brushing (Figure 4.3-F2). However, brushing alone (TWD/TWD/B) was not sufficient to damage bacteria cells as indicated by similar bacterial populations as TWD/TWD (Table 4.3).

When trisodium phosphate dip and hot water dip and brushing were used together (TSP/HWD/B), SEM images showed that the surface was similar to the skin after TWD/TSP, but with more and larger fat globules and cooked-like connective tissue (Figure 4.3-G1). Brushing during TSP/HWD/B treatment seemed to penetrate deeper areas, exposing more fat globules and connective tissues, with the connective tissue shrinking after hot water exposure (Figure 4.3-G1). The larger fat globules after TSP/HWD/B are expected to be from deeper dermis or hypodermis - the deepest skin layer, which are fat storehouses. After TSP/HWD/B, some microscopic fields of the histologically stained skin sections showed that major portions of the stratum compactum were sloughed off as no major blood vessels were visible indicating deep penetration by TSP/HWD/B (Figure 4.3-G2). In other fields, TSP/HWD/B-induced damage appeared to be similar to that after TSP/HWD treatment i.e., deep damage in the stratum compactum of the

dermis but no sloughing off of the dermal layers (Figure 4.3-G3). Like the TSP/HWD treatment, the high pH of TSP/HWD/B treatment resulted in lysis of red blood cells (arrows in Figure 4.3-G3). The percentage of representative microscopic fields of histologically stained skin sections with an appearance similar to the description above is shown in Table 4.7.

The thickness of broiler skin is roughly 700 to 1500 μm (Kondjoyan and Portanguen, 2008). Bacteria can penetrate up to 50 μm with a maximum population observed between 0 to 10 μm (Chantarapanont et al., 2003; and Jang et al., 2007). Since TSP/HWD and TSP/HWD/B can penetrate up to 100 μm or more (Figure 4.3-E2 and 4.3-G3) and expose bacteria in the stratum compactum of the dermis, the treatments are expected to be effective against bacteria in deeper skin locations.

Despite microscopic evidence showing deepest penetration using TSP/HWD/B, there was no difference between TSP/HWD and TSP/HWD/B in reductions of *E. coli*, total coliforms or *Salmonella* (Table 4.3). These results can be explained by the low populations of *E. coli* and total coliforms (0.7 log CFU/g), and low prevalence of *Salmonella* (23%) that remained on the skin after TSP/HWD. Another possible reason would be decreased exposure of bacteria to trisodium phosphate in deeper areas, making subsequent hot water exposure in presence of brushing less effective. Sampathkumar et al. (2004) also indicated that exposure of *Salmonella* to a sublethal dose of 1.5% TSP or pH 10.0 resulted in a significant increase in thermo tolerance.

Figure 4.3 Scanning electron microscope (A1 - G1, 10000X magnification) and light microscope (A2 - G2 and G3, 20X magnification) images of skin surface and histologically stained skin sections, respectively, for reference and treated skins.

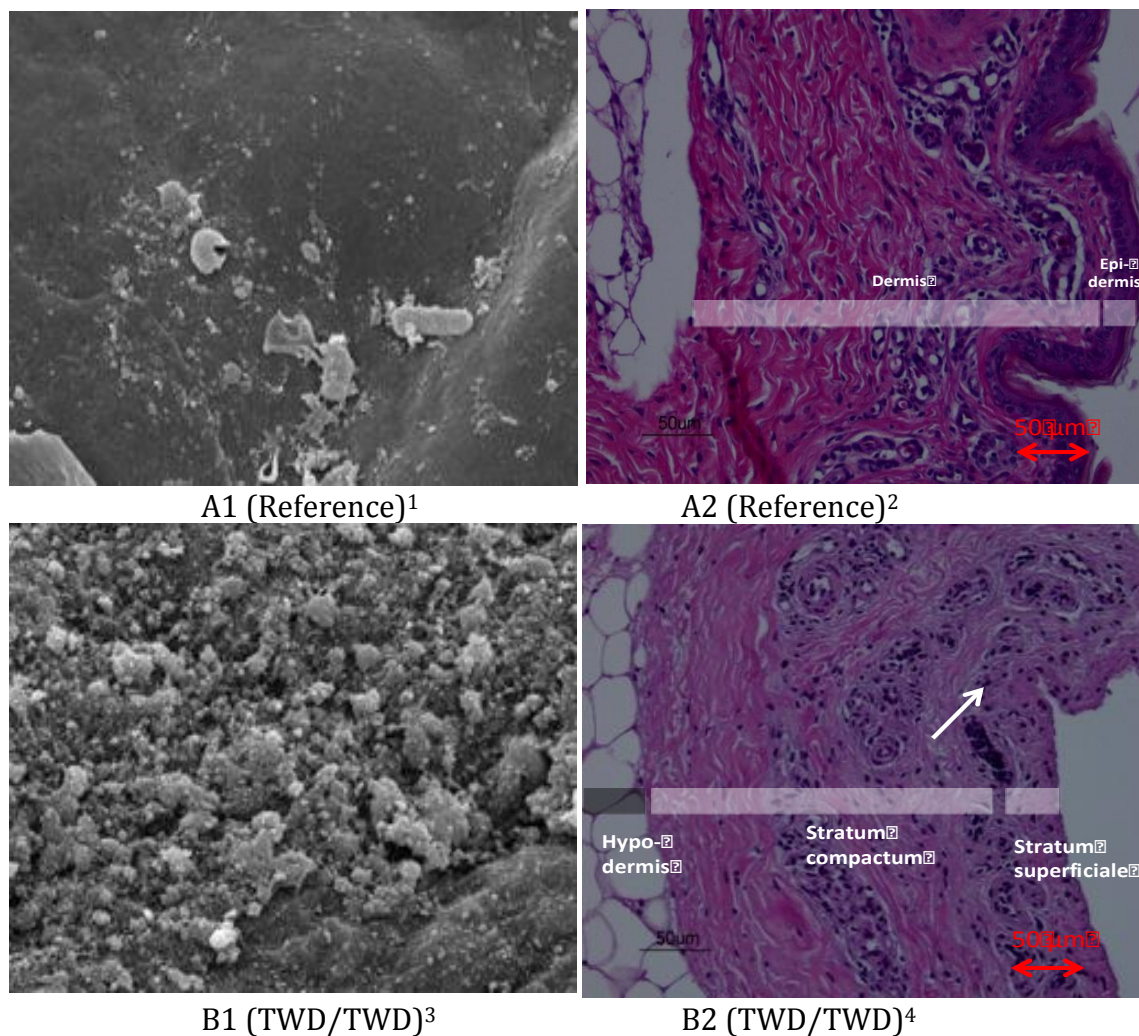


Figure 4.3 (cont'd)

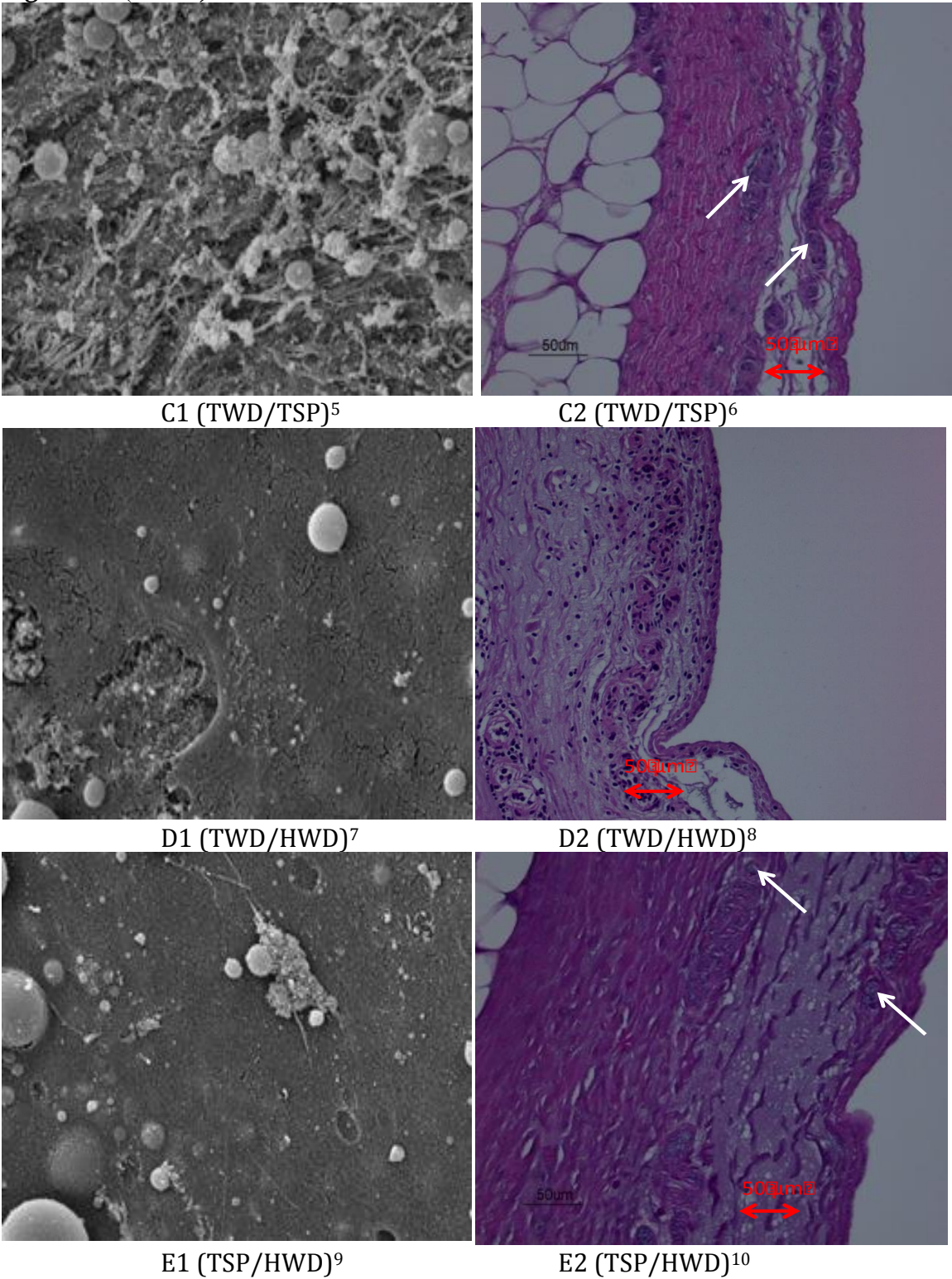
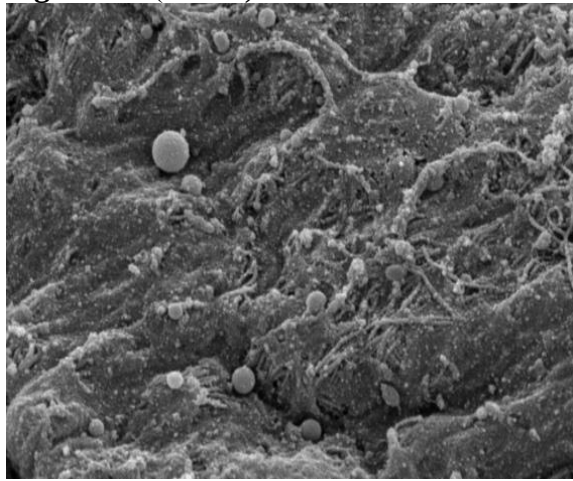
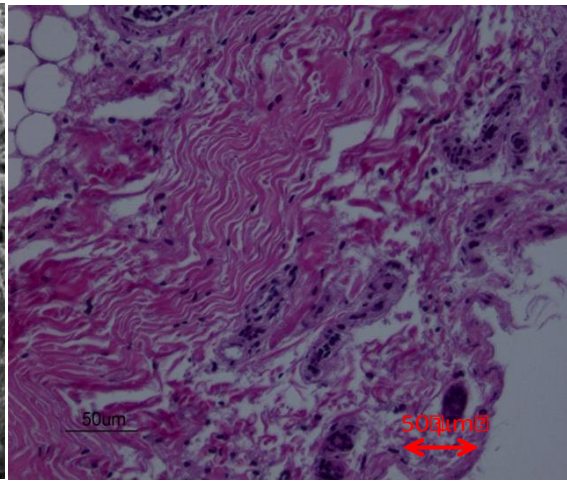


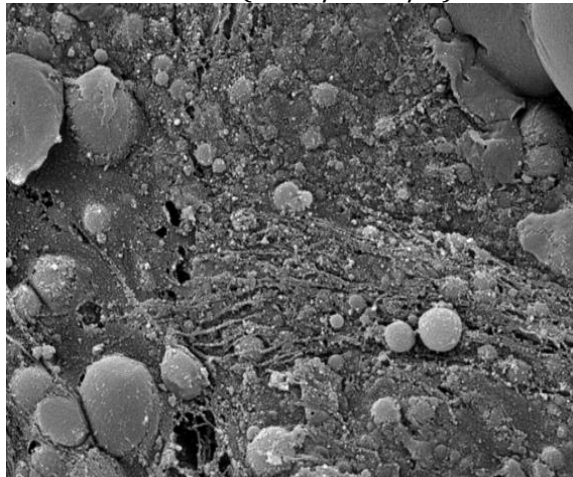
Figure 4.3 (cont'd)



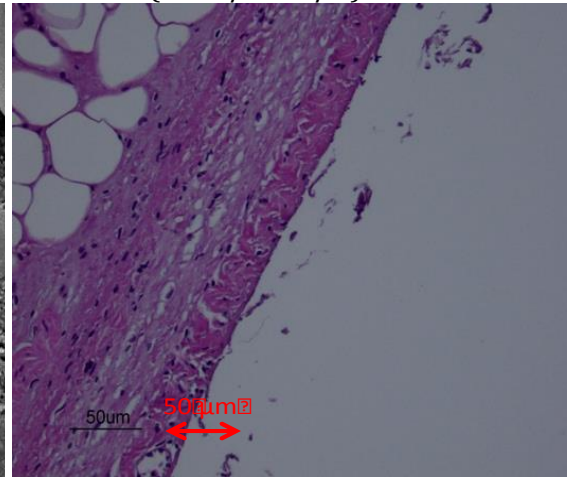
F1 (TWD/TWD/B)¹¹



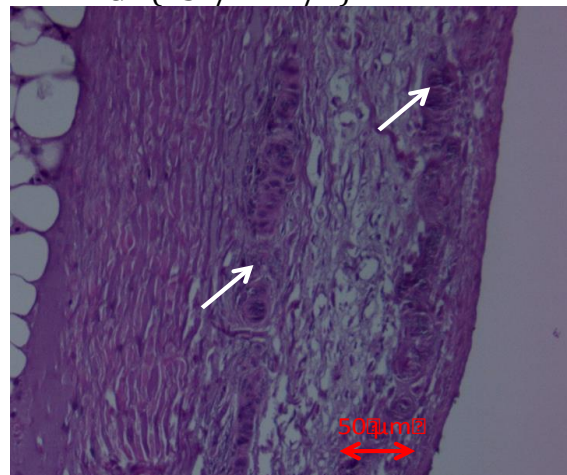
F2 (TWD/TWD/B)¹²



G1 (TSP/HWD/B)¹³



G2 (TSP/HWD/B)¹⁴



G3 (TSP/HWD/B)¹⁵

^{1,2}Reference-broiler breast skin after bleeding

^{3,4}TWD/TWD treated-broiler breast skin after scalding and evisceration

^{5,6}TWD/TSP treated-broiler breast skin after scalding and evisceration

^{7,8}TWD/HWD treated-broiler breast skin after scalding and evisceration

Figure 4.3 (cont'd)

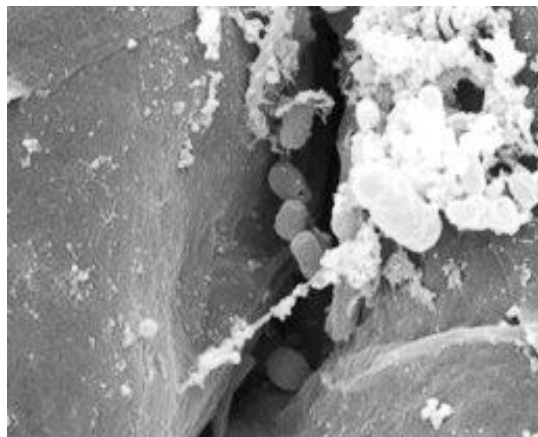
^{9,10}TSP/HWD treated-broiler breast skin after scalding and evisceration

^{11,12}TWD/TWD/B treated-broiler breast skin after scalding and evisceration

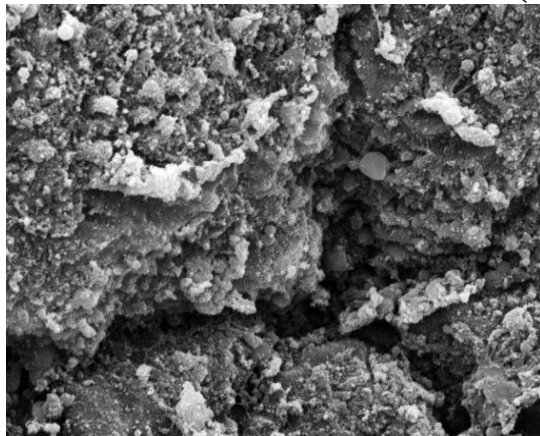
^{13,14,15}TSP/HWD/B treated-broiler breast skin after scalding and evisceration

White arrows point towards red blood cells

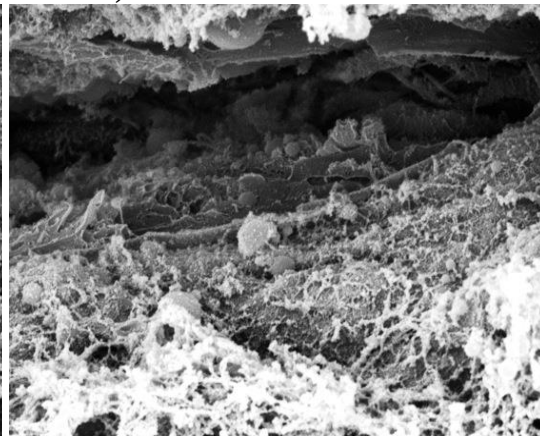
Figure 4.4 Scanning electron microscope images (10000X magnification) of skin crevices for reference and treated skins



A (Reference)¹

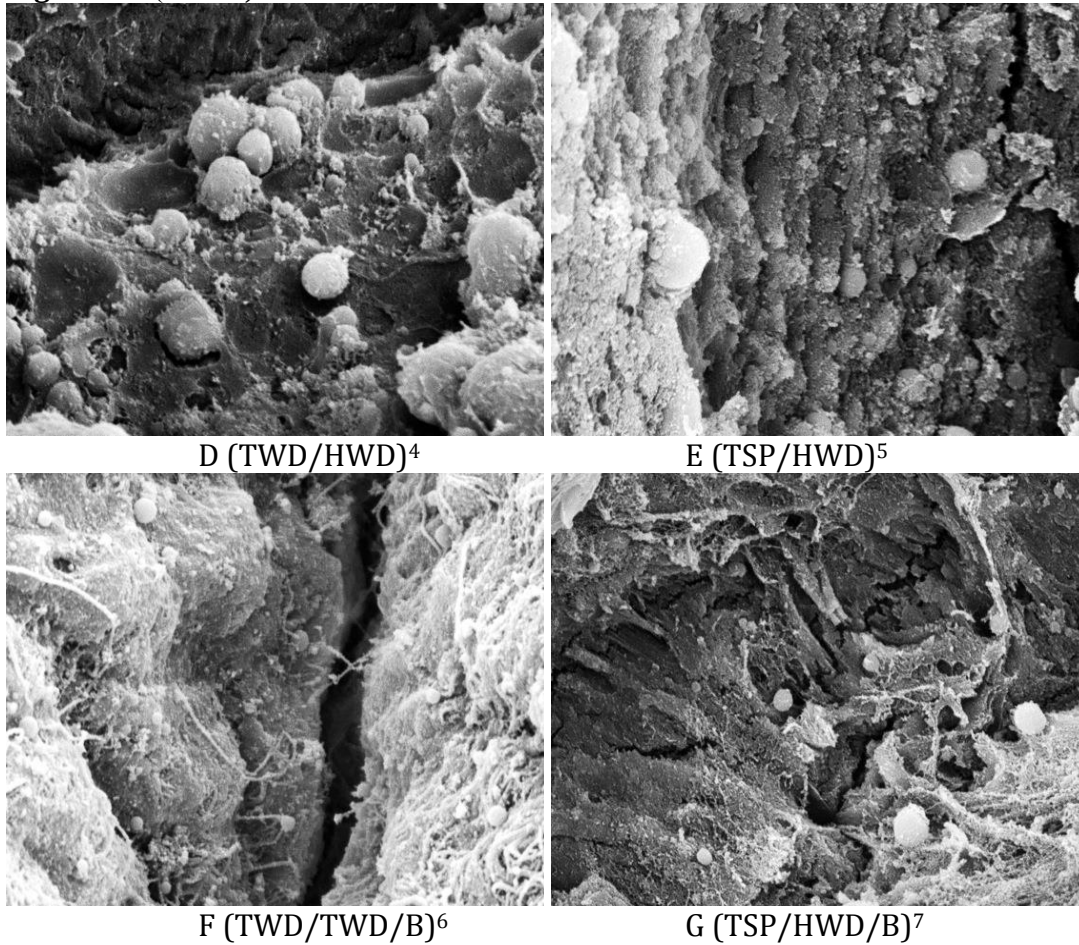


B (TWD/TWD)²



C (TWD/TSP)³

Figure 4.4 (cont'd)



¹Reference-broiler breast skin after bleeding

²TWD/TWD treated-broiler breast skin after scalding and evisceration

³TWD/TSP treated-broiler breast skin after scalding and evisceration

⁴TWD/HWD treated-broiler breast skin after scalding and evisceration

⁵TSP/HWD treated-broiler breast skin after scalding and evisceration

⁶TWD/TWD/B treated-broiler breast skin after scalding and evisceration

⁷TSP/HWD/B treated-broiler breast skin after scalding and evisceration

Table 4.7 Observations on skin structure before and after treatments based on light microscopy of histologically stained skin sections and percentages of representative microscopic fields with appearances corresponding to the observations described

Treatment¹	Observations	Representative microscopic fields (%)	
Reference	Intact epidermis and dermis layers	100	
TWD/TWD	Epidermis removed and dermis intact	100	
TWD/TSP	Corrosion of entire st. superficiale	90	
	Lysis of red blood cells	100	
TWD/HWD	Tissue discoloration	80	
	Partial corrosion of st. superficiale	50	
TSP/HWD	Deep damage in st. compactum	100	
	Lysis of red blood cells	100	
TWD/TWD/B	Disorganization in structure	56	
TSP/HWD/B	Appearance similar to TSP/HWD	62	Mutually exclusive
	Sloughing off of entire st. superficiale and part of st. compactum	38	

¹Total number of fields observed for each treatment = 48

¹Reference-broiler breast skin after bleeding

¹TWD/TWD treated-broiler breast skin after scalding and evisceration

¹TWD/TSP treated-broiler breast skin after scalding and evisceration

¹TWD/HWD treated-broiler breast skin after scalding and evisceration

¹TSP/HWD treated-broiler breast skin after scalding and evisceration

¹TWD/TWD/B treated-broiler breast skin after scalding and evisceration

¹TSP/HWD/B treated-broiler breast skin after scalding and evisceration

SEM images of skin crevices (Figure 4.4-A to 4.4-G) showed a similar pattern of structural changes as seen on the skin surface (Figure 4.3-A1 to 4.3-G1), although intensity of the changes was reduced. Reference images of crevices of skin samples, obtained from carcasses after bleeding, showed an intact epidermis with larger numbers of bacteria (Figure 4.4-A) than those seen on the smooth surface (Figure 4.3-A1). The epidermis layer in crevices was removed

along with bacteria after scalding and evisceration (Figure 4.4-B to 4.4-G). Pictures, taken before and after scalding, support the hypothesis that bacteria, predominantly gram positive, colonize the epidermal layer, which is removed during scalding to expose fresh dermis vulnerable to be colonized by gut pathogens (Thomas and McMeekin, 1980). Similar to the surface, debris in skin crevices after TWD/TWD (Figure 4.4-B) was removed to expose some connective tissue and clean fat globules after TWD/TWD/B (Figure 4.4-F). More connective tissue and fat globules were exposed after TWD/TSP (Figure 4.4-C) while surface smoothness increased with no visual connective tissue after TWD/HWD (Figure 4.4-D). TSP/HWD treated skin (Figure 4.4-E) looked similar to TWD/HWD treated skin (Figure 4.4-D) except for having a less smooth surface. After TSP/HWD/B (Figure 4.4-G), the skin was not smooth and contained less connective tissue than the TWD/TWD/B and TWD/TSP treatments.

In conclusion, both TSP and the hot water dip are common intervention strategies against bacteria in poultry and red meat processing facilities. The combination of TSP and hot water dipping has not been implemented as frequently compared to single applications. Brushing of broiler carcasses before scalding is recommended for reducing debris and fecal material. However, the effect of brushing on the microbiological quality of eviscerated carcasses has not been sufficiently studied. In the comparison of single and combined treatments, the combination of three treatments (trisodium phosphate, hot water, and brushing) resulted in the best decontamination of broiler carcasses, with intermediate results observed for trisodium phosphate or hot water dip, compared to the control. However, the combination treatments increased yellowness of the carcass skin. Based on these results, additional research is needed to elucidate the effects of trisodium phosphate, hot water, and brushing parameters including time, temperature, pressure, and bristle properties on improvement of broiler carcass safety and

optimization of carcass appearance.

CHAPTER 5

CHARACTERISTICS OF *SALMONELLA* ASSOCIATION AND PENETRATION TO BROILER SKIN DURING EXPOSURE WITH/WITHOUT STOMACHING

5.1 Abstract

The objectives of this study were to evaluate *Salmonella* association to broiler skin during refrigerated exposure and location on broiler skin after stomaching. In the first experiment, broiler skins from eviscerated carcasses were immediately taken and exposed to a cocktail containing green fluorescent protein (GFP) tagged *Salmonella* Enteritidis (1×10^9 CFU/ml) for 0.5, 6h, 12h, and 24h for 48h at 4°C. After each exposure, two stomachings (1 min each) and grinding of the stomached skin were conducted to quantify loosely associated (from stomachings) and tightly associated (from grinding) *Salmonella* on the skin, respectively. Following the *Salmonella* exposure to the skin for 24 and 48 h, confocal images were taken before and after the two stomachings. After the 1st stomaching, about 71% of the *Salmonella* cells were recovered, whereas 17 and 12% of the *Salmonella* cells were recovered after the 2nd stomaching and the final grinding, respectively, regardless of incubation time. Confocal images showed that two stomachings removed most *Salmonella* cells from the skin but a few cells were observed at an average depth of 29 μm compared to 9 μm for skin before stomaching. In the second experiment, broiler skins were either dip-exposed for 2 min or stomached for 2 min, using the same GFP-*Salmonella* cocktail diluted in phosphate buffer saline to obtain 1×10^8 cells/ml. *Salmonella* cells on flat surfaces of dipped and stomached skins were observed up to 10 and 62 μm depths, respectively, and *Salmonella* cells inside crevices of dipped and stomached skins were found up to average depths of 68 and 132 μm depths, respectively. *Salmonella* cells were found floating freely in entrapped water in skin crevices, indicating that water might play an important role in entrapment and localization of bacteria in skin crevices.

5.2 Introduction

Many studies have been conducted to evaluate various physical and chemical sanitizing agents for improving microbiological quality of broiler carcasses during processing, but little research has been carried out to elucidate different aspects of bacterial attachment, such as attachment affinity and location on broiler skin. Arritt et al. (2002) mentioned that given the right conditions, bacteria take only minutes to attach tightly to broiler skin, with no difference in bacterial populations between 0.5 and 10 min of exposure. Nayak et al. (2001) made direct visual observations using scanning electron microscope to study *Salmonella* attachment and detachment on broiler skin.

Green fluorescent protein (GFP) has been used for visualizing localization and survival of bacterial cells in biological systems (Ling et al., 2000; Burnett and Beuchat, 2001). A major advantage of using GFP tagged bacterial cells is simpler sample preparation that does not require additional substrate for fluorescence. Since fluorescence does not depend on access to substrate, fluorescence can be visualized in all cells, irrespective of their location/depth in the skin, using z-stacking procedures of confocal microscopy (Chantarapanont et al., 2003). However, some disadvantages of GFP-tagged cells are possible loss of plasmid during cell division and denaturation of fluorescent protein upon exposure to sanitizers (Burnett and Beuchat, 2001). As a result, the technique is primarily used for studies that do not use sanitizers or disinfectants with sample incubation under refrigeration for slow cell division after inoculation. Using this technique, Chantarapanont et al. (2003) investigated survival of *Campylobacter jejuni* cells at specific sites and various depths on chicken skin during storage. In this chapter, *Salmonella* association strength, location, and penetration in broiler skin were investigated using the

stomaching and grinding methods for sample processing, GFP-tagged *Salmonella* cells, and confocal scanning laser microscopy.

5.3 Materials and methods

5.3.1 *Salmonella* strains

Two GFP labeled, ampicillin resistant *Salmonella* Enteritidis strains (ME18 and H4717) were obtained from Center for Food Safety, University of Georgia (Athens, GA). In Experiment I described below, the two GFP labeled strains were compared for their attachment abilities with a strain of *Salmonella* Thompson (FSIS 120) isolated from chicken. All strains had been preserved at -80°C in trypticase soy broth (TSB) containing 0.6% (wt/vol) yeast extract (YE) (BD, Sparks, MD) and 20% glycerol.

5.3.2 Experiment I: Time required for *Salmonella* to associate tightly to broiler skin

5.3.2.1 Microtiter plate attachment assay

A modified microtiter plate assay was conducted in triplicate to compare attachment abilities of the two GFP labeled *Salmonella* Enteritidis strains and a strain of *Salmonella* Thompson (FSIS 120) isolated from chicken (Stepanovic et al., 2000). Stock cultures of the strains were streaked onto trypticase soy agar (TSA) plates with plates containing 100 µg/ml of ampicillin (Sigma-Aldrich, MO) for the two GFP labeled strains, and incubated at 37°C for 24 h. A single colony was transferred to 9 ml of TSB with TSB containing 100 µg/ml of ampicillin for the two GFP labeled strains, followed by overnight incubation at 37°C. Overnight cultures of the

strains were serially diluted to 10^7 CFU/ml in TSB. After vortexing, 200 μ l of the diluted bacterial suspensions were transferred to three wells of a 96 well non-pyrogenic polystyrene microtiter tissue culture plate (Corning Inc., NY). Three wells per plate containing 200 μ l of sterile TSB served as negative controls. After 48 h of incubation under refrigeration (4°C), the microtiter plates were emptied, rinsed three times with 250 μ l of sterile phosphate buffer to remove unattached cells, and left to air dry. The remaining cells were fixed to the well by adding 200 μ l of 99% methanol (Fisher Chemicals, Fair Lawn, NJ). The methanol in wells was decanted after 15 min, and plates were left to dry in air. After drying, the wells were stained for 5 min by adding 200 μ l of 2% crystal violet. Excess stain was removed by placing the plates under running sterile deionized water. After the plates were air dried, 160 μ l of 33% (vol/vol) glacial acetic acid (Sigma Chemical Company) was added to solubilize the dye bound to cells attached to the wells. Optical density of each well was measured at 570 nm using a EnSpire® Multimode Plate Reader (PerkinElmer, MA).

5.3.2.2 Broiler skin model

Fresh skins from the breast area of broiler carcasses were excised immediately after evisceration and before chemical spraying at a commercial broiler processing plant. Using the skin, a broiler skin model was prepared according to the method of Kim et al. (1996b) with a slight modification. Briefly, the bottom end of a 50-ml Nalgene conical-shaped centrifuge tube was first cut for inoculum addition. Broiler skin was then mounted on the open mouth at the top end of the tube such that the outside of the skin surface faced towards the inside of the tube. The skin was tightly held in position using a rubber band and covered with sterile aluminum foil to avoid drying.

5.3.2.3 Storage of broiler skin after *Salmonella* inoculation

For this study, the frozen stock cultures of the two GFP labeled strains were transferred to TSB containing 100 µg/ml ampicillin (Sigma-Aldrich, MO) for 24 h at 37°C, pelleted by centrifugation at 3,100 x *g* for 15 min at 4°C, and then re-suspended in sterile phosphate buffer saline (PBS, pH 7.4). The optical density (OD) of both cell suspensions was measured at 600 nm, suspensions were adjusted to the same OD value, and mixed together in equal volumes to obtain a two-strain *Salmonella* Enteritidis cocktail containing 1 x 10⁹ CFU/ml. The *Salmonella* population in the inoculum was confirmed by plating appropriate dilutions on trypticase soy agar (TSA; Difco, BD) containing 100 µg/ml ampicillin and incubating for 24 h at 37°C.

For microbial analysis, ten skin samples, in each of the two replications, were prepared and inoculated with the cocktail of GFP labeled ampicillin resistant *Salmonella* Enteritidis strains (ME18 and H4717) at 10⁹ CFU/2 cm². The samples were then equally distributed for five different storage durations at 4°C (2 samples/treatment): for 0.5, 6, 12, 24, and 48 h.

For confocal imaging, during each of the two replications, six skin samples were prepared and inoculated as explained previously. The samples were then equally distributed for two different storage durations at 4°C (3 samples/treatment): for 24, and 48 h.

5.3.2.4 Skin sampling for microbiological analysis

At the end of each storage time, the tubes containing the *Salmonella* cocktail were decanted and the skins were gently rinsed 3 times with 4 ml of sterile water to remove unattached *Salmonella* cells. The skin, which was exposed to the cocktail during storage, was cut from the centrifuge tube and stomached for 1 min after transferring to a stomacher bag containing 9 ml of sterile phosphate buffer saline (PBS). The skin was then transferred to a fresh

stomacher bag containing sterile 9 ml of PBS and stomached again for 1 min. The two stomachings were conducted to enumerate *Salmonella* that were expected to be loosely associated (Singh et al., 2015). Following the two stomachings, the skin was finally ground with sterile 9 ml of PBS to enumerate *Salmonellae* that were expected to be tightly associated (Singh et al., 2015).

5.3.2.5 Skin sampling for confocal imaging

At the end of each storage duration, the tubes were decanted, and the skins were gently rinsed before cutting the inoculated area as explained before. The resulting skins were then stomached two times using fresh buffer as before. Microscopic observations of *Salmonella* cells on skins before and after the two stomachings were made using a confocal microscope (Olympus, Tokyo, Japan).

5.3.2.6 Microbiological analysis

Serial 10-fold dilutions of the stomached and final-ground broiler skins were plated on TSA supplemented with ampicillin (100 µg/ml). Bacterial colonies on the plates were enumerated after incubation for 24 h at 37°C.

5.3.2.7 Confocal microscopy

The surfaces of broiler skins were visualized using an Olympus FluoView FV1000 Confocal Laser Scanning Microscope (Olympus, Tokyo, Japan), 60X oil objective and a pinhole aperture of 1.2. Fluorescent *Salmonella* cells were visualized using an excitation wavelength of 488 nm at 10% intensity and a BA505-525 emission filter. The emitted light was

assigned a green color to envision green cells. *Salmonella* cells at different depths were visualized by imaging optical sections using a z-stacking procedure with a step size of 2 μm . Each optical section contained 1024 X 1024 pixels.

5.3.3 Experiment II: Effect of stomaching on *Salmonella* penetration in broiler skin

5.3.3.1 Skin treatment

For each of two replications, four pieces (1 g/piece) of broiler breast skin were obtained from a local organic grocery store and randomly divided between two treatments: 1) Dip for 2 min in 9 ml GFP-tagged *Salmonella* Enteritidis cocktail (1×10^8 CFU/ml), 2) Stomach for 2 min in 9 ml GFP-tagged *Salmonella* Enteritidis cocktail (1×10^8 CFU/ml).

5.3.3.2 Confocal microscopy

Skin samples were prepared as described previously, except that triple rinsing with sterile water after exposure was not done, and *Salmonella* cells on the skin were visualized by a z-stacking procedure using confocal microscopy as described previously (5.3.1.6). Twelve to twenty microscopic fields were observed for each treatment.

5.3.4 Statistical analysis

In experiment 1, bacterial counts were converted to $\log \text{CFU}/6 \text{ cm}^2$. Two-factorial analysis (sampling method x storage time) was conducted to compare bacterial counts. With no interaction between the two factors, data were pooled for storage time. For comparison of absorbance readings from microtiter plate attachment assay, *Salmonella* counts among the three

sampling methods in experiment 1 (1st stomaching, 2nd stomaching, and grinding), and *Salmonella* penetration depth among the treatments in experiments 1 and 2, GLM and Duncan's multiple range test were used at $P < 0.05$ (SAS 9.4, 2013, SAS Institute Inc.).

5.4 Results and discussion

5.4.1 Experiment I: Time required for *Salmonella* to associate tightly to broiler skin

In microtiter plate attachment assay, the two GFP labeled *Salmonella* Enteritidis and the chicken isolated *Salmonella* Thomson strains showed similar attachment ($P > 0.05$), with average optical density values of 0.135, 0.141, and 0.127 for *Salmonella* Enteritidis ME18, *Salmonella* Enteritidis H4717, and *Salmonella* Thomson FSIS 120, respectively.

In case of the broiler skin model test, the microbial population data were pooled since no interaction was seen between storage treatment and sampling method. *Salmonella* recovery was greater after the initial skin stomaching (71%) compared to the second stomaching (17%) and final grinding (12%) of the same skin (Table 5.1). These results differed from those of our previous study, which indicated that 65% of mesophilic aerobic bacteria (MAB) were recovered after final grinding, while 17 and 18% of MAB were recovered after the 1st and the 2nd-through-10th stomachings, respectively (Singh et al., 2015). These differences were expected for two reasons: First, the bacteria enumerated in the current study were *Salmonella* Enteritidis which were artificially exposed to broiler skin, whereas in previous study, bacteria enumerated were naturally occurring MAB on the skin. Secondly, the exposure time of 48 h might be insufficient for *Salmonellae* to associate tightly with the skin, whereas in the previous study, most natural MAB have presumably habituated for a long time and associated tightly with the skin. Several

studies have suggested that bacteria can associate tightly/firmly with broiler skin before birds arrive at processing plants (Labellec et al., 1985; Lillard et al., 1990; Nayak et al., 2001).

In the analysis of confocal images, *Salmonella* cells were present in skin up to an average depth of 9 µm before-stomaching (Table 5.2). Most of the *Salmonella* cells were removed after double stomaching but a few remaining cells were observed up to an average depth of 29 µm ($P < 0.05$, Table 5.1 and 5.2). Figure 5.1 shows that after 48 h of refrigerated storage, *Salmonellae* are present on broiler skins up to depths of 10 and 38 µm before and after double stomaching, respectively. Figure 5.1 supports the finding that compared to before stomaching, fewer cells, but up to a deeper depth, were observed after stomaching. Few if any cells were observed on surface of skin before stomaching, possibly because of triple washing with sterile water.

Table 5.1 *Salmonella* population¹ (SD)/percent² recovery from two stomachings and one grinding of broiler skin after refrigerated exposure to a *Salmonella* cocktail (1 x 10⁹ CFU/ml)

Treatment	<i>Salmonella</i> count/percentage recovered
Stomach 1	7.6 ^a (0.4)/71%
Stomach 2	6.8 ^b (0.5)/17%
Grind	6.5 ^b (0.7)/12%

^{a,b}Means with the column with no common superscript are different ($P < 0.05$)

¹Log CFU/6cm² (Standard deviation)

²Percentage of *Salmonella* recovered from 2 stomachings and grinding using non-log transformed data

^{1,2}Data are pooled for incubation times

^{1,2}n = 16

Table 5.2 Average (SD) and range of depth of *Salmonella* cells on broiler skin before and after two 1 min stomachings in a *Salmonella* cocktail (1 x 10⁹ CFU/ml)

Treatment	Average <i>Salmonella</i> depth (µm)	Range (min – max) of <i>Salmonella</i> depth (µm)
Before stomach	9 ^a (3)	6 - 14
After stomach	29 ^b (27)	6 - 86

^{a,b}Means within a column with no common superscript are different ($P < 0.05$)

Number of observations = 6

Figure 5.1 Confocal image of uninoculated control skin (A) and images taken before (B, C, D and E) and after (F, G, H and I) two 1 min stomachings of broiler skin inoculated with a *Salmonella* cocktail (1×10^9 CFU/ml) and incubated at 4°C for 48 h

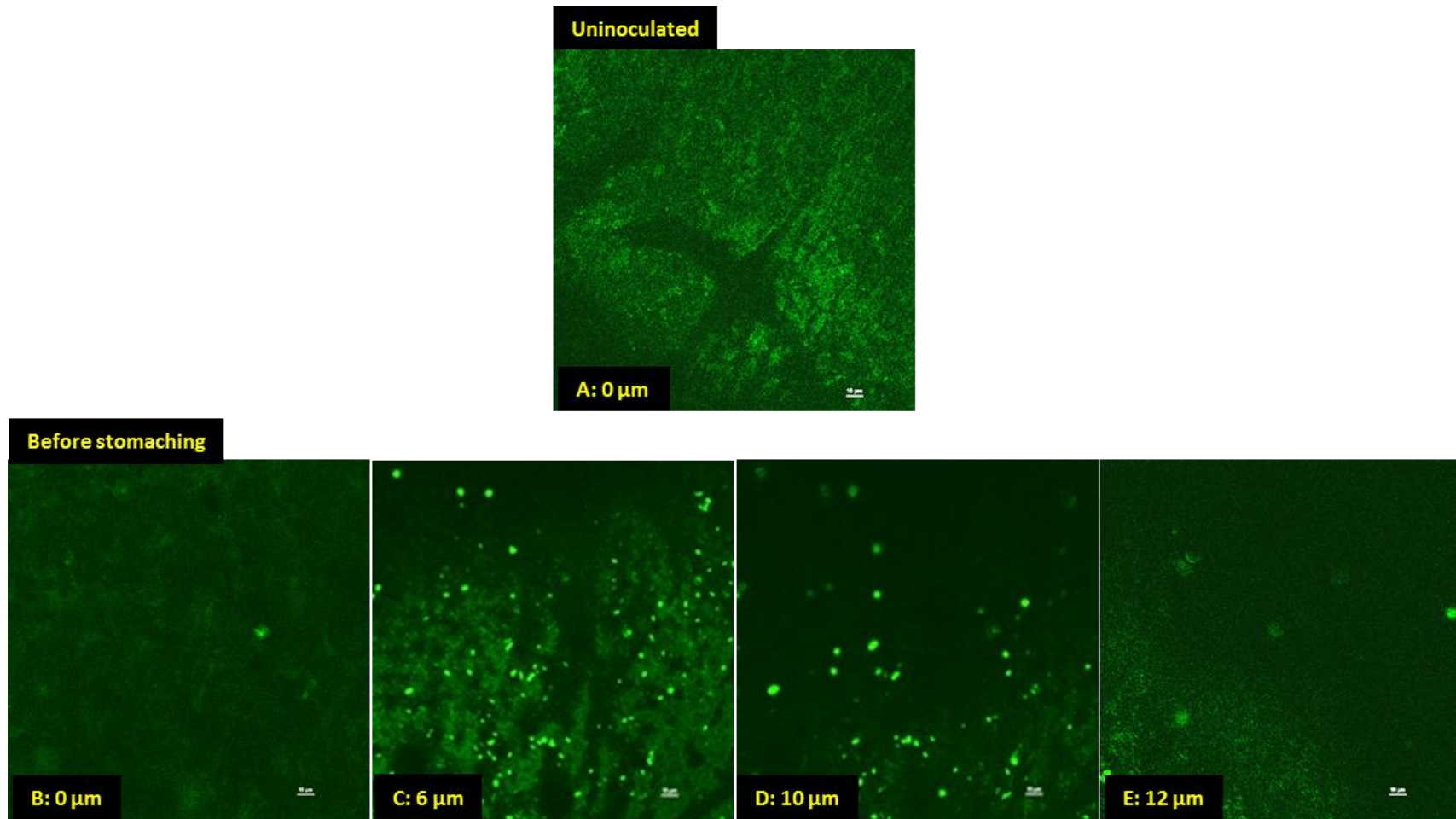
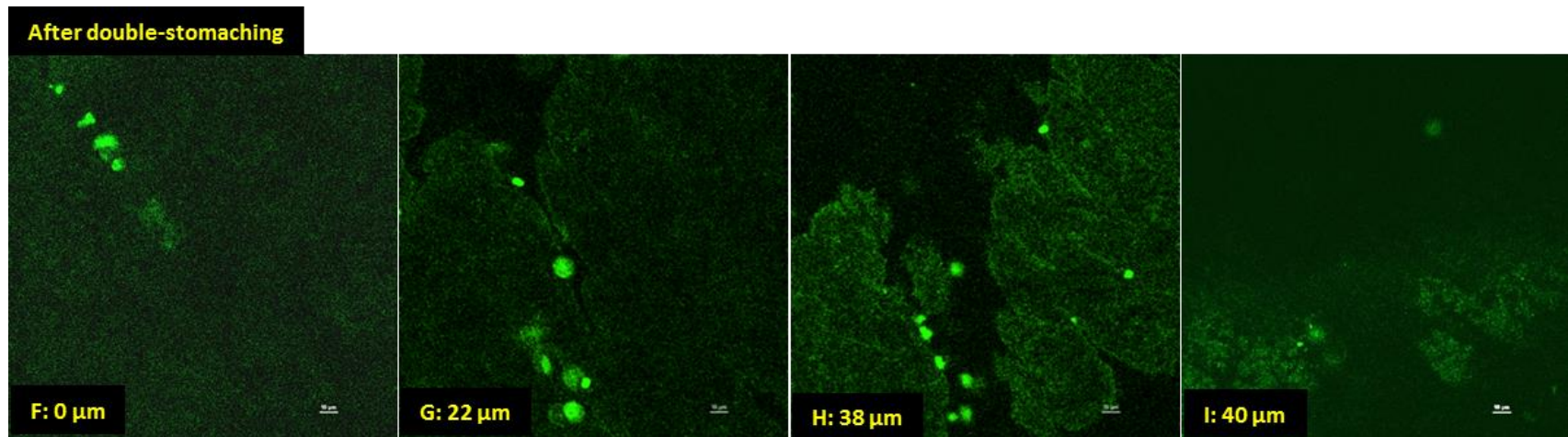


Figure 5.1 (Cont'd)



5.4.2 Experiment II: Effect of stomaching on *Salmonella* penetration in broiler skin

Following the observation of *Salmonella* penetration after double stomaching, additional test was conducted to determine *Salmonella* penetration after 2 min of exposure with and without stomaching of broiler skin in a *Salmonella* cocktail buffer containing 10^8 CFU/ml. *Salmonellae* were observed up to an average depth of 10 μm on the flat surfaces of skins held for 2 min without stomaching. However, *Salmonellae* were able to penetrate into the crevices up to an average depth of 68 μm without stomaching (Table 5.3). Using confocal microscopy, Chantarapanont et al. (2003) reported similar results for *Campylobacter*, which was seen at depths of 8.4 μm and 50 μm on the broiler skin surface and inside crevices, respectively. Jang et al. (2007) found *Campylobacter* up to a depth of 30 μm inside crevices of artificially inoculated chicken skin.

Figure 5.2 shows representative images of the control uninoculated skin surface (A), flat skin surfaces (B, C, D), and skin crevices (E, F, G); which were used to quantify *Salmonella* penetration depth at different locations on broiler skin in Table 5.3. No *Salmonellae* were observed on the uninoculated control skin (Figure 5.2-A). For both the dipped and stomached skins, *Salmonellae* penetration was greater in crevices than on flat surfaces (Table 5.3). On flat skin surfaces and inside skin crevices, *Salmonellae* were present on dipped samples up to average depths of 10 and 68 μm , respectively, with stomaching increasing the average depths to 62 and 132 μm , respectively ($P < 0.05$) (Table 5.3).

Most of the *Salmonella* cells located deep inside crevices were floating freely in water and were not attached to the surfaces/walls of the crevices (Figure 5.2-E, 5.2-F, and 5.2-G). Kim et al. (1996c) also observed free-floating *Salmonellae* in chicken feather follicles at depths as high as 142 μm . Thomas and McMeekin (1982) reported that broiler processing caused skin

swelling due to water absorption, exposing deep crevices and channels on the skin surface. Finer crevices have sufficient space and surface tension to trap water along with bacteria. These physical structures play a greater role in *Salmonella* entrapment/association than interactions between the crevice and bacterial surfaces. Kim et al. (1996b) also concluded that attachment of *Salmonella* to poultry skin is non-specific and cell surface factors are not important in the attachment process.

Table 5.3 Average (SD) and range of depth of *Salmonella* cells on flat surfaces and inside crevices of broiler skin after a 2 min immersion (no stomaching) and 2 min of stomaching in a *Salmonella* cocktail (1×10^8 CFU/ml)

Treatment	Type of surface	Average <i>Salmonella</i> depth (μm)	Range (min – max) of <i>Salmonella</i> depth (μm)
Dip	Flat	10 ^c (3)	6 - 16
	Crevice	68 ^b (39)	28 - 150
Stomach	Flat	62 ^b (22)	30 - 84
	Crevice	132 ^a (60)	58 - 200

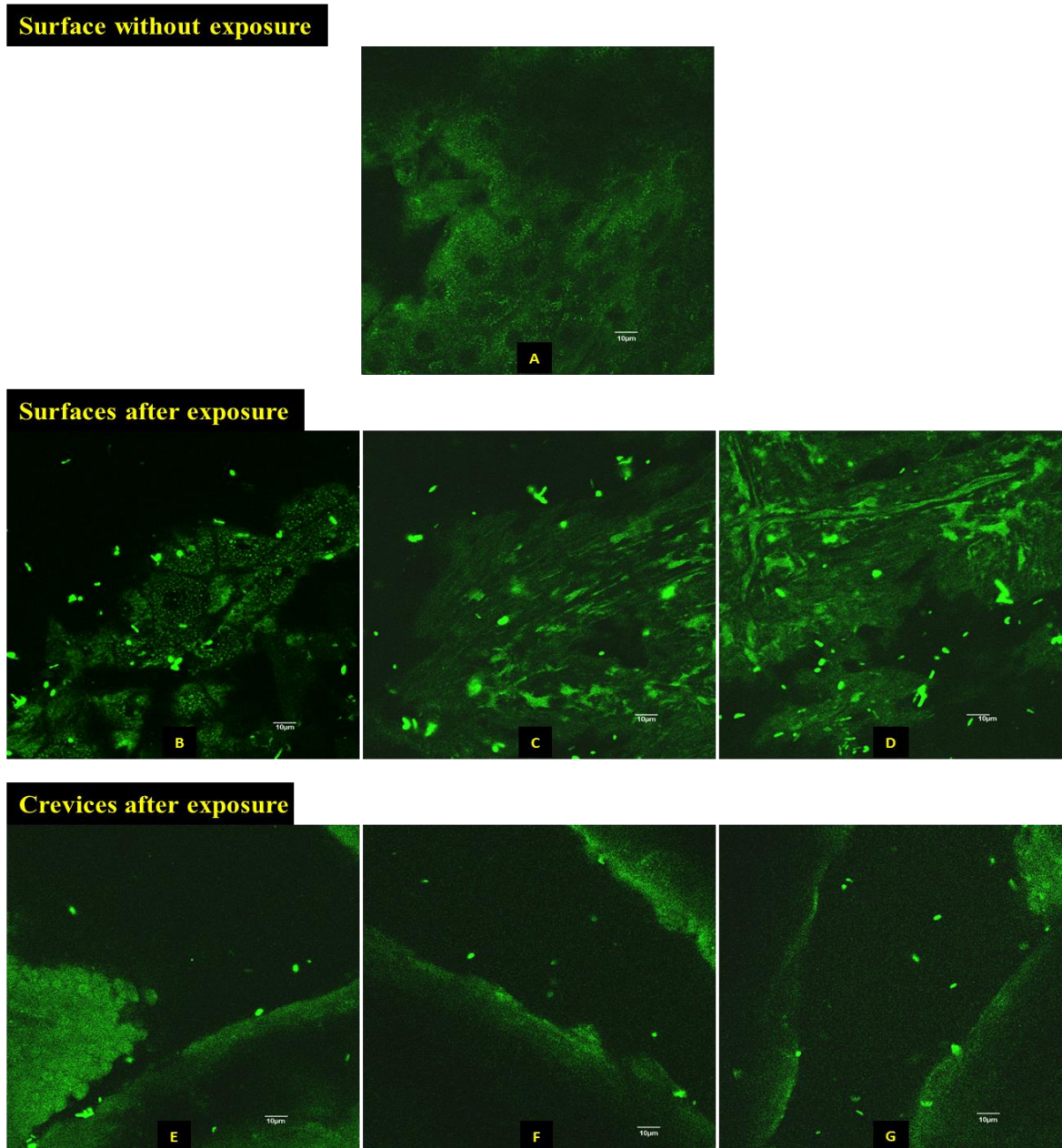
^{a-c}Means within a column with no common superscript are different ($P < 0.05$)

Number of observations = 6 to 10

In this study, almost 88 % of the *Salmonella* inoculum was easily recovered after two stomachings indicating that most newly introduced bacteria, unless they are tightly associated, can be removed by processing interventions. Most *Salmonella* cells located inside crevices were found floating freely in water indicating the role of trapped water in localization of bacteria deep inside crevices and cracks. Since stomaching introduced some cells into deeper locations that may be difficult to access by antimicrobial treatments, the impact of such broiler processing steps as brushing, scalding, de-feathering and evisceration needs to be further studied in terms of pathogen location and survivability. Based on these results, destructive sampling methods, such

as grinding, are recommended for maximum recovery of pathogens from broilers, especially when the level of contamination is low.

Figure 5.2 Representative confocal images of skin surface without *Salmonella* exposure (A)¹, flat skin surfaces after *Salmonella* exposure (B, C, and D)¹, and skin crevices after *Salmonella* exposure (E, F, and G)¹



¹A, B, C, D, E, F and G are images taken from different skin samples

CONCLUSIONS AND FUTURE RECOMMENDATIONS

Broiler skin was found to be inhabited by two categories of bacteria; one was loosely associated which stomaching or swabbing could easily quantify and other was tightly associated which could only be completely quantified by grinding. Tightly associated populations of mesophilic aerobic bacteria, *E. coli* and total coliforms appeared to be similar or larger than those loosely associated. Loosely associated bacteria can easily transfer from one surface to another leading to increased risk of cross contamination, whereas tightly associated bacteria are more likely to survive washing and disinfection steps during processing. While the significance of these bacterial categories is debatable, processing interventions should desirably be able to control both loosely and tightly associated bacteria.

This study found that hot water spraying (HWS) of eviscerated carcasses was effective in reducing mesophilic aerobic bacteria (MAB) but not *Salmonella* or *Campylobacter* on broiler carcasses. Hot water spray followed by chlorine immersion chilling reduced the prevalence of loosely, intermediately, and tightly associated *Salmonella*, but not intermediately or tightly associated *Campylobacter*. Application of HWS at 71°C for 1 min caused undesirable partially cooked carcass appearance.

Bacteria loosely and tightly associated to broiler skin were further quantified by stomaching, swabbing, and grinding. Most mesophilic aerobic bacteria, *E. coli*, and total coliforms were found to be tightly associated with skin and were non recoverable after ten consecutive stomachings or swabbings but could be recovered by grinding.

Hot water dipping, trisodium phosphate dipping, and brushing were more effective when combined than when any single treatment was used alone to decontaminate broiler carcasses. Only the combination of hot water dip and trisodium phosphate dip, with (TSP/HWD/B) or without (TSP/HWD) brushing, significantly ($P < 0.05$) reduced *Salmonella* prevalence on

carcasses. Greater reductions using the combined treatments (TSP/HWD and TSP/HWD/B) was also observed for MAB, *E. coli* and total coliforms. These combination treatments also penetrated deeper into the stratum compactum of the skin dermis, indicating activity against bacteria hidden in deep crevices and cracks of the dermal layer. However, these combination treatments (TSP/HWD and TSP/HWD/B) resulted in some changes in broiler skin color, especially an increase in yellowness, which was the most prominent change.

A broiler skin model was prepared and exposed to GFP tagged *Salmonella* mimicking bacterial contamination on natural skin. Results indicated that inoculated *Salmonella* on the model skin surface, unlike naturally occurring bacteria, were not associated tightly in large numbers after up to 48 h of exposure at 4°C. Therefore, 48 h exposure at 4°C is not sufficient for tight association of *Salmonella*, which might take months to associate tightly to broiler skin under natural farm conditions. The extent of association is also dependent on bacterial species and strain. While stomaching was able to remove most bacteria from broiler skin, some cells were pushed into deeper locations in the skin. Confocal images showed that deep water filled crevices held free-floating bacteria. Hence, processing water appears to be a medium that can trap bacteria in deeper locations inside crevices. Structural conformations of broiler skin that lead to water retention might also be more significant in holding bacteria in crevices than bacterial or skin surface properties.

There is need to optimize treatments, especially TSP, as tested in this study to reduce issues related to wastewater management and equipment corrosion while preserving antimicrobial activity and product quality. Previously, our lab demonstrated that a VitB1 derivative, named thiamine dilauryl sulphate (TDS), had synergistic activity with TSP against *Salmonella* in broth culture (unpublished data). Application of TDS to TSP and hot water might

be one way to reduce the TSP concentration while still maintaining the antimicrobial efficacy of the combined treatment.

Further research is needed to improve methodology for sampling of tightly associated bacteria. Methods that involve sampling of entire carcass skin along with skin shredding need to be developed. Cross contamination from one sample to other might be a key concern in equipment designed to shred the skin. Developing economic disposable sample holding units in such equipment can be one solution.

Effect of brushing when used in combination with TSP and hot water was not sufficiently elucidated in the current study due to the low level of contamination on broiler carcasses. The current study needs to be repeated using highly contaminated or inoculated carcasses to improve resolution. While brushing is expected to remove tightly associated bacteria, it can also be a source of cross contamination when used in large-scale industrial settings. Further research is needed to develop brush decontamination strategies to reduce the cross contamination risk associated with the process.

Despite the consumer perception that small-scale, pastured poultry production systems produce safer, healthier products than conventional poultry system, the limited data that exists on the microbial safety of small-scale, pastured poultry suggests (i) these systems do not produce broilers with lower foodborne pathogen loads, and (ii) by-products from on-farm processing are contaminated with foodborne pathogens at levels that present a risk of cross-contamination to farm environment. Research is needed to improve safety of on-farm poultry processing by developing appropriate pathogen reduction treatments during scalding, chilling and by-product, wastewater and offal, disposal.

Genetic barcode tagged *Salmonella* isolates have been used in attempt to elucidate the routes of *Salmonella* contamination at the farm and in processing plant. Such studies can also improve our current understanding about the contribution of the farm environment and the length of time needed for bacteria to tightly associate to broiler skin.

With next generation sequencing becoming cheaper and easier to perform, underlying genetic differences between loosely and tightly associated bacterial isolates can help understand “if” and “what” genetic factors contribute to tight association.

BIBLIOGRAPHY

BIBLIOGRAPHY

- ACMSF (Advisory Committee on the Microbiological Safety of Food). 2005. Second Report on Campylobacter. Food Standards Agency, London.
- Allen, V. M., D. B. Tinker, M. H. Hinton, and C. M. Wathes. 2003. Dispersal of micro organisms in commercial defeathering systems. *British Poultry Science*. 44:53-59.
- Allen, V.M., S.A. Bull, J.E.L. Corry, G. Domingue, F. Jørgensen, J.A. Frost, R. Whyte, A. Gonzalez, N. Elviss, and T.J. Humphrey. 2007. Campylobacter spp. contamination of chicken carcasses during processing in relation to flock colonization. *International Journal of Food Microbiology* 113:54-61.
- Alonso-Hernando, A., C. A.-Calleja, and R. Capita. 2015. Effect of various decontamination treatments against gram-positive bacteria on chicken stored under differing conditions of temperature abuse. *Food Control* 47:71-76.
- Altekruse, S.F., M. E. Berrang, H. Marks, B. Patel, W. K. Shaw, Jr., P. Saini, P. A. Bennett, and J. S. Bailey. 2009. Enumeration of *Escherichia coli* cells on chicken carcasses as a potential measure of microbial process control in a random selection of slaughter establishments in the United States. *Applied and Environmental Microbiology* 75:3522-3527.
- Altekruse, S.F., N.J. Stern, P.I. Fields, D.L. Swerdlow. 1999. *Campylobacter jejuni* - an emerging foodborne pathogen. *Emerging Infectious Diseases* 5:28-35.
- Alter, T., F. Gaull, A. Froe, and K. Fehlhaber. 2005. Distribution of *Campylobacter jejuni* strains at different stages of a turkey slaughter line. *Food Microbiology* 22:345-351.
- Araujo, E. A., N. J. Andrade, L. H. M. Silva, A. F. Carvalho, C. A. Silva, A. M. Ramos. 2010. Control of microbial adhesion as a strategy for food and bioprocess technology. *Food Bioprocess Technology* 3:321-332.
- Arnold, J. W., and G. W. Bailey. 2000. Surface finishes on stainless steel reduce bacterial attachment and early biofilm formation: scanning electron and atomic force microscopy study. *Poultry Science* 79:1839-1845.

- Arnold, J. W., D. H. Boothe, O. Suzuki, and G. W. Bailey. 2004. Multiple imaging techniques demonstrate the manipulation of surfaces to reduce bacterial contamination and corrosion. *Journal of Microscopy* 216:215–221.
- Arritt, F.M., J. D. Eifert, M. D. Pierson and S. S. Sumner. 2002. Efficacy of antimicrobials against *Campylobacter jejuni* on chicken breast skin. *Journal of Applied Poultry Research* 11:358-366.
- Avans J. S., S. N. Albright, A. S. Morton, B. E. Prewitt, P. A. Kendall, and J. N. Sofos. 2002. Destruction of microorganisms on chicken carcasses by steam and boiling water immersion. *Food Control* 13:445-450.
- Avens, J. S., and B. F. Miller. 1970. Quantifying bacteria on poultry carcass skin. *Poultry Science* 49:1309-1315.
- Bautista, D., N. Sylvester, S. Barbut, and M. Griffiths. 1997. The determination efficacy of antimicrobial rinses on turkey carcasses using response surface designs. *International Journal of Food Microbiology* 34:279-292.
- Benedict R. C., F. J. Schultz, and S. B. Jones. 1991. Attachment and removal of *Salmonella* spp. on meat and poultry tissues. *Journal of Food Safety* 11:135-148.
- Berrang, M.E., and J.S. Bailey. 2009. On-line brush and spray washers to lower numbers of *Campylobacter* and *Escherichia coli* and presence of *Salmonella* on broiler carcasses during processing. *Journal of Applied Poultry Science* 18:74-78.
- Berrang, M. E., J. A. Dickens, and M. T. Musgrove. 2000. Effects of hot water application after defeathering on the levels of *Campylobacter*, coliform bacteria, and *Escherichia coli* on broiler carcasses. *Poultry Science* 79:1689-1693.
- Berrang, M. E., N. A. Cox, and B. B. Oakley. 2014. Sampling by sponge wipe or skin excision for recovery of inoculated *Salmonella* and *Campylobacter* from defeathered broiler carcasses. *Journal of Food Protection* 77:824-827.
- Berrang, M. E., R. J. Buhr, J. A. Cason, and J. A. Dickens. 2001. Broiler carcass contamination with *Campylobacter* from feces during defeathering. *Journal of Food Protection* 64:2063-2066.

- Bolder, N.M. 1997. Decontamination of meat and poultry carcass. Trends in Food Science and Technology 8:221-227.
- Bolton, D. J., H. Meredith, D. Walsh, and D. A. McDowell. 2014. The effect of chemical treatments in laboratory and broiler plant studies on the microbial status and shelf-life of poultry. Food Control 36:230-237.
- Boyd, R. D., J. Verran, M. V. Jones, and M. Bhakoo. 2002. Use of atomic force microscope to determine the effect of substratum surface topography on bacteria adhesion. Langmuir 18:2343-2346.
- Boysen, L., and H. Rosenquist. 2009. Reduction of thermotolerant *Campylobacter* species on broiler carcasses following physical decontamination at slaughter. Journal of Food Protection 72:497-502.
- Boysen, L., N. S. Wechter, and H. Rosenquist. 2013. Effects of decontamination at varying contamination levels of *Campylobacter jejuni* on broiler meat. Poultry Science 92:1425-1429.
- Bryan, F. L., and M. P. Doyle. 1995. Health risks and consequences of *Salmonella* and *Campylobacter jejuni* in raw poultry. J. of Food Prot. 58(3):326-344.
- Buhr, R.J., D. V. Bourassa, J. K. Northcutt, A. Hinton, Jr., K. D. Ingram, and J. A. Cason. 2005. Bacteria Recovery from Genetically Feathered and Featherless Broiler Carcasses after Immersion Chilling. Poultry Science 84:1499-1504.
- Burnett, S. L., and L. R. Beuchat. 2002. Comparison of methods for fluorescent detection of viable, dead, and total *Escherichia coli* O157: H7 cells in suspensions and on apples using confocal scanning laser microscopy following treatment with sanitizers. International journal of food microbiology 74:37-45.
- Capita, R., C. A. Calleja, M. C. G. Fernández, and B. Moreno. 2002a. Activity of trisodium phosphate compared with sodium hydroxide wash solutions against *Listeria monocytogenes* attached to chicken skin during refrigerated storage. Food Microbiology 19:57-63.

- Capita, R., C.A. Calleja, M.C.G. Fernandez, and B. Moreno. 2002b. Review: Trisodium Phosphate (TSP) treatment for decontamination of poultry. *Food Science and Technology International* 8:11-24.
- Capita, R., M. Prieto, and C. Alonso-Calleja. 2004. Sampling methods for microbiological analysis of red meat and poultry carcasses. *Journal of Food Protection* 67:1303-1308.
- Cason, J. A., A. Hinton Jr, and K. D. Ingram. 2000. Coliforms, *Escherichia coli*, and *Salmonellae* concentrations in a multiple-tank, counterflow poultry scalding. *Journal of Food Protection* 63:1184-1188.
- CDC (Centers for Disease Control and Prevention), Agency for toxic substances and disease registry (ATSDR). Division of toxicology. 1997. Chloroform. Accessed on Feb 21, 2013 from <http://www.atsdr.cdc.gov/toxprofiles/tp6-c1.pdf>
- CDC (Center for Disease Control and Prevention). FoodNet Report. 2012. Accessed on June 7 2015 from http://www.cdc.gov/foodnet/PDFs/2012_annual_report_508c.pdf
- CDC. 2014. Food Safety Progress Report. Accessed on Feb 24, 2016 from <http://www.cdc.gov/foodnet/trends/tables-2014.html>
- Chain, A., E. Arnaud, A. Kondjoyan, A. Collignan and S. Sarter. 2013. Effect of steam and lactic acid treatments on the survival of *Salmonella* Enteritidis and *Campylobacter jejuni* inoculated on chicken skin. *International Journal of Food Microbiology* 162:276-282.
- Chantarapanont, W., M. Berrang, and J. F. Frank. 2003. Direct microscopic observation and viability determination of *Campylobacter jejuni* on chicken skin. *Journal of Food Protection* 66:2222-2230.
- Chantarapanont, W., M. Berrang, and J. F. Frank. 2004. Direct microscopic observation of viability of *Campylobacter jejuni* on chicken skin treated with selected chemical sanitizing agents. *Journal of Food Protection* 67:1146-1152.
- Cherrington, C. A., V. Allen, and M. Hinton. 1992. The influence of temperature and organic matter on the bactericidal activity of short-chain organic acids on salmonellas. *Journal of Applied Bacteriology* 72:500-503.

- Christensen, K. D., N. G. Zimmermann, C. L. Wyatt, T. N. Goodman, R. J. Buhr, and P. Twining. 1994. Dietary and environmental factors affecting skin strength in broiler chickens. *Poultry Science* 73:224-235.
- Clouser, C. S., S. Doores, M. G. Mast, and S. J. Knabel. 1995. The role of defeathering in the contamination of turkey skin by *Salmonella* species and *Listeria monocytogenes*. *Poultry Science* 74:723-731.
- Colin P. and Salvat G. 1996. Decontamination of poultry carcasses using trisodium phosphate treatment. In: Hinton M.H. and Rowlings C. (eds.), Factors affecting the microbial quality of meat, Vol. 4. Microbial methods for the meat industry. Concerted Action CT94-1456. Bristol: University of Bristol Press. pp. 227–237.
- Cook, A., J. Odumeru, S. Lee, and F. Pollari. 2012. *Campylobacter*, *Salmonella*, *Listeria monocytogenes*, Verotoxigenic *Escherichia coli*, and *Escherichia coli* prevalence, enumeration, and subtypes on retail chicken breasts with and without skin. *Journal of Food Protection* 75:34-40.
- Corry, J. E. L., and H. I. Atabay. 2001. Poultry as a source of *Campylobacter* and related organisms. *Journal of Applied Microbiology* 90:96S-114S.
- Corry, J. E. L., S. J. James, G. Purnell, C. S. Barbedo-Pinto, Y. Chochois, M. Howell, and C. James. 2007. Surface pasteurization of chicken carcasses using hot water. *Journal of Food Engineering* 79:913-919.
- Cox, N. A., A. J. Mercuri, B. J. Juven, J. E. Thomson, and V. Chow. 1974a. Evaluation of succinic acid and heat to improve the microbiological quality of poultry meat. *Journal of Food Science* 39:985-987.
- Cox, N. A., A. J. Mercuri, J. E. Thomson, and D. W. Gregory Jr. 1974b. Quality of broiler carcasses as affected by hot water treatments. *Poultry Science* 53:1566-1571.
- Cox, N. A., A. J. Mercuri, J. E. Thomson, and V. Chew. 1976. Swab and excised tissue sampling for total and *Enterobacteriaceae* counts of fresh and surface-frozen broiler skin. *Poultry Science* 55:2405-2408.
- Cox, N. A., A. J. Mercuri, D. A. Tanner, M. O. Carson, J. E. Thomson, and M. S. Bailey. 1978. Effectiveness of sampling methods for *Salmonella* detection on processed broilers. *Journal of Food Protection* 41:341-343.

- Cox, N. A., L. J. Richardson, J. A. Carson, R. J. Ruhr, Y. Vizzier-Thaxton, D. P. Smith, P. J. Fedorka-Cray, C. P. Romanenghi, L. V. B. Romanenghi, and M. P. Doyle. 2010. Comparison of neck skin excision and whole carcass rinse sampling methods for microbiological evaluation of broiler carcasses before and after immersion chilling. *Journal of Food Protection* 73:976-980.
- D'aoust, J. Y., P. Stotland, and A. Boville. 1982. Sampling methods for detection of *Salmonella* in raw chicken carcasses. *Journal of Food Science* 5:1643-1644.
- Davis, M.A., M. Singh, and D.E. Conner. 2010. Poultry-borne pathogens: Plant considerations. Pages 175-203 in *Poultry Meat Processing*. C.M. Owens, C.Z. Alvarado, and A.R. Sams, eds. CRC Press Taylor & Francis Group, Boca Raton, FL.
- De Ledesma, A. M. R., H. P. Riemann, and T. B. Farver. 1996. Short-time treatment with alkali/or hot water to remove common pathogenic and spoilage bacteria from chicken wing skin. *Journal of Food Protection* 59:746-750.
- del Río, E., M. Panizo-Morán, Prieto, C. Alonso-Calleja, and R. Capita. 2007. Effect of various chemical decontamination treatments on natural microflora and sensory characteristics of poultry. *International Journal of Food Microbiology* 115:268-280.
- Dickens, J. A., and A. D. Whittemore. 1994. The effect of acetic acid and air injection on appearance, moisture pickup, microbiological quality, and *Salmonellae* incidence on processed poultry carcasses. *Poultry Science* 73:582-586.
- Dickens, J.A., and A. D. Whittemore. 1995. The effects of extended chilling times with acetic on the temperature and microbiological quality of processed poultry carcasses. *Poultry Science* 74:1044-1048.
- Dickens, J. A., and A. D. Whittemore, 1997. Effects of Acetic acid and Hydrogen Peroxide application during defeathering on the microbiological quality of broiler carcasses prior to evisceration. *Poultry Science* 76:657-660.
- Dickens, J.A., B.G. Lyon, A.D. Whittemore and C.E. Lyon. 1994. The effect of an acetic acid dip on carcass appearance, microbiological quality, and cooked breast meat texture and flavor. *Poultry Science* 73:576-581.

- Dickins, M. A., S. Franklin, R. Stefanova, G. E. Shutze, K. D. Eissenach, I. Wesley, and D. Cave. 2002. Diversity of *Campylobacter* isolates from retail poultry carcasses and from humans as demonstrated by pulsed-field gel electrophoresis. *Journal of Food Protection* 65:957-962.
- Dincer, A.H., and T. Baysal. 2004. Decontamination techniques of pathogen bacteria in meat and poultry. *Critical Reviews in Microbiology* 30:197-204.
- Dunnick, J.W., and R.L. Melnick. 1993. Assessment of the carcinogenic potential of chlorinated water: experimental studies of chlorine, chloramines, and trihalomethanes. *Journal of National Cancer Institute* 85:817-822.
- EFSA panel on Biological Hazards (BIOHAZ). 2010. Scientific opinion on quantification of the risk posed by broiler meat to human *campylobacteriosis* in the EU. *EFSA J.* 8:1437. doi:10.2903/j.efsa.2010.1437.
- Ellerbroek, L.I., J.-A. Lienau, and G. Klein. 2010. *Campylobacter* spp. in Broiler Flocks at Farm Level and the Potential for Cross-Contamination During Slaughter. *Zoonoses and Public Health* 57:e81-88.
- Estrela, C., C. R. A. Estrela, E. L. Barbin, J. C. E. Spano, M. A. Marchesan, and J. D. Pecora. 2002. Mechanism of action of sodium hypochlorite. *Brazilian Dental Journal* 13:113-117.
- European Food Safety Authority (EFSA). 2008. A quantitative microbiological risk assessment on *Salmonella* in meat: source attribution for human *Salmonellosis* from meat. *The EFSA Journal* 625:1-32. Accessed on June 7 2015 from <http://www.efsa.europa.eu/en/scdocs/doc/625.pdf>
- Fabrizio, K. A., R. R. Sharma, A. Demirci, and C. N. Cutter. 2002. Comparison of electrolyzed oxidizing water with various antimicrobial interventions to reduce *Salmonella* species on poultry. *Poultry Science* 81:1598-1605.
- FAO/WHO. 2009. Microbiological Risk Assessment, Series 19. *Salmonella* and *Campylobacter* in chicken meat. Meeting Report. Accessed on Feb 24, 2016 from <http://www.who.int/foodsafety/publications/micro/MRA19.pdf>
- Firstenberg-Eden, R. 1981. Attachment of bacteria to meat surfaces: a review. *Journal of Food Protection* 44:602-607.

- Gast, R. K. 1997. *Salmonella* infections / paratyphoid infections. Pages 97–121 in Diseases of Poultry. 10th ed. B. W. Calnek, H. J. Barnes, C. W. Beard, L. R. McDougald, Y. M. Saif, ed. Iowa State Univ. Press, Ames.
- Ghafir, Y., and G. Daube. 2008. Comparison of swabbing and destructive methods for microbiological pig carcass sampling. *Letters in Applied Microbiology* 47: 322-326.
- Gill, C. O., M. Badoni, L. F. Moza, S. Barbut, and M. W. Griffiths. 2005. Microbiological sampling of poultry carcass portions by excision, rinsing, or swabbing. *Journal of Food Protection* 68:2718-2720.
- Göksoy, E. O., C. James, J. E. L. Corry, and S. J. James. 2001. The effect of hot-water immersions on the appearance and microbiological quality of skin-on chicken breast pieces. *International Journal of Food Science and Technology* 36:61-69.
- Goksoy, E. O., S. Kirkan, and F. Kok. 2004. Micro- biological quality of broiler carcasses during processing in two slaughterhouses in Turkey. *Poultry Science* 83:1427-1432.
- Gorman, B. M., J. N. Sofos, J. B. Morgan, G. R. Schmidt, and G. C. Smith. 1995. Evaluation of hand-trimming, various sanitizing agents, and hot water spray-washing as decontamination interventions for beef brisket adipose tissue. *Journal of Food Protection* 58:899-907.
- Guerin, M.T., C. Sir, J. M. Sargeant, L. Waddell, A. M. O'Connor, R. W. Wills, R. H. Bailey, and J. A. Byrd. 2010. The change in prevalence of *Campylobacter* on chicken carcasses during processing: A systematic review. *Poultry Science* 89:1070-1084.
- Haake, A., G. A. Scott, and K. A. Holbrook. 2001. Structure and function of the skin: overview of the epidermis and dermis. In R. K. Freinkel, & D. T. Woodley (Eds.), *The biology of the skin* (pp. 19-45). New York. CRC Press.
- Hannah, J. F., D. L. Fletcher, N. A. Cox, D. P. Smith, J. A. Cason, J. K. Northcutt, L. J. Richardson, and R. J. Buhr. 2008. Effect of sand and shaking duration on the recovery of aerobic bacteria, coliforms, and *Escherichia coli* from prechill broiler whole carcass rinsates. *Journal of Applied Poultry Research* 17:272-277.

- Hannah, J. F., J. A. Cason, J. R. Richardson, N. A. Cox, A. Hinton Jr., R. J. Buhr and D. P. Smith. 2011. Effect of stomaching on numbers of bacteria recovered from chicken skin. *Poultry Science* 90:491-493.
- Hathcox, A. K., C. A. Hwang, A. V. A. Resurreccion, and L. R. Beuchat. 1995. Consumer evaluation of raw and fried chicken after washing in trisodium phosphate or lactic acid/sodium benzoate solutions. *Journal of Food Science* 60:604-605.
- Hayama, Y., T. Yamamoto, F. Kasuga and T. Tsutsui. 2011. Simulation Model for *Campylobacter* Cross-Contamination During Poultry Processing at Slaughterhouses. *Zoonoses and Public Health* 58:399-406.
- Hinton, A., and J. A. Cason. 2008. Bacterial flora of processed broiler chicken skin after successive washings in mixtures of potassium hydroxide and lauric acid. *Journal of Food Protection* 71:1707-1713.
- Hoffmann, S., P. Fischbeck, A. Krupnick, and M. McWilliams. 2007. Using expert elicitation to link foodborne illnesses in the United States to foods. *Journal of Food Protection* 70:1220-1229.
- Izat, A. L., F. A. Gardner, J. H. Denton, and F. A. Golan. 1988. Incidence and level of *Campylobacter jejuni* in broiler processing. *Poultry Science* 67:1568-1572.
- Izat, A.L., M. Colbert, M.H. Adams, M.A. Reiber, and P.W. Waldroup. 1989. Production and processing studies to reduce the incidence of *Salmonella* on commercial broilers. *Journal of Food Protection* 52:670-673.
- Izat, A. L., W. Yamaguchi, S. Kaniawati, J. P. McGinnis, S. G. Raymond, R. E. Hierholzer, and J. M. Kopek. 1991. Use of consecutive carcass rinses and a most probable number procedure to estimate salmonellae contamination of inoculated broilers. *Poultry Science* 70:1448-1451.
- James, C., C. Vincent, T. I. de Andrade Lima, and S. J. James. 2006. The primary chilling of poultry carcasses-a review. *International Journal of Refrigeration* 29:847-862.
- James, C., E. O. Goksoy, J. E. L. Corry, and S. J. James. 2000. Surface pasteurization of poultry meat using steam at atmospheric pressure. *Journal of Food Engineering* 45:111-117.

- James, C., S. J. James, N. Hannay, G. Purnell, C. Barbedo-Pinto, H. Yaman, and M. Araujo., M Gonzalez, L., J. Calvo, M. Howell, and J. E. L. Corry. 2007. Decontamination of poultry carcasses using steam or hot water in combination with rapid cooling, chilling or freezing of carcass surfaces. *International Journal of Food Microbiology* 114:195-203.
- James, W.O., R.L. Brewer, J.C. Prucha, and W.O. Williams. 1992. Effects of chlorination of chill water on the bacteriological profile of raw chicken carcasses and giblets. *Journal of American Veterinary Medical Association* 200:60-63.
- Jang, K.I., M.G. Kim, S.D. Ha, K.S. Kim, K.H. Lee, D.H. Chung, C.H. Kim, and K.Y. Kim. 2007. Morphology and adhesion of *Campylobacter jejuni* to chicken skin under varying conditions. *Journal of Microbiology and Biotechnology* 17:202-206.
- Jeong, J. Y., K. K. Janardhanan, A. M. Booren, D. M. Karcher, and I. Kang. 2011. Moisture content, processing yield, and surface color of broiler carcasses chilled by water, air, or evaporative air. *Poultry Science* 90:687-693.
- Kasschau, M. R., M. P. Byam-Smith, D. S. Gentry, and F. N. Watson. 1995. Influence of pH and temperature on hemolysis by adult *Schistosoma mansoni* membranes. *Journal of Experimental Zoology* 271:315-322.
- Kegode, R.B., D.K. Doetkott, M.L. Khaita, and I.V. Wesley. 2008. Occurrence of *Campylobacter* species, *Salmonella* species and generic *Escherichia coli* in meat products from retail outlets in the fargo metropolitan area. *Food Safety* 28:111-125.
- Kim, J. W., and M. F. Slavik. 1996a. Cetylpyridinium chloride (CPC) treatment on poultry skin to reduce attached *Samonella*. *Journal of Food Protection* 59:322-326.
- Kim, J. W., M. F. Slavik, F. G. Bender, 1994. Removal of *Salmonella* Typhimurium attached to chicken skin by rinsing with trisodium phosphate solution: scanning electron microscopic examination. *Journal of Food Safety* 14:77-84.
- Kim, K. Y., H. S. Lillard, J. F. Frank, and S. E. Craven. 1996b. Attachment of *Salmonella* Typhimurium to poultry skin as related to cell viability. *Journal of Food Science* 61:439-441.
- Kim, K.Y., J. F. Frank, and S. E. Craven. 1996c. Three-dimensional visualization of *Salmonella* attachment to poultry skin using confocal scanning laser microscopy. *Letters in Applied*

Microbiology 22:280-282.

- Kondjoyan, A., and S. Portanguen. 2008. Effect of superheated steam on the inactivation of *Listeria innocua* surface-inoculated onto chicken skin. *Journal of Food Engineering* 87:162-171.
- Koolman, L. P. Whyte, J. Meade, J. Lyng, and D. Bolton. 2014. A combination of chemical and ultrasonication treatments to reduce *Campulobacter jejuni* on raw poultry. *Food Bioprocess Technology* 7:3602-3607.
- Korber, D. R., A. Choi, G. M. Wolfaardt, S. C. Ingham, and D. E. Caldwell. 1997. Substratum topography influences susceptibility of *Salmonella enteritidis* biofilms to Trisodium Phosphate. *Applied and Environmental Microbiology* 63:3352-3358.
- Kotula, K.L, and Y. Pandya. 1995. Bacterial contamination of broiler chickens before scalding. *Journal of Food Protection* 58:1326-1329.
- Kramer, J. M., J. A. Frost, F. J. Bolton, and D. R. A. Wareing. 2000. *Campylobacter* contamination of raw meat and poultry at retail sale: identification of multiple types and comparison with isolates from human infection. *Journal of Food Protection* 63:1654-1659.
- Lahellec, C., and P. Colin. 1985. Relationship between serotypes of *Salmonellae* from hatcheries and rearing farms and those from processed poultry carcasses. *British Poultry Science* 26:179-186.
- Lecompte, J-Y, A. Kondjoyan, S. Sarter, S. Portanguen, and A. Collignan. 2008. Effects of steam and lactic acid treatments on inactivation of *Listeria innocua* surface-inoculated on chicken skin. *International Journal of Food Microbiology* 127:155-161.
- Lee, N. Y., S. Y. Park, I. S. Kang, and S. D. Ha. 2014. The evaluation of combined chemical and physical treatments on the reduction of resident microorganisms and *Salmonella* Typhimurium attached to chicken skin. *Poultry Science* 93:208-215.
- Liao, CH and Cooke PH. 2001. Response to trisodium phosphate treatment of *Salmonella* Chester attached to fresh-cut green pepper slices. *Canadian Journal of Microbiology* 47:25-32.

- Li, J. W., Z. Yu, Z. Cai, M. Gao, and F. Chao. 1996. Trihalomethanes formation in water treated with chlorine dioxide. *Water Research* 30:2371-2376.
- Lillard, H. S. 1986. Role of fimbriae and flagella in the attachment of *Salmonella typhimurium* to poultry skin. *Journal of Food Science* 51:54-56.
- Lillard, H. S. 1988. Comparison of sampling methods and implications for bacterial decontamination of poultry carcasses by rinsing. *Journal of Food Protection* 51:405-408.
- Lillard, H. S. 1989a. Factors affecting the persistence of *Salmonella* during the processing of poultry. *Journal of Food Protection* 52:829-832.
- Lillard, H. S. 1989b. Incidence and recovery of Salmonellae and other bacteria from commercially processes poultry carcasses at selected pre- and post-evisceration sites. *Journal of Food Protection* 52:88-91.
- Lillard, H. S. 1990. The impact of commercial processing procedures on the bacterial contamination and cross-contamination of broiler carcasses. *Journal of Food Protection* 53:202-207.
- Lillard, H. S., L. C. Blankenship, J. A. Dickens, S. E. Craven, and A. D. Shackelford. 1987. Effect of acetic acid on the microbiological quality of scalded, picked and unpicked broiler carcasses. *Journal of Food Protection* 50:112-114.
- Lin, D., M. Yan, S. Lin, and S. Chen. 2014. Increasing prevalence of hydrogen sulfide negative *Salmonella* in retail meats. *Food Control* 43:1-4.
- Ling, S. H. M., X. H. Wang, L. Xie, T. M. Lim, and K. Y. Leung. 2000. Use of green fluorescent protein (GFP) to study the invasion pathways of *Edwardsiella tarda* in in vivo and in vitro fish models. *Microbiology* 146:7-19.
- Li, Y., H. Hang, and B. L. Swem. 2002. Effect of high-temperature inside-outside spray on survival of *Campylobacter jejuni* attached to prechill chicken carcasses. *Poultry Science* 81:1371-1377.
- Li, Y., J-W Kim, M. F. Slavik, C. L. Griffis, J. T. Walker, and H. Wang. 1994. *Salmonella typhimurium* attached to chicken skin reduced using electrical stimulation and inorganic salts. *Journal of Food Science* 59:23-25.

- Li, Y., M. F. Slavik, J. T. Walker, and H. Xiong. 1997. Pre-Chill Spray of Chicken Carcasses to Reduce *Salmonella typhimurium*. *Journal of Food Science* 62:605-607.
- Magnusson. 1982. Hydrophobic interaction-a mechanism of bacterial binding. *Scandinavian Journal of Infectious Diseases Supplementum* 33:32-36.
- Mariott, N.G. 1999. *Principles of Food Sanitation*, 4th edition. Aspen publishing Inc. Gaithersburg, MD.
- Martinez, B., M. F. Celda, B. Anastasio, I. Garcia, and M. C. Lopez-Mendoza. 2010. Microbiological sampling of carcasses by excision or swabbing with three types of sponge or gauze. *Journal of Food Protection* 73:81-87.
- Mazengia, E., M. Samadpour, H. W. Hill, K. Greeson, K. Tenney, G. Liao, X. Huang, and J. S. Meschke. 2014. Prevalence, concentrations, and antibiotic sensitivities of *Salmonella* serovars in poultry from retail establishments in Seattle, Washington. *Journal of Food Protection* 77:885-893.
- McEvoy, J. M., C. W. Nde, J. S. Sherwood, and C. M. Logue. 2005. An evaluation of sampling methods for the detection of *Escherichia coli* and *Salmonella* on turkey carcasses. *Journal of Food Protection* 68:34-39
- McKee, S. 2004. Effective use of chlorine in food processing environments. Auburn University Department of Poultry Science. Accessed on Feb 14, 2013 from <http://www.ag.auburn.edu/poul/virtuallibrary/pdf/mckeeeffectivechlorine.pdf>.
- McMeekin, T. A., and C. J. Thomas. 1978. Retention of bacteria on chicken skin after immersion in bacterial suspensions. *Journal of Applied Microbiology* 45:383-387.
- McMeekin, T. A., C. J. Thomas, and D. McCall. 1979. Scanning electron microscopy of microorganisms on chicken skin. *Journal of Applied Microbiology* 46:195-200.
- McMeekin, T. A., C. J. Thomas, and P. I. Pennington. 1984. Contamination and decontamination of poultry carcass neck tissue. *Journal of Food Safety* 6:79-88.

- Mead, G.C. 2004. Current trends in the microbiological safety of poultry meat. *World's Poultry Science Journal* 60:112-118.
- Medilanski, E., K. Kaufmann, L. Y. Wick, O. Wanner, and H. Harms. 2002. Influence of the surface topography of stainless steel on bacterial adhesion. *Biofouling* 18:193-203.
- Meredith, H., D. McDowell, and D. J. Bolton. 2013. An evaluation of trisodium phosphate, citric acid, and lactic acid cloacal wash treatments to reduce *Campylobacter*, total viable counts (TVC) and total enterobacteriaceae counts (TEC) on broiler carcasses during processing. *Food Control* 32:149-152.
- Miwa, N., Y. Takegahara, K. Terai, H. Kato, and T. Takeuchi. 2003. *Campylobacter jejuni* contamination on broiler carcasses of *C. jejuni*-negative flocks during processing in a Japanese slaughterhouse. *International Journal of Food Microbiology* 84:105-109.
- Moore, G., I. S. Blair, and D. A. McDowell. 2007. Recovery and transfer of *Salmonella* Typhimurium from four different domestic food contact surfaces. *Journal of Food Protection* 70:2273-2280.
- Morild, R. K., J. E. Olsen, and S. Aabo. 2011. Change in attachment of *Salmonella* Typhimurium, *Yersinia enterocolitica*, and *Listeria monocytogenes* to pork skin and muscle after hot water and lactic acid decontamination. *Food Control* 145:353-358.
- Morris, C. A., L. M. Lucia, J. W. Savell, and G. R. Acuff. 1997. Trisodium phosphate treatment of pork carcasses. *Journal of Food Science* 62:402-403.
- Morrison, G. J., and G. H. Fleet. 1985. Reduction of *Salmonella* on chicken carcasses by immersion treatments. *Journal of Food Protection* 48:939-943.
- Mountney, G.J., and J. O'Malley. 1965. Acids as poultry meat preservatives. *Poultry Science* 44:582-586.
- Mulder R. W. A. W., L. W. J. Dorrestein, and J. Van Der Broek. 1978. Cross contamination during the scalding and plucking of broilers. *British Poultry Science* 190:61-70.
- Nassar, T.J., A. S. Al-Mashhadi, A. K. Fawal, and A. F. Shalhat. 1997. Decontamination of chicken carcasses artificially contaminated with *Salmonella*. *Revue Scientifique Technique (International Office of Epizootics)* 16:891-897.

- National Advisory Committee on Microbiological Criteria for Foods. 1997. Generic HACCP application in broiler slaughter and processing. *Journal of Food Protection* 60:579–604.
- National Antimicrobial Resistance Monitoring System (NARMS) Retail Meat Report 2011. FDA. Accessed on Feb 24, 2016 from <http://www.fda.gov/downloads/AnimalVeterinary/SafetyHealth/AntimicrobialResistance/NationalAntimicrobialResistanceMonitoringSystem/UCM334834.pdf>
- National Antimicrobial Resistance Monitoring System (NARMS) Integrated Report 2012-2013. Accessed on Feb 24, 2016 from <http://www.fda.gov/downloads/AnimalVeterinary/SafetyHealth/AntimicrobialResistance/NationalAntimicrobialResistanceMonitoringSystem/UCM453398.pdf>
- Nayak, R., P.B. Kenney, and G.K. Bissonnette. 2001. Inhibition and reversal of *Salmonella* Typhimurium attachment to poultry skin using zinc chloride. *Journal of Food Protection* 64:456-461.
- Northcutt, J., D. Smith, K. D. Ingram, A. Jr Hinton, and M. Musgrove. 2007. Recovery of bacteria from broiler carcasses after spray washing with acidified electrolyzed water or sodium hypochlorite solutions. *Poultry Science* 86:2239-2244.
- Northcutt, J. K., J. A. Cason, D. P. Smith, R. J. Buhr, and D. L. Fletcher. 2006. Broiler carcass bacterial counts after immersion chilling using either a low or high volume of water. *Poultry Science* 85:1802-1806.
- Notermans, S., and E. H. Kampelmacher. 1974. Attachment of some bacterial strains to the skin of broiler chickens. *British Poultry Science* 15:573-585.
- Notermans, S., and E. H. Kampelmacher. 1975. *Heat destruction of some bacterial strains attached to broiler skin*. *British Poultry Science* 16:351-361.
- Notermans, S., E. H. Kampelmacher, and M. Van Schothorst. 1975. Studies on sampling methods used in the control of hygiene in poultry processing. *Journal of Applied Microbiology* 39:55-61.
- Notermans, S., F. M. Van Leusden, and M. V. Schothorst. 1977. Suitability of different bacterial groups for determining faecal contamination during post scalding stages in the processing

- of broiler chickens. *Journal of Applied Bacteriology*. 43:383-389.
- OECD/FAO. 2014. OECD-FAO Agricultural Outlook 2014. DOI 10.1787/agr_outlook-2014-en, p-175. Retrieved on Jan 8, 2015 from [https://www.embrapa.br/documents/1024963/1025740/OECD-FAO Agricultural Outlook 2014-2023/20082926-0f88-4159-970a-2a1c65795c47](https://www.embrapa.br/documents/1024963/1025740/OECD-FAO+Agricultural+Outlook+2014-2023/20082926-0f88-4159-970a-2a1c65795c47)
- Olsen, J.E., D.J. Brown, M. Madsen, and M. Bisgaard. 2003. Cross-contamination with *Salmonella* on a broiler slaughterhouse line demonstrated by use of epidemiological markers. *Journal of Applied Microbiology* 94:826-835.
- Oosterom, J., S. Notermans, H. Karman, and G. B. Engels. 1983. Origin and prevalence of *Campylobacter jejuni* in poultry processing. *Journal of Food Protection* 46:339-344.
- Oyarzabal, O. A., C. Hawk, S. F. Bilgili, C. C. Warf, and G. K. Kemp. 2004. Effects of post-chill application of acidified sodium chlorite to control *Campylobacter* spp. and *Escherichia coli* on commercial broiler carcasses. *Journal of Food Protection* 67:2288-2291.
- Pepperell, R., C. A. Reid, S. N. Solano, M. L. Hutchison, L. D. Walters, A. M. Johnston, and S. Buncic. 2005. Experimental comparison of excision and swabbing microbiological sampling methods for carcasses. *Journal of Food Protection* 68: 2163-2168.
- Peyrat, M.B., C. Soumet, P. Maris, and P. Sanders. 2008. Recovery of *Campylobacter jejuni* from surfaces of poultry slaughterhouses after cleaning and disinfection procedures: Analysis of a potential source of carcass contamination. *International Journal of Food Microbiology* 124:188-194.
- Posch, J., G. Feierl, G. Wuest, W. Sixl, S. Schmidt, Du. Haas, F.F. Reinthaler, and E. Marth. 2006. Transmission of *Campylobacter* spp. in a poultry slaughterhouse and genetic characterisation of the isolates by pulsed-field gel electrophoresis. *British Poultry Science* 47:286-293.
- Purnell, G., C. James, S. J. James, M. Howell, and J. E. L. Corry. 2014. Comparison of acidified sodium chlorite, chlorine dioxide, peroxyacetic acid, and tri-sodium phosphate spray washes for decontamination of chicken carcasses. *Food Bioprocess Technology* 7:2093-2101.
- Purnell, G., K. Mattick, and T. Humphrey. 2004. The use of 'hot wash' treatments to reduce the number of pathogenic and spoilage bacteria on raw retail poultry. *Journal of Food*

Engineering 62:29-36.

- Ramirez, A. J., G. R. Acuff, L. M. Lucia, and J. W. Savell. 2001. Lactic acid and trisodium phosphate treatment of lamb breast to reduce bacterial contamination. *Journal of Food Protection* 64:1439-1441.
- Rasschaert, G., K. Houf, and L. De Zutter. 2007. Impact of the slaughter line contamination on the presence of *Salmonella* on broiler carcasses. *Journal of Applied Microbiology* 103:333-341.
- Rasschaert, G., K. Houf, C. Godard, C. Wildemaue, M. Pastuszcak-Frak, and L. DeZutter. 2008. Contamination of carcasses with *Salmonella* during poultry slaughter. *Journal of Food Protection* 71:146-152.
- Rasschaert, G., K. Houf, J. Van Hende, and L. De Zutter. 2006. *Campylobacter* contamination during poultry slaughter in Belgium. *Journal of Food Protection* 69:27-33.
- Ricke, S. C., M. M. Kunder, D. R. Miller, and J. T. Keeton. 2005. Alternatives to antibiotics: chemical and physical antimicrobial interventions and foodborne pathogen response. *Poultry Science* 84:667-675.
- Rodriguez de Ledesma, A. M., H. P. Riemann, and T. B. Farver. 1996. Short-time treatment with alkali and/or hot water to remove common pathogenic and spoilage bacteria from chicken wing skin. *Journal of Food Protection* 59:746-750.
- Rosenquist, H., H. M. Sommer, N. L. Nielsen, and B. B. Christensen. 2006. The effect of slaughter operations on the contamination of chicken carcasses with thermotolerant *Campylobacter*. *International Journal of Food Microbiology* 108:226-232.
- Rosenquist, H., N. L. Nielsen, H. M. Sommer, B. Norrung, and B. B. Christensen. 2003. Quantitative risk assessment of human campylobacteriosis associated with thermophilic *Campylobacter* species in chickens. *International Journal of Food Microbiology* 83:87-103.
- Russell, Scott M. 2009. Chlorine: Still the most popular sanitizer in the poultry industry. Featured online article, The Poultry Site. Accessed on Feb 24, 2016 from <http://www.thepoultrysite.com/articles/1383/chlorine-still-the-most-popular-sanitizer-in-the-poultry-industry>

- Sakhare, P. Z., N. M. Sachindra, K. P. Yashoda, and D. N. Rao. 1999. Efficacy of intermittent decontamination treatments during processing in reducing the microbial load on broiler chicken carcass. *Food Control* 10:189-194.
- Salvat, P. C., J. C. Allo, S. Fenner, M. J. Laisney, M. T. Toquin, F. Humbert, and P. Colin. 1997. Effects of AvGard™ treatment on the microbiological flora of poultry carcasses. *British Poultry Science* 38:489-498.
- Sampathkumar, B., G. G. Khachatourians, and D. R. Korber. 2004. Treatment of *Salmonella enterica* serovar Enteritidis with a sublethal concentration of trisodium phosphate or alkaline pH induces thermotolerance. *Applied and Environmental Microbiology* 70:4613-4620.
- Sanchez, M. X., W. M. Fluckey, M. M. Brashears, and S. R. McKee. 2002. Microbial profile and antibiotic susceptibility of *Campylobacter* spp. and *Salmonella* spp. in broilers processed in air-chilled and immersion-chilled environments. *Journal of Food Protection* 65:948-956.
- Sarlin, L. L., E. T. Barnhart, D. J. Caldwell, R. W. Moore, J. A. Byrd, D. Y. Caldwell, D. E. Corrier, J. R. Deloach, and B. M. Hargis. 1998. Evaluation of alternative sampling methods for *Salmonella* critical control point determination at broiler processing. *Poultry Science* 77:1253-1257.
- SAS Institute. 2002. SAS User's Guide. SAS Inst. Inc., Cary, NC.
- Scott R. M. 2012. Proper use of chlorine in poultry processing plants. In S. M. Russell (Ed.), *Controlling Salmonella in Poultry Production and Processing*. Page 221. Taylor and Francis Group, FL.
- SCVPH. 1998. Report on benefits and limitations of antimicrobial treatments for poultry carcasses, adopted on 30 October 1998. Retrieved on June7, 2015 from http://ec.europa.eu/food/fs/sc/scv/out14_en.pdf
- Shackelford, A. D., A. D. Whittemore, C. M. Papa, and R. L. Wilson. 1992. Development of a prototype carcass cleaning machine. *Journal of Applied Poultry Research* 1:235-241.

- Shane, S. M. 1997. Campylobacteriosis. Pages 235–245 in Diseases of Poultry. 10th ed. B. W. Calnek, H. J. Barnes, C. W. Beard, L. R. McDougald, Y. M. Saif, ed. Iowa State Univ. Press, Ames.
- Sharpe, A.N., C.I.B. Kingombe, P. Watney, L.J. Parrington, I. Dudas, and M.P. Diotte. 1996. Efficient nondestructive sampler for carcasses and other surfaces. *Journal of Food Protection* 59:757-763.
- Sheng, X., Y. P. Ting, and S. O. Pehkonen. 2008. The influence of ionic strength, nutrients and pH on bacterial adhesion to metals. *Journal of Colloid and Interface Science* 321:256-264.
- Sinde, E. and J. Carballo. 2000. Attachment of *Salmonella* spp. and *Listeria monocytogenes* to stainless steel, rubber and polytetrafluorethylene: the influence of free energy and the effect of commercial sanitizers. *Food Microbiology* 17:439-447.
- Singh, P., H. C. Lee, K. B. Chin, S. D. Ha, and I. Kang. 2015. Quantification of loosely associated and tightly associated bacteria on broiler carcass skin using swabbing, stomaching, and grinding methods. *Poultry Science* 94:3034-3039.
- Sinhamahapatra, M., S. Biswas, A. K. Das, and D. Bhattacharyya. 2004. Comparative study of different surface decontaminants on chicken quality. *British Poultry Science* 45:624-630.
- Snijders, J.M.A., M.H.W. Janssen, G.E. Gerats, and G.P. Corstiaensen. 1984. A comparative study of sampling techniques for monitoring carcass contamination. *International Journal of Food Microbiology* 1:229-236.
- Sofos, J. N. 2008. Challenges to meat safety in the 21st Century. *Meat Technol.* 78:3-13.
- Stepanović, S., D. Vuković, I. Dakić, B. Savić, and M. Švabić-Vlahović. 2000. A modified microtiter-plate test for quantification of staphylococcal biofilm formation. *Journal of microbiological methods* 40:175-179.
- Stopforth, J. D., R. O'Connor, M. Lopes, B. Kotapalli, W. E. Hill, and M. Samadpour. 2007. Validation of individual and multiple-sequential interventions for reduction of microbial populations during processing of poultry carcasses and parts. *Journal of Food Protection* 70:1393-1401.

- Tamblyn, K.C., and D.E. Conner. 1997. Bactericidal activity of organic acids against *Salmonella* Typhimurium attached to broiler chicken skin. *Journal of Food Protection* 60:629-633.
- Tamblyn, K.C., D.E. Conner, and S.F. Bilgili. 1997. Utilization of the skin attachment model to determine the antimicrobial efficacy of potential carcass treatments. *Poultry Science* 76:1318-1323.
- Teotia, J.S., and B.F. Miller. 1975. Destruction of *Salmonella* on poultry meat with lysozyme, EDTA, X-ray, microwave and chlorine. *Poultry Science* 54:1388-1394.
- Thomas, C. J., and T. A. McMeekin. 1980. Contamination of broiler carcass skin during commercial processing procedures: an Electron Microscope study. *Applied and Environmental Microbiology* 40:133-144.
- Thomas, C. J., and T.A. McMeekin. 1982. Effect of water immersion on the microtopography of the skin of chicken carcasses. *Journal of the Science of Food and Agriculture* 33:549-554.
- Thomson, J.E., N.A. Cox, W.K. Whitehead, and A.J. Mercuri. 1974. Effect of hot spray washing on broiler carcass quality. *Poultry Science* 53:946-952.
- Tompkins, NM, JS Avens, PA Kendall, and MD Salman. 2008. Effect of boiling water carcass immersion on aerobic bacteria counts of poultry skin and processed ground poultry meat. *Zoonoses Public Health*. 55:235-241.
- Trimble, L. M., W. Q. Alali, K. E. Gibson, S. C. Ricke, P. Crandall, D. Jaroni, and M. Berrang. 2013. *Salmonella* and *Campylobacter* prevalence and concentration on pasture-raised broilers processed on-farm, in a mobile processing unit, and at small USDA-inspected facility. *Food Control* 34:177-182.
- Tsai, L. S., J.E. Schade, and B. T. Molyneux. 1992. Chlorination of poultry chiller water: chlorine demand and disinfection efficiency. *Poultry Science* 71:188-196.
- Upsher, F.J., and L.E. Fletcher. 1996. Review of chlorine and organohalides and their significance to the Royal Australian Navy. DSTO Aeronautical and Maritime Research Laboratory, Melbourne Victoria. Accessed on Feb 24, 2016 from <http://www.dtic.mil/dtic/tr/fulltext/u2/a311578.pdf>

- USDA ERS. 2000. Food safety efforts accelerated in the 1990s.
<http://webarchives.cdlib.org/sw1tx36512/http://www.ers.usda.gov/publications/foodreview/septdec00/FRsept00h.pdf>
- USDA FSIS. 2002 The use of trisodium phosphate as an antimicrobial agent in poultry processing in the United States. USDA-FSIS, Office of International Affairs. November 2002.
- USDA FSIS. 2006. Safe and suitable ingredients used in the production of meat and poultry products. Accessed on Feb 24, 2016 from,
http://www.fsis.usda.gov/OPPDE/rdad/FSISDirectives/7120.1_Amend_6.pdf?redirecthttp=true
- USDA FSIS (US Department of Agriculture-Food Safety and Inspection Services). 2003. Use of chlorine to treat poultry chilled water.
- Vaclavik, V. A., and E. W. Christian. 2014. Meat, Poultry, Fish, and Dry Beans. In Essentials of Food Science (pp. 133-172). Springer New York.
- Van der Wal, A, W. Norde, A. J. B. Zehnder, and J. Lyklema. 1997. Determination of the total charge in the cell walls of gram-positive bacteria. Colloids and Surfaces Biointerfaces 9:81-100.
- Vellinga A. and Van Loock F. 2002. The dioxin crisis as experiment to determine poultry-related *Campylobacter* enteritis. Emerging Infectious Diseases 8:19-22.
- Venkata, L.P.P., S. Backert, S.L. Vieira, and O.A. Oyarzabal. 2007. Evaluation of Logistic Processing To Reduce Cross-Contamination of Commercial Broiler Carcasses with *Campylobacter* spp. Journal of Food Protection 70:2549-2554.
- Vorst, K. L., and Todd, E. C. D., and E. T. Ryser. 2004. Improved quantitative recovery of *Listeria monocytogenes* from stainless steel surfaces using a one-ply composite tissue. Journal of Food Protection 67:2212-2217.
- Wang, W-C, Y. Li, M. F. Slavik, and H. Xiong. 1997. Trisodium phosphate and cetylpyridinium chloride spraying on chicken skin to reduce attached *Salmonella typhimurium*. Journal of Food Protection 60:992-994.

- Weir, K. A., and C. A. Lunam. 2004. A histological study of emu (*Dromaius novaehollandiae*) skin. *Journal of Zoology* 264:259-266.
- Wempe, J. M., C. A. Genigeorgis, T. B. Farver, and H. I. Yusufu. 1983. Prevalence of *Campylobacter jejuni* in two California chicken processing plants. *Applied and Environmental Microbiology* 45:355-359.
- Whyte, P., J. D. Collins, K. McGill, C. Monahan, and H. O'Mahony. 2001. Quantitative investigation of the effects of chemical decontamination procedures on the microbiological status of broiler carcasses during processing. *Journal of Food Protection* 64:179-183.
- Whyte, P., K. McGill, and J. D. Collins. 2003. An assessment of steam pasteurization and hot water immersion treatments for the microbiological decontamination of broiler carcasses. *Food Microbiology* 20:111-117.
- Wilson, D. L., V. A. Rathinam, W. Qi, L. M. Wick, J. Landgraf, J. A. Bell, A. Plovanich-Jones, J. Parrish, R. L. Finley, L. S. Mansfield, and J. E. Linz. 2010. Genetic diversity in *Campylobacter jejuni* is associated with differential colonization of broiler chickens and C57BL/6J IL10-deficient mice. *Microbiology* 156:2046-2057.
- Wu, Diezhang, W. Q. Alali, M. A. Harrison, and C. L. Hofacre. 2014. Prevalence of *Salmonella* in neck skin and bone of chickens. *Journal of Food Protection* 77:1193-1197.
- Xiong, H., Y. Li, M. F. Slavik, and J. T. Walker. 1998. Spraying chicken skin with selected chemicals to reduce attached *Salmonella typhimurium*. *Journal of Food Protection* 61:272-275.
- Yang, H., Y. B. Li, and M. G. Johnson. 2001. Survival and death of *Salmonella* Typhimurium and *Campylobacter jejuni* in processing water and on chicken skin during poultry scalding and chilling. *Journal of Food Protection* 64:770-776.
- Yang, Z., Y. Li, M. Slavik. 1998. Use of antimicrobial spray applied with an inside-outside birdwasher to reduce bacterial contamination on prechilled chicken carcasses. *Journal of Food Protection* 61:829-832.
- Zhang, L., J. Y. Jeong, K. K. Janardhanan, E. T. Ryser, and I. Kang. 2011. Microbiological quality of water immersion-chilled and air-chilled broilers. *Journal of Food Protection* 74:1531-1535.

- Zhang, L., P. Singh, H.C. Lee, and I. Kang. 2013. Effect of hot water spray on broiler carcasses for reduction of loosely attached, intermediately attached, and tightly attached pathogenic (*Salmonella* and *Campylobacter*) and mesophilic aerobic bacteria. *Poultry Science* 92:804-810.
- Zhang, Q. Q., K. P. Ye, X. L. Xu, G. H. Zhou, and J. X. Cao. 2012. Comparison of excision, swabbing and rinsing sampling methods to determine the microbiological quality of broiler carcasses. *Journal of Food Safety* 32:134-139.
- Zhao, C., B. Ge, J. De Villena, R. Sudler, E. Yeh, S. Zhao, D. G. White, D. W. and J. Meng. 2001. Prevalence of *Campylobacter* spp., *Escherichia coli* and *Salmonella* serovars in retail chicken, turkey, pork and beef from the greater Washington, D.C., Area. *Applied and Environmental Microbiology* 67:5431-5436.