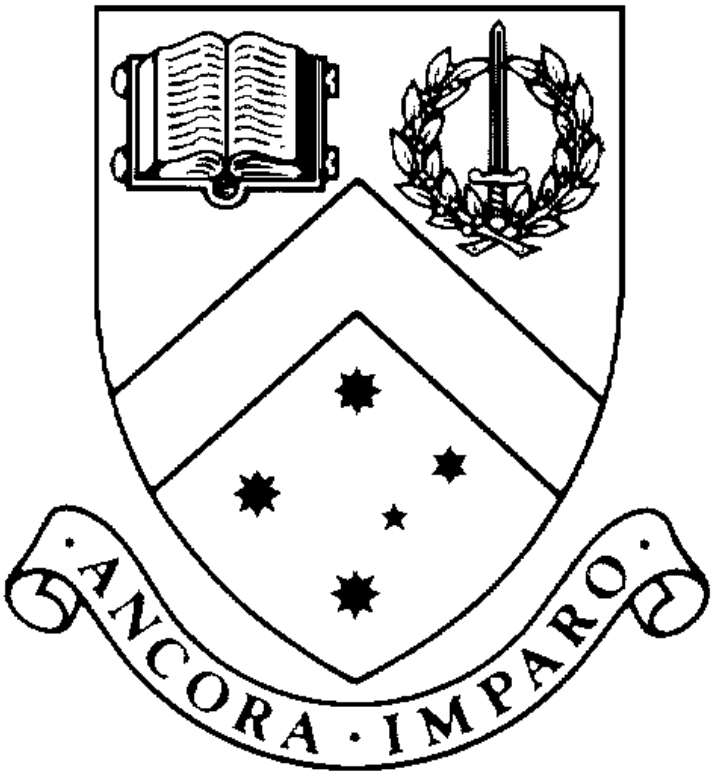

Monash University

Effect of Acids on the Survival of *Salmonella* Attached to Chicken Meat

Sin Mei Tan

A Thesis submitted in fulfillment of the requirement for the degree of
Doctor of Philosophy (PhD)
School of Science
Monash University Malaysia 2014

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Notice 1

Notice 2

General Declaration

In accordance with Monash University Doctorate Regulation 17.2 Doctor of Philosophy and Research Master's Regulations the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes one original paper published in peer reviewed journal and two unpublished manuscripts. The core theme of the thesis is the Effect of Acids on the Survival of *Salmonella* Attached to Chicken Skin and Meat. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of mine, the candidate, working within the School of Science, under the supervision of Professor Gary Dykes.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledgers in the team-based research. In the case of Chapter 2, Chapter 3, Chapter 4 and Chapter 5, my contribution to the work involved the following:

Thesis chapter	**Publication title**	**Publication status**	**Nature and Extent of candidate's contribution**
Chapter 2 and 3	Buffering effect of chicken skin and meat protects *Salmonella enterica* against hydrochloric acid but not organic acid treatment	Published	I was responsible for 100% of data analysis and 90% of drafting and writing.
Chapter 4	Fat contributes to the buffering capacity of chicken skin and meat but enhances the vulnerability of attached *Salmonella* cells to acetic acid treatment	Accepted	I was responsible for 100% of data analysis and 90% of drafting and writing.
Chapter 5	Acetic acid induces pH independent cellular energy depletion in *Salmonella enterica*	Accepted	I was responsible for 100% of data analysis and 90% of drafting and writing.

I have not renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Signed: …….

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Acknowledgements

I would like to express my sincerest appreciation to my supervisors, Professor Gary Dykes and Dr. Sui Mae Lee for the consistent guidance and support. Special thanks to Professor Gary Dykes for his professionalism in scientific research, guidance in writing skills, suggestion and ability to smooth over difficult situations. I could not have asked for a better person to walk me through this PhD process.

I would also like to express my sincerest appreciation to the following people, without whom completing this challenge would be more difficult. Members of my Review Panel committee: Dr. Siow Lee Fong, Dr. Choo Wee Sim, Professor Sadequr Rahman, and Professor Chris Austin for their advices and suggestions. Administrative staff: Ms. Bawani Veeriah, Ms Irdawani B.M. Selamat and Ms. Masitah M. Salleh for their administrative assistance and support. School of Science, Monash University Malaysia for the financial support throughout the 3.5 years of PhD candidature.

Many thanks to the former and current lab mates of Professor Dykes' research team for their kind assistances that made the long hours in the lab fun. Last but not least, I thank my parents and brother for their endless encouragement and support all the way through this process. I couldn't have achieved this degree without the love, encouragement, understanding and support these people offered.

Abstract

Salmonella is one of the most common pathogens of concern on poultry meat and products. Research on effective interventions to reduce *Salmonella* on these products is of significant current research interest. Marination is one such intervention which has been suggested to enhance the safety of meat by inhibiting the growth of microorganisms. The buffering effect of poultry meat may, however, neutralize the acidic pH of marinades and nullify their antimicrobial effects against *Salmonella*. There is limited literature on the influence of buffering effect on the survival of *Salmonella* attached to marinated chicken skin and meat.

The first part of this project was an investigation into the buffering effect of chicken skin and meat with particular reference to the role it plays in protecting *Salmonella* against acidic pH induced by HCl. The results indicated that chicken meat buffered better than chicken skin and that the buffering effect of chicken skin and meat protected four strains of *Salmonella* against pH stress. Since the presence of organic acids specifically (in addition to pH alone) play an important role in the antimicrobial activity of marinades on meat, the effect of organic acids on the survival of *Salmonella* on chicken skin and meat was also examined. Of four acids examined, acetic acids resulted in the highest reduction of viable count of *Salmonella* on chicken (in a range of 5.79 - 10.63 log CFU/ml(or g)/pH-unit; $p < 0.001$), followed by citric acid (4.08 - 7.80 log CFU/ml(or g)/pH-unit, $p < 0.05$), lactic acid (4.92 - 8.79 log CFU/ml(or g)/pH-unit, $p < 0.05$) and hydrochloric acid (HCl; 2.92 - 6.52 log CFU/ml(or g)/pH-unit, $p < 0.001$). Results also showed that the buffering effect of chicken protecting *Salmonella* against HCl did not provide sufficient protection in the case of organic acids. This indicated that marinades with organic acids should be

effective in reducing *Salmonella* on chicken despite the buffering effect of chicken skin and meat.

In the second part of this study the role played by fat in chicken skin and meat play on their buffering capacity was investigated. In addition, the survival of *Salmonella* attached to chicken skin and meat with or without fat and treated with acetic acid was determined. Results showed that chicken skin has a higher fat content as compared to chicken meat. The extracted fat and skin remnants (without fat) did not have a strong buffering capacity (7.0 mmol H^+/ (pH*kg) and 6.9 mmol H^+/ (pH*kg) respectively; $p > 0.05$). When the components occurred together, however, a strong buffering capacity (13 mmol H^+/ (pH*kg); $p < 0.05$) was observed. This indicates that fat contributes to some extent to the buffering capacity of chicken skin and meat. However, *Salmonella* Typhimurium ATCC 33062 was better protected when attached to skin remnants without fat (~3.5 log CFU/g; $p < 0.05$) from acetic acid treatment than skin (no viable count), extracted fat (no viable count), meat remnants (~1.5 log CFU/g; $p < 0.05$) and meat (~2.5 log CFU/g; $p < 0.05$). *Salmonella* cells attached to chicken skin (with fat) were more susceptible to acetic acid than those attached to skin remnants (without fat). It is suggested that fat in chicken skin may enhance the vulnerability of attached cells to acetic acid treatments.

The third part of this project was carried out with the objective of investigating the antimicrobial mode of action of acetic acid against *Salmonella* with respect to cellular membrane disruption and the role of undissociated acid molecules in cellular energy depletion. It was established using a nucleic acid/protein assay and a SEM study that acetic acid did not cause leakage of intracellular components from the *Salmonella*

strains. Some elongated cells observed in the micrographs indicated a possibility of acetic acid may inhibit DNA synthesis in *Salmonella* cells. Using an ATP assay it was found that at a neutral pH acetic acid caused energy depletion with ADP/ATP ratio in the range between 0.48 to 2.63 ($p < 0.05$) for four strains of *Salmonella*, probably due to the action of undissociated acid molecules. The antimicrobial effect of acetic acid was better under acidic conditions (ratio of 5.56 ± 1.27; $p < 0.05$) where the role of both acidic pH and undissociated acid molecules may act together. It was concluded that the inhibitory effect of acetic acid is not solely attributable to acidic pH but also the undissociated acid molecules.

Taken together the results of this project suggested that marinades or other acetic acid treatments should be effective against *Salmonella* on chicken because of the acetic acid induced-energy depletion and inhibition of DNA synthesis in the bacterial cells. This should happen regardless of the buffering effect of skin and meat on the acidic pH. The fat in chicken skin and meat was suggested to support the antimicrobial activity of acetic acid against *Salmonella* attached to them. The presence of fat together with the application of organic acids may contribute to an effective intervention in reducing *Salmonella* on poultry meat.

Keywords

Buffering capacity, Cellular energy depletion, Chicken meat, Chicken skin, Fat, Organic acid, pH, *Salmonella*

List of Abbreviations

ADP	Adenosine diphosphate
ANOVA	Analysis of variance
ATCC	American type culture collection
ATP	Adenosine triphosphate
BC	Buffering capacity
BPW	Buffered peptone water
CFU	Colony forming unit
DNA	Deoxyribonucleic acid
HCl	Hydrochloric acid
NaOH	Sodium hydroxide
PBS	Phosphate buffered saline
SD	Standard deviation
SEM	Scanning electron microscope
TAL	Thin agar layer
TSA	Tryptic soy agar
TSB	Tryptic soy broth
XLD	Xylose lysine deoxycholate agar

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Chapter 1

Literature Review

1.1 Introduction

Controlling foodborne pathogens at all levels of production has been a challenge to the poultry industry in order to maintain food safety (Harris et al., 2006; Lynch et al., 2006; Zhao et al., 2009). *Salmonella* is one of the most common foodborne pathogens found on poultry and related products. This pathogen is able to colonize the gastrointestinal tract of poultry and the resulting contaminated poultry products have been identified as the most important source of *Salmonella* transmission to humans (Foley et al., 2011; Lynch et al., 2006). Marination has been used at retail level to reduce pathogens and extend shelf-life of poultry products. Commercially available marinade sauces are typically oil-water emulsions that contain salt, sugar, organic acids, thickening agents and spices (Ramnani et al., 2010). It is reported that the acidic pH and organic acids assist in the antimicrobial effect of marinades (Birk et al., 2010). However, a study found that the buffering capability of raw meat was able to neutralize acidic pH and nullify the antimicrobial effect of marinades against foodborne pathogens (Björkroth, 2005). The buffering capability of meat is the ability of meat to neutralize acidic and alkaline pH and to withstand pH fluctuations (Goli et al., 2007). There are a limited number of studies on the buffering capability of meat in relation to the effect of acidic pH and organic acids in marinades against *Salmonella*. The objective of this thesis and its literature review is to study and examine the association between the effect of different acids on *Salmonella* attached to chicken and the buffering capacity of chicken.

1.2 Poultry consumption

Poultry meat is a major source of protein worldwide. It is the second most consumed meat in the European Union with an annual per capita consumption of 23.1 kg in 2012 (AVEC, 2013), while in the United States the annual per capita consumption of poultry is estimated to be 45 kg (USDA, 2014). The poultry industry has grown tremendously in recent years. The import of poultry into the European Union has been increased from 30,000 tons in 2005 to 89,000 tons in 2013 (AVEC, 2013). Broiler and turkey consumption in the United States was reported to be 78,000 tons in 2009 and 89,000 tons in 2013 (USDA, 2014). The growth of poultry industry also affected the international meat market. From 2009 to 2013, international poultry production had an average annual growth of 13.3%, whereas pork production has grown by 9.4% and beef production by 2.5% (USDA, 2014). The poultry meat industry has been influenced by a consumer demand for greater variety and convenience which has led to the production of processed poultry products, rather than the simple delivery of slaughtered animals for sale. In countries such as China, Indonesia and India, where further processed poultry comprises only 5 to 10% of the market, a demand for these products has been increased as convenience and variety of food products has become important (Mandava & Hoogenkamp, 1999). In addition, the growth of further processed poultry products in Western food and fast-food outlets have resulted in products such as chicken nuggets, chicken salads and chicken sandwiches being added to menus. In the United States, the food service segment of meat industry has increased from 25% in 1970 to approximately 42% of every food dollar spent in 2005 (National Chicken Council, 2005).

1.3 Salmonella

Poultry meat is known to harbour a variety of microorganisms that are able to cause foodborne illnesses and pose potential health hazards. Foodborne diseases are often associated with the consumption of contaminated poultry meats and their processed products. *Salmonella* have been recognized as a major foodborne pathogen, and raw poultry is considered as a very important source of the bacteria (Capita et al., 2001; Jørgensen et al., 2002). It is the primary pathogen considered in this study.

1.3.1 Physiology and taxonomy

Salmonella species belong to the family Enterobacteriaceae and are Gram-negative, facultative anaerobic, non-spore forming rods. Most *Salmonella* are motile by peritrichous flagella and are chemoorganotrophic, with the ability to metabolize nutrients by both respiratory and fermentative pathways. They usually do not ferment lactose, sucrose and salicin, but are able to ferment glucose and other monosaccharides with the production of gas (D' Aoust & Maurer, 2007; Jay, 2000). *Salmonella* are resilient microbes that readily adapt to extreme environments such as acidic pH (pH 3) and elevated temperatures (≤ 54°C). The pH range for *Salmonella* growth is from pH 4.0 to 9.0 with the optimum growth at pH 7.0 ± 0.2 (D' Aoust & Maurer, 2007). Studies have shown that *Salmonella* serovars contain acid-adaptation system which enables the microorganisms to survive and proliferate at pH as low as 2.5 (Baik et al., 1996; Waterman & Small, 1998). These organisms are able to grow well within 24 hours at 37 ℃ on culture media. The lowest temperatures for *Salmonella* growth has been reported to be 5.3 ℃ for *S.* Heidelberg and 6.2 ℃ for *S.* Typhimurium (Matches & Liston, 1968). *Salmonella* Seftenberg is the most heat resistant of all *Salmonella* serovars (Ng et al., 1969). It has been reported to survive at

44 ℃ and was 30 times more heat resistant as compared to *S*. Typhimurium (Ng et al., 1969). *Salmonella* can be conditioned to tolerate a wider range of temperature, pH as well as salt concentration (D' Aoust & Maurer, 2007).

Although the 2,555 *Salmonella* serovars are viewed by some as individual species, all *Salmonella* are currently categorized into two species, *Salmonella enterica* and *Salmonella bongori,* based on molecular investigations (Grimont & Weill, 2007). *Salmonella enterica* is divided into the following subspecies: *enterica* (I); *salamae* (II); *arizonae* (IIIa); *diarizonae* (IIIb); *houtenae* (IV), and *indica* (VI), whereas *S. bongori* (previously a subspecies of *S. enterica*) is denoted with the Roman numeral V (D' Aoust & Maurer, 2007; Jay, 2000). For epidemiological purposes, *Salmonella* are categorized into three groups: (1) those that infect humans only such as *S.* Typhi, *S.* Paratyphi A, and *S.* Paratyphi C; (2) the host-adapted serovars which include *S.* Gallinarum (poultry), *S.* Dublin (cattle), *S.* Abortus-equi (horses), *S.* Abortus-ovis (sheep) and *S.* Choleraesuis (swine), and (3) the unadapted serovars with no host preference but which are pathogenic to humans including most of the foodborne serovars (Jay, 2000). The serotypes of *Salmonella* are differentiated by the O, H and Vi antigens using the Kaufmann-White scheme and then categorized into subgroups according to common antigenic factors (Grimont & Weill, 2007; Jay, 2000).

1.3.2 Role as foodborne pathogen and incidences of salmonellosis

Salmonella are found primarily in the intestinal tracts of animals such as birds, reptiles, farm animals and humans. As part of the intestinal flora, the microorganisms are excreted together with faeces from which they may be transmitted through a vector to other animals or into water. When contaminated food or water is consumed

by humans and other animals, *Salmonella* grow and mature inside the host until they are once again shed through faecal matter to continue the cycle (Jay, 2000). International shipping of animal products and feeds is largely responsible for the widespread dissemination of salmonellosis and its consequences (Jay, 2000).

Most of foodborne outbreaks of disease caused by *Salmonella* are due to exposure to livestock, cross-contamination of processed food by raw ingredients, poor sanitation of kitchen surfaces and equipment, as well as poor handling practices (Cunningham, 1987; Varnam & Evans, 1996). Meat and poultry animals are infected by *Salmonella* transmitted from the environment via insects, rodents, feeds, other animals and humans (Jay, 2000). Animal feed is one of the major sources of animal infected by *Salmonella* in the poultry industry, with the practice of 'recycling' waste proteins, such as feathers and animal parts that are not used for human food, increasing the risk of *Salmonella* infections of the poultry. Once *Salmonella* have infected a primary breeding flock, other units in the same house can be infected via the eggs through to the progeny and also via horizontal transmission (Wray et al., 1999). Young chickens are particularly susceptible and can be infected within a few hours of hatching (Xu et al., 1988).

Once *Salmonella* enters a susceptible host, it penetrates and passes through the gut lumen into epithelium of the small intestine where it proliferates and invades the ileum and colon. The infection typically elicits an inflammatory response (Forsythe, 2000). *Salmonella* infections lead to salmonellosis resulting in enteric fever (also known as typhoid fever) if infections are caused by *S.* Typhi and gastroenteritis or diarrhea if infections are caused by non-typhoidal *Salmonella*. Typhoid and

paratyphoid fever remain as an important cause of morbidity worldwide. Regional typhoid fever incidences reported from Europe and North America were 0.1 and 0.8 per 100,000 populations respectively. Incidences of typhoid fever in Africa and Asia were reported to be 724.6 and 77.4 per 100,000 populations in 2010 (Buckle et al., 2012). Additional research is needed in developing countries and particularly in Africa, Latin America and Southeast Asia as these countries are not wealthy enough to support highly developed national typhoid fever surveillance systems. Non-typhoidal salmonellosis is one of the leading causes of acute gastroenteritis (Majowicz et al., 2010). The infective dose of *Salmonella* for humans is as few as 15 to 20 cells depending on the age and health of the host, and symptoms can be severe in the cases of elderly, infants and immune-compromised individuals (USFDA, 2003). The onset of symptoms occurs after an incubation time of at least 8 hours (Willey et al., 2008). Symptoms of salmonellosis consist of nausea, vomiting, abdominal pain, headache, chills and diarrhea (which sometimes may be bloody) (Willey et al., 2008). The global human health impact of non-typhoidal salmonellosis is high with an estimation of 93.8 million cases of gastroenteritis of which approximately 85% of the cases are foodborne, resulting in 155,000 deaths annually (CDC, 2014). In 2009, a total of 108,614 confirmed salmonellosis cases (23.7 per 100,000 populations) were reported from the European Union. The two most commonly reported *Salmonella* serovars were *S. enterica* serovar Enteritidis and *S. enterica* serovar Typhimurium representing 52.3% and 23.3% of all salmonellosis confirmed cases respectively (EFSA, 2011). Both serovars were commonly associated with the infections caused by consumption of contaminated poultry meat and eggs.

1.3.3 Prevalence of Salmonella *on poultry*

Salmonella is commonly found on poultry carcasses worldwide (Jay, 2000). For example, a total of 38.3% of poultry carcasses have been reported to be *Salmonella* positive in wet markets and processing plants in Malaysia (Rusul et al., 1996). In Korea, *Salmonella* was detected in 25.9% of raw broilers, with *Salmonella* Enteritidis, *Salmonella* Virchow, and *Salmonella* Virginia, the main serovars isolated (Chang, 2000). The prevalence of *Salmonella* isolated from raw poultry at retail level in six provinces and two cities in China was 52.2% (Yang et al., 2011). *Salmonella* was successfully isolated from 40% of broilers in Turkey with the predominant serovar of being *S*. Enteritidis (81.5%) (Carli et al., 2001). In the United States, a total of 825 samples of retail chicken meat randomly selected from 59 stores in Greater Washington, D.C. area were examined for the presence of *Salmonella*. It was found that 70.7% of the chicken samples were contaminated with *Salmonella* (Zhao et al., 2001). In addition, *Salmonella* was isolated from 22% of conventionally raised (n=141) and 20.8% organically raised (n=53) chickens in Louisiana. The predominant serovars isolated were *S*. Enteritidis, *S*. Hadar and *S*. Kentucky (Lestari et al., 2009). The prevalence of *Salmonella* on chicken meat for sale in retail outlets and supermarkets in Castilla and León of Spain was 35.83% and the predominant serovars were *S*. Enteritidis (47.88%) and *S*. hadar (25.35%) (Domínguez et al., 2002). Another study in Northwestern Spain also reported that *Salmonella* were isolated from 17.9% of chicken samples (60 out of 336 carcasses), and *S*. Enteritidis was the most common strain (Capita et al., 2007). From these studies, it is apparent that *Salmonella* Enteritidis is often the leading cause of foodborne disease associated with poultry meat. As raw poultry meats are the potential vehicles for transmitting foodborne

illness, hazard analysis critical control point (HACCP) systems should be implemented not only at the retail level but at the farms and processing plants.

1.4 Organic acids in marination

Food-safety concerns nowadays are exacerbated since food quality is the most important criterion from the consumer's point of view (Califano et al., 1997). In order to extend the shelf-life and assure the quality of poultry products, the need for control over food spoilage and foodborne pathogens has become paramount in food industry. Marination is a practice which enhances the safety and shelf life of meat products by inhibiting the growth of microorganisms while at the same time improving flavor and tenderness of the meat products (Björkroth, 2005). Marination involves exposing the meat to an acidic or alkaline solution in order to alter the pH of the tissue (Cannon et al., 1993). Acidic marination in particular is more widely used because it effectively reduces bacterial growth and prevents spoilage of meat products (Mendonca et al., 1989; Reynolds & Carpenter, 1974). A wide range of marinades are available commercially for domestic and industrial use. Commercially available marinade sauces are typically oil-water emulsions that contain salt, sugar, acids (e.g. acetic and citric), thickening agents (e.g. xanthan gum and guar gum), spices and aroma enhancers (Ramnani et al., 2010).

Organic acids have a long history of being used as food additives and preservatives for preventing food spoilage and extending the shelf life of food. Organic acids are saturated straight chain carboxylic acids and their respective derivatives which exist in unsaturated, hydroxylic and phenolic forms (Cherrington et al., 1991). In the food industry, marinades are made using weak acids, such as acetic acid, lactic acid and

citric acid, and are generally supplemented with NaCl (Goli et al., 2007). It has been established that the growth of microbes in marinated products can be suppressed by the low pH caused by the presence of organic acids in marinades (Björkroth, 2005). Acetic acid and lactic acid are commonly used in meat marination to inhibit bacteria and pathogens such as *Bacillus* sp., *Salmonella* sp. and *Pseudomonas* sp. (Berge et al., 2001; Davidson, 2001). A study by Siragusa and Dickson (1993) reported that the level of *Salmonella* Typhimurium after acetic acid treatment on poultry was reduced by 1.11 log compared with control. Smaoui et al. (2011) also demonstrated that the addition of 1% lactic acid in marinated chicken can delay the growth of microbes and undesirable chemical appearance.

1.4.1 Organic acids and poultry

Chicken carcasses may harbour a large number of bacteria and foodborne pathogens after processing (Mani-López et al., 2012; Northcutt et al., 2003). For this reason, antimicrobial chemicals are commonly used during processing to reduce pathogen loads on carcasses in some countries. The most common antimicrobial treatment used for decontamination of poultry meat is sodium hypochlorite (Mountney & O'Malley, 1965). While sodium hypochlorite may be sufficient to control *Salmonella* cross-contamination between most poultry carcasses, the difficulties in optimizing the disinfectant properties of chlorine and the unpleasant and harmful odours due to the production of chlorine gas and trichloramines have been an issue in the poultry processing industry (Hinton et al., 2007; Northcutt et al., 2005; Northcutt et al., 2008). For these reasons, the use of alternative methods, such as organic acids, which are generally recognized as safe (GRAS) to disinfect poultry carcasses have been widely studied (Mani-López et al., 2012). Organic acids used to spray or dip poultry

carcasses have been reported to reduce the viable count of *Salmonella* by at least 3 log (Harris et al., 2006; Hinton & Ingram, 2005; Kubena et al., 2001; Lu et al., 2005).

1.4.2 The antimicrobial mode of action of organic acids

The antimicrobial mechanism of action of organic acids depends on the type of acid and the target bacteria (Kubena et al., 2001). For example, the minimal inhibitory concentration of acetic acid for *Bacillus subtilis* is 250 times lower than for *Lactobacillus* spp. (Hsiao & Siebert, 1999). A number of explanations have been offered to account for the antimicrobial activity of organic acids. The capability of these acids in their undissociated form to diffuse across the cell membrane and acidify the cytoplasm is one of these explanations (Leeson et al., 2005; Van Immerseel et al., 2006). The acidification of the cytoplasm could lead to the inhibition of bacterial growth through the presence of H^+ ions produced from the dissociation of acid molecules, membrane disruption, inhibition of metabolic reactions, accumulation of toxic anions, energy exhaustion to maintain homeostasis, and alteration of important biomolecules such as enzymes, proteins, and DNA (Islam, 2012; Mani-López et al., 2012). Some organic acids (malic and citric acids) have been shown to have the ability to chelate and disrupt the bacterial cell membrane (Mani-López et al., 2012). Most of the antimicrobial properties of organic acids are related to changes in pH. However, the inhibitory effects of organic acid may, however, vary depending on their carbon chain, hydroxyl groups, and double bonds (Hsiao & Siebert, 1999). For example, the variability of carbon chain of an organic acid is proposed to be an important factor in determining if the acid has a bactericidal or bacteriostatic effect. Van Immerseel et al. (2006) reported that a dose of 25 mM of a medium chain fatty acid was bacteriostatic to a *Salmonella* Enteritidis strain but no inhibitory effect was

observed for a short chain fatty acid. These variables may explain the inconsistent results regarding the administration of organic acids and their antimicrobial effects in chickens (Cengiz et al., 2012).

1.5 Buffering capacity of meat

The buffering capacity of meat is the ability of meat to neutralize acidic pH towards neutral and its ability to withstand rapid pH fluctuations (Goli et al., 2007). The greater the buffering capacity, the greater the quantity of acid or base needed to alter the pH. However, buffering capacity has its limit and once it reaches a saturation point, the pH of the medium changes rapidly as acids or bases are added. The pH of meat products is important because of its major influence on the water holding capacity, tenderness and juiciness of the meat and on the interaction of the meat with different salts (Bendall, 1979; Rao et al., 1989). It also determines the stability of the meat with respect to autolytic and microbial degradation (Saunders, 1994). Organic acids are commonly used in meat marination, and a large number of protons are required to reduce the pH of the meat (Goli et al., 2007). Several components in meat act as the donors or receptors of protons which affect the buffering capacity of the meat. Immersion in acidic marinades will cause a reduction in pH of meat, along with the leakage of meat components such as lactate, protein and non-protein nitrogen compounds. Phosphate groups, proteins and dipeptides are responsible for the buffering effect mainly at a pH close to neutrality while the amino acids and lactates account for the buffering capacity observed at a marination pH of 3-5 (Gault, 1991; Kyl ä-Puhju et al., 2004).

Bate-Smith (1938) published the first analysis of the buffering capacity of ox and pork meats by titrating with dilute acid and base. Since then several authors have studied related variables that influence the buffering capacity such as the roles of proteins, effect of heating and different muscle types in meat (Hamm & Deatherage, 1960; Honikel & Hamm, 1974; Sayre et al., 1963). It was not until Svensson and Tornberg (1998) proposed the method of titrating minced beef with a strong acid (as strong acid can be completely dissociated) to better determine the buffering capacity of the meat. This study was based on determining the balance of chemical compounds at equilibrium in the presence of a given quantity of strong acid solution. It is assumed that the meat matrix will act as a base that will be protonated by the acid added in the pH range, usually that of marinades used (Goli et al., 2007). A number of studies have determined the values for the buffering capacity of meat in weak or strong acids and bases at different pH range. Sayre et al. (1963) reported that the buffering capacity for pork *longissimus* muscle was 55 mmol H^+/ (pH*kg meat) mmol at pH's ranging from 4.8 to 7. Honikel and Hamm (1974) reported that the mean buffering capacity for beef muscle was 49 mmol H^+/ (pH*kg meat), at pH's ranging from 4 to 9. Puolanne and Kivikari (2000) reported that the buffering capacities for pork and beef *triceps brachii* and *longissimus* muscles were 45, 52, 48 and 51 mmol H^+/ (pH*kg meat), respectively at pH's ranging from 5.5 to 7. Due to the different kinds of acids and dilution ratios used in these studies, it is difficult to compare the results between them.

1.6 Research objectives

There is limited literature on the influence of buffering on the survival of *Salmonella* attached to chicken skin and meat treated with different acids. Studies carried out previously (Björkroth, 2005; Tan, 2010) suggested that the buffering capability of

chicken meat rapidly neutralized the acidic pH of the marinade leading to a reduction in effectiveness of the antimicrobial activity of marinades. The following research questions were raised by previous work: 1. Does the buffering capability of skin and meat which can neutralize the acidic pH of marinades have an effect on the *Salmonella* that attached to skin and meat? 2. If organic acids were present in the marinades, would the buffering capability of skin and meat have the same effect on *Salmonella* as it would if acidified by inorganic acids? 3. Does the fat composition of chicken skin and meat contribute to their buffering capability? 4. Are there other antimicrobial mechanisms other than acidic pH associated with organic acids which may responsible for reducing the number of *Salmonella* on chicken?

Based on these questions, the research objectives for this project were as follow:

(1) To investigate the buffering effect of chicken skin and meat as well as the role this plays in protecting *Salmonella* against pH stress (Chapter 2)

(2) To examine the comparative effect of organic acids and inorganic acid on the survival of *Salmonella* attached to chicken skin and meat (Chapter 3)

(3) To investigate the role of fat associated with chicken skin and meat in their buffering capacity and on the survival of *Salmonella* attached to them treated with acetic acid (Chapter 4)

(4) To study the antimicrobial mode of action of acetic acid against *Salmonella* on chicken (Chapter 5)

Chapter 2

Effect of pH and the Influence of Buffering Effect of Chicken Skin and Meat on the Survival of *Salmonella* Attached to Them

The work presented in this chapter represents a part of the following peer reviewed publication:

Tan, S.M., Lee, S.M., & Dykes, G.A. (2014) Buffering effect of chicken skin and meat protects *Salmonella enterica* strains against hydrochloric acid but not organic acid treatment. *Food Control*, 42, 329-334.

Declaration for Thesis Chapter 2

Declaration by candidate

In the case of Chapter 2, the nature and extent of my contribution to the work was the following:

Nature of contribution	**Extent of contribution (%)**
I was responsible for 100% of data analysis and 90% of drafting and writing.	90%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	**Nature of contribution**	**Extent of contribution (%)**
Sui Mae Lee	SL was responsible for 2% of writing and the review of the publication.	2%
Gary Dykes	GD was responsible for 8% of writing and the review of the publication.	8%

Candidature's Signature		**Date**

Declaration by co-authors

The undersigned hereby certify that:

(1) The above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors;

(2) They meet the criteria for authorship in that they have participated in the conception, execution or interpretation, of at least that part of the publication in their field of expertise;

(3) They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;

(4) There are no other authors of the publication according to these criteria;

(5) Potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and

(6) The original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	**School of Science, Monash University Malaysia**	
Signature 1	[redacted]	**Date**
Signature 2	[redacted]	**Date**

2.1 Introduction

Chicken meat is consumed worldwide as a major source of protein and is known to harbour a variety of microorganisms that are able to cause foodborne illness (Capita et al., 2001). *Salmonella* is recognized as a major foodborne pathogen and is considered as one of the most common encountered on poultry and related products (Capita et al., 2001; Jørgensen et al., 2002). *Salmonella* are resilient microbes that readily adapt to extreme environments such as acidic pH (pH 3) and elevated temperature (≤54 ℃). The range for *Salmonella* growth is from pH 4.0 to 9.0 with an optimum at pH 7.0 ± 0.2 (D'Aoust & Maurer, 2007). Studies have shown that some *Salmonella* serovars contain acid-adaptation systems which enable the microorganisms to survive and proliferate at a pH as low as 2.5 (Baik et al., 1996; Waterman & Small, 1998). Individuals develop *Salmonella* infections, known as salmonellosis, due to the consumption of contaminated food and water. In 2009 a total of 108,614 confirmed salmonellosis cases (23.7 per 100,000 populations) were reported from the European Union. The two most commonly reported *Salmonella* serovars were *Salmonella enterica* serovar Enteritidis and *S. enterica* serovar Typhimurium representing 52.3% and 23.3% of all confirmed salmonellosis cases (EFSA, 2011). Infections by both serovars were commonly associated with the consumption of contaminated poultry meat and eggs.

In order to extend the shelf-life and ensure the quality of poultry products the need to control food spoilage micro-organisms and foodborne pathogens is important. Marination is a practice which, it is claimed, enhances the safety and shelf life of meat products by inhibiting the growth of microorganisms, while at the same time improving flavor and tenderness of the products (Björkroth, 2005). It is a process

which involves soaking meat in complex sauces typically known as ‘marinades’ which consist of salt, sugar, organic acids, thickening agents (e.g. xanthan gum and guar gum), spices and aroma enhancers (Ramnani et al., 2010).

As indicated above, the low pH of marinades is one of the factors that reportedly contribute to a reduction in the number of microbes associated with chicken meat. The pH of meat products is important because of its major influence on their water holding capacity, tenderness and juiciness, as well as their interaction with different salts (Bendall, 1979; Rao et al., 1989). The pH of meat also determines its stability with respect to autolytic and microbial degradation (Saunders, 1994). The buffering effect of meat is the ability of meat to neutralize acidic or alkaline pH towards neutral and also to withstand rapid pH fluctuations (Goli et al., 2007). Studies by Björkroth (2005) and Ramnani et al. (2010) reported that there is a buffering effect of raw meat on the acidic pH in marinades which may neutralize their acidic pH and nullify their antimicrobial effects.

There is limited literature on the influence of buffering on the survival of *Salmonella* attached to marinated chicken skin and meat. Here we investigate the buffering effect of chicken skin and meat as well as the role this plays in protecting *Salmonella* against acidic pH.

2.2 Materials and methods

2.2.1. Preparation of chicken skin and meat samples

Chicken breasts with skin from freshly slaughtered chickens were purchased from a local wet market. Sub-samples of chicken skin and meat were aseptically cut from the

breasts to a weight of 5.0 g ± 0.2 g each and placed in polyethylene bags for further use.

2.2.2 The buffering effect of chicken skin and meat

In order to investigate the buffering capability of chicken skin and chicken meat, both components were incubated in 1 x phosphate buffered saline (PBS; 2.7 mM KCl, 10 mM Na_2HPO_4, 17 mM KH_2PO_4, 137 mM NaCl; 1st-Base, Singapore) at different initial pH levels. Changes to the pH of the buffer after 24 hours were recorded as the buffering effect of the chicken skin and meat. The 1 x PBS used was adjusted with 1M HCl (Merck, Germany) and 1M NaOH (Merck, Germany) to initial pH levels of 2 ± 0.05, 3 ± 0.05, 5 ± 0.05, 7 ± 0.05, 9 ± 0.05 and 11 ± 0.05, respectively. The pH of PBS before and after incubation was measured using a calibrated pH meter (Sartorius, Germany). A 10 ml aliquot of PBS at each of the respective pH's was added to each of the skin and meat samples. Treated skin and meat samples were incubated for 24 hours at 4 ℃. These incubation conditions were used to mimic typical commercial or home marination. The mean and standard deviation of the pH was calculated from triplicate independent experiments.

2.2.3 Preparation of Salmonella *inoculum*

Salmonella enterica serovar Typhimurium (ATCC 33062 and ATCC 14028) and *Salmonella enterica* serovar Enteritidis (ATCC 13076 and ATCC 49216) were used in the study because these two serovars have been commonly found on chicken. Each of the *Salmonella* strains was streaked on Tryptic Soy Agar (TSA) (Oxoid, UK) from pure glycerol stock cultures obtained from the American Type Culture Collection (Manassas, USA) and incubated for 24 hours at 37 ℃. The *Salmonella* strains were

then streaked on Xylose Lysine Deoxycholate Agar (XLD) (Oxoid, UK) to verify their characteristics. *Salmonella* colonies growing on this medium will produce black colonies. A colony of each *Salmonella* strain was taken from the XLD and inoculated into 10 ml of Tryptic Soy Broth (TSB) (Oxoid, UK) and incubated for another 24 hours at 37 ℃. After incubation, the cultures were centrifuged at 5000 x *g* (Hettich, USA) for 10 minutes. The supernatant was discarded and the pellet was re-suspended in 10 ml of 1 x PBS. A total of 110ml of the suspension was prepared for all the inoculation.

2.2.4 Inoculation of skin and meat samples

A 5 ml aliquot of *Salmonella* inoculum was inoculated onto chicken skin and meat samples. The *Salmonella* cells were allowed to adhere to the skin and meat samples for 1 hour at room temperature. The skin and meat samples were then rinsed three times with 1 x PBS to ensure all unattached cells had been removed. Rinsed samples were then assigned to different treatments.

2.2.5 Protection of Salmonella *against pH stress by chicken skin and meat*

Volumes of 1 x PBS were adjusted to the same initial pH values listed above. Inoculated chicken skin and meat was placed into pH adjusted PBS and incubated at 4 ℃ for 24 hours. The pH of the PBS was measured before and after incubation. *Salmonella* on chicken was enumerated before and after exposure to PBS at the different pH's and compared to *Salmonella* in corresponding pH adjusted PBS without chicken. To investigate the effect of pH on the survival of *Salmonella* in the PBS solution without chicken, 5 ml of the cell suspension prepared as described above was treated with 10 ml of pH adjusted PBS and was incubated at 4 ℃ for 24

hours. The mean and standard deviation of the viable counts of *Salmonella* from different pH treatments was calculated from triplicates independent experiments.

2.2.6 Enumeration of Salmonella *on treated chicken skin and meat samples*

The thin agar layer (TAL) method was used for this study as it has a better bacterial recovery rate than XLD (Kang & Fung, 2000; Wu & Fung, 2001). The TAL was prepared according to Kang and Fung (2000). Tryptic Soy Agar was used as non-selective medium while XLD was used as selective medium. Both the selective and non-selective media were prepared according to the manufacturer's instructions. Approximately 25 ml of sterilized XLD agar was solidified in a petri dish (8.5 cm diameter) as a base and overlaid by 14 ml of sterilized TSA.

Treated samples were transferred into sterile stomacher bags and 50 ml of Buffered Peptone Water (BPW) (Oxoid, UK) was added to each sample respectively. Samples were homogenized for 2 minutes. Serial decimal dilutions were conducted and 0.1 ml of appropriately diluted samples was inoculated directly onto the TAL medium using spread plate technique. The plates were counted after incubation for 24 hours at 37 ℃ and results recorded as colony forming units per ml (CFU/ml (or g)).

2.2.7 Statistical analyses

The buffering effects of chicken components were represented as '1 - gradient of slopes of linear regression equations' generated from transformed data (pH to log pH) with a linear equation $y = m x + c$, where m represents the slope of the equation. Significant differences in buffering among chicken components (skin and meat) were determined using one-way ANOVA. The viable counts of *Salmonella* from different

pH treatments and on different chicken components (skin and meat) were compared using two-way ANOVA. Post-hoc Tukey's test was used for pairwise comparison to determine the significant difference between samples at $p < 0.05$. All tests were conducted using SPSS software version 16.0 (PASW Statistics 20; SPSS Inc.).

2.3 Results and Discussion

2.3.1 The buffering effect of chicken skin and meat

The buffering effect of chicken skin and meat was determined based on the changes from the initial pH of the PBS solutions after exposure to these chicken components. The buffering effect of chicken skin and meat was represented as '1 - gradient of slopes of linear regression equations' generated from transformed data (pH to log pH). Three linear regression equations were generated from three sets of transformed data and the graphs are shown in Appendix I. Figure 1 is a summarizing graph which includes the averages of all three data sets of each chicken skin, meat, and PBS alone. A buffering effect towards neutrality was observed in the presence of chicken skin and/or meat. For example, pH 2 changed to 4.74 ($p < 0.001$) and pH 11 to pH 8.8 ($p < 0.001$) after exposure to chicken components. The pH of PBS after incubation without meat or skin did not differ ($p > 0.05$) from the initial adjusted pH (e.g. pH 2 changed to pH 2.03 ± 0.10). While dilution of the buffer components at the higher and lower pH's may have played some role in the observed trends, the data suggests that this was minor and therefore this possibility was not investigated in the current study.

The gradient of the slope of PBS without chicken was regarded as 1.0 at which no buffering effect was observed. Chicken meat had a higher buffering effect (1 - gradient of slope = 0.979, $p < 0.05$) as compared to chicken skin (1 - gradient of slope

= 0.961, $p < 0.05$). This could be because of a higher level of dipeptides in chicken meat as compared to chicken skin. Dipeptide content is known to be the primary contributing factor to the difference in the buffering capacity in meat products (Puolanne & Kivikari, 2000). Plowman and Close (1988) found that the dipeptide content of chicken meat is twice as high as beef and pork. Regardless of the meat species, variations in the physiological characteristics across the same carcass, for example chicken breast and thigh muscles, could result in different buffering capacity. Chicken breast muscle contains 87.5 to 100% white fibers while chicken thigh muscle contains a mixture of red fibers (25-30%), intermediate fibers (40-50%) and white fibers (20-30%) (Papinaho et al., 1996). The buffering capacity of chicken breast and chicken thigh muscles at pH's ranging from 5.5 to 7.0 are 58 and 41 mmol H^+/(pH*kg), respectively (Puolanne & Kivikari, 2000). Other factors contributing to the buffering effect of chicken meat include phosphate, proteins and lactates (Plowman & Close, 1988). In addition, the buffering effect of chicken skin may be because of its complex matrix of protein, lipids and mucopolysaccharides (Kim et al., 1993).

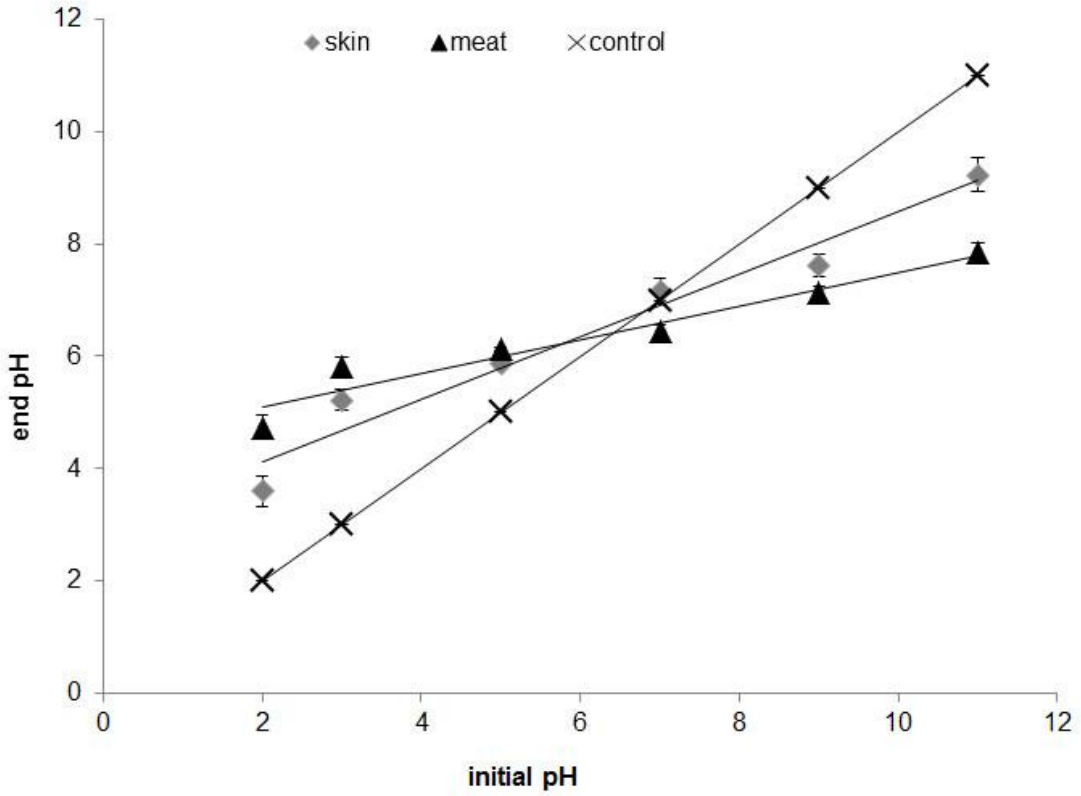

Figure 1. Relationship between pH before (initial pH) and after (end pH) incubated with chicken skin and meat for 24 hours at 4 ℃. The buffering effect of chicken skin and meat was represented as '1-slopes of each linear regression equation'; the linear equations of (a) chicken skin: $y = 0.039x + 0.548$, $R^2 = 0.897$; (b) chicken meat: $y = 0.021x + 0.670$, $R^2 = 0.902$; (c) PBS without chicken: $y = x$, $R^2 = 1$.

2.3.2 Protection of Salmonella *against pH stress by chicken skin and meat*

In order to investigate the degree to which chicken skin and meat protect *Salmonella* against the effects of pH in marinades we used PBS to simulate a typical marinade because it contains phosphate and salt but excludes other components such as organic acids, herbs and spices that may contribute to additional antimicrobial effects. In this study we did not examine the effect of the background microbial flora and its potential effect on the buffering capacity or survival of *Salmonella*. Future studies

should investigate this as another potential variable influencing the safety of marinated foods.

As a similar trend was observed for all four *Salmonella* strains, the effects of pH on the survival of *Salmonella* Typhimurium ATCC 14028 attached to chicken skin, meat and in PBS solution are presented as an example in Figure 2. The data for *S.* Typhimurium ATCC 33062, *S.* Enteritidis ATCC 13076 and *S.* Enteritidis ATCC 49216 are presented in Appendix II. Viable counts of *Salmonella* (~7-9 log CFU/g) on chicken skin and meat samples treated with PBS adjusted to pH 3, pH 5, pH 7 and pH 9 did not differ ($p > 0.05$) from the viable counts on non-treated samples. The viable counts of *Salmonella* inoculated into PBS solutions adjusted to pH 3, pH 5, and pH 9 were reduced ($p < 0.05$) to ~6-8 log CFU/ml as compared to non-treated samples (~9 log CFU/ml). These observations were consistent with D'Aoust and Maurer (2007) who found that *Salmonella* could only survive between pH 3 and pH 9. No viable bacteria were present in PBS solutions adjusted to pH 2 and pH 11 using HCl for all *Salmonella* strains. This indicates that pH 2 and pH 11 conditions are bactericidal to these pathogens. On the other hand, a high number of *Salmonella* (~6-7 log CFU/g, $p < 0.001$) survived when chicken skin and meat samples were present in PBS of initial pH 2 and 11. This indicates that the buffering effect of chicken skin and meat protected the *Salmonella* strains against the effects of pH stress (Björkroth, 2005; Ramnani et al., 2010). Birk et al. (2010) found that when the pH of meat was lowered a subsequent rapid rise in pH was observed within few minutes which to some extent neutralized the antibacterial effect of the initial lowered pH. When meat is immersed in acidic and alkaline marinades, it causes a reduction in the pH of the meat, along with leakage of meat components such as lactate, protein and non-protein nitrogen

compounds (Goli et al., 2011). Phosphate groups, proteins and dipeptides act mainly at a pH close to neutrality while the amino acids and the lactates account for the buffering capacity observed at a marination pH in a range of 3-5 (Gault, 1985; Kylä-Puhju et al., 2004). The presence of a buffering effect in meat suggests that the inhibitory action of marinades should not be fully attributed to the effect of acidic and alkaline pH of the marinades.

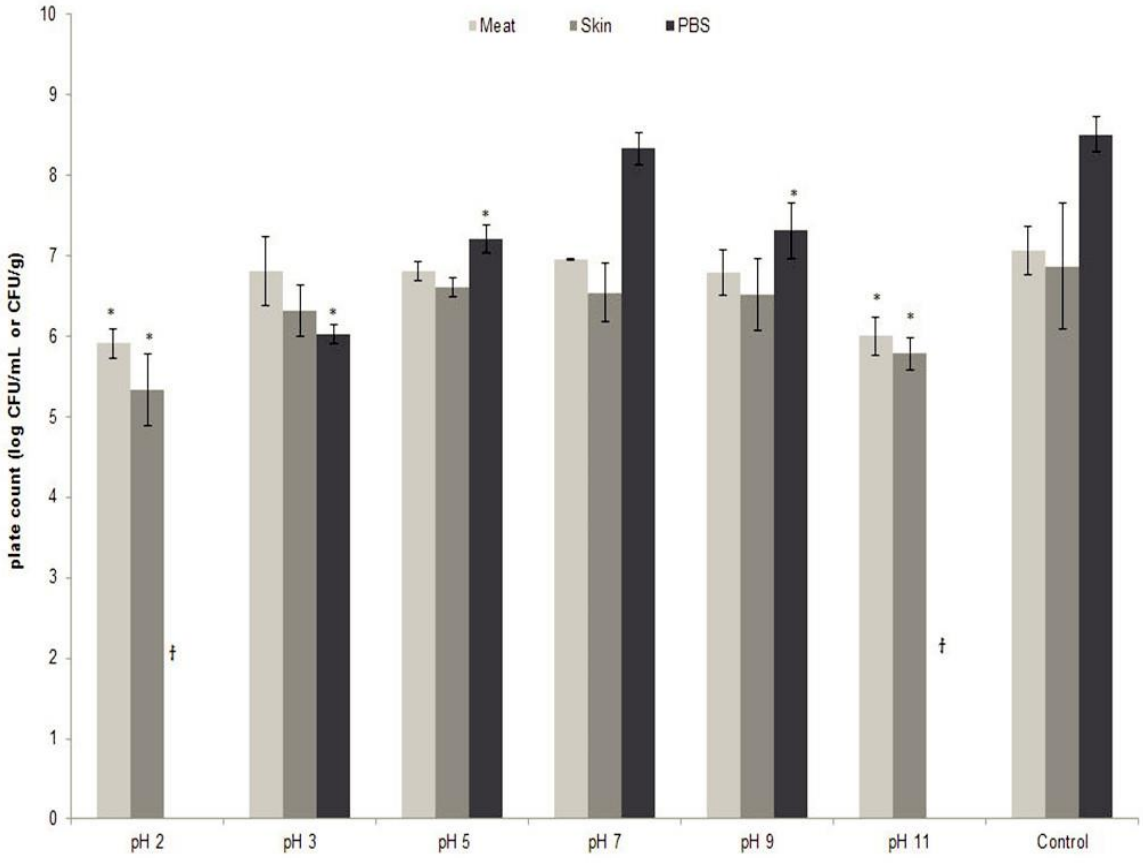

Figure 2. The effect of pH on the survival of *S.* Typhimurium ATCC 14028 attached to chicken meat and skin, and in PBS alone. The control is *Salmonella* attached to chicken meat and skin, and in PBS alone, with no treatment applied. Results are presented as mean ± SD where n = 3; * indicates a significant difference between pH treatments and the control; ***t*** indicates no viable count was observed for the pH treatments.

2.4 Conclusion

Chicken meat showed a better buffering effect as compared to chicken skin. The buffering effect of chicken skin and meat was able to protect *Salmonella* on chicken against pH stress. This suggested that the inhibitory effect of marinades should not be fully attributed to the effect of pH of the marinades.

Chapter 3

Effect of Organic Acids and the Influence of Buffering Effect of Chicken Skin and Meat on the Survival of *Salmonella* Attached to Them

The work presented in this chapter represents a part of the following peer reviewed publication:

Tan, S.M., Lee, S.M., & Dykes, G.A. (2014) Buffering effect of chicken skin and meat protects *Salmonella enterica* strains against hydrochloric acid but not organic acid treatment. *Food Control*, 42, 329-334.

Declaration for Thesis Chapter 3

Declaration by candidate

In the case of Chapter 3, the nature and extent of my contribution to the work was the following:

Nature of contribution	**Extent of contribution (%)**
I was responsible for 100% of data analysis and 90% of drafting and writing.	90%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	**Nature of contribution**	**Extent of contribution (%)**
Sui Mae Lee	SL was responsible for 2% of writing and the review of the publication.	2%
Gary Dykes	GD was responsible for 8% of writing and the review of the publication.	8%

Candidature's Signature		**Date**

Declaration by co-authors

The undersigned hereby certify that:

(1) The above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors;

(2) They meet the criteria for authorship in that they have participated in the conception, execution or interpretation, of at least that part of the publication in their field of expertise;

(3) They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;

(4) There are no other authors of the publication according to these criteria;

(5) Potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and

(6) The original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	**School of Science, Monash University Malaysia**	
Signature 1	[redacted]	**Date**
Signature 2	[redacted]	**Date**

3.1 Introduction

Salmonella is one of the leading causes of some zoonotic enteric infections worldwide. In the European Union (EU), over 100,000 salmonellosis cases are reported each year. From 2007 to 2013, 328,537 *Salmonella* outbreak cases were reported caused by *S.* Enteritidis by 27 countries in EU, mainly associated with broilers and egg products (ECDC & EFSA, 2014). *Salmonella* is estimated to cause more than 1.2 million infections, with more than 23,000 hospitalizations and 450 deaths reported in the United States (CDC, 2014). A more accurate estimate of numbers of salmonellosis is difficult to determine because only large outbreaks are investigated while sporadic cases are under-reported. Data on salmonellosis in many countries in Asia and Africa is limited with only 1 to 10% of the cases reported (Hanes, 2003; Portillo, 2000). Salmonellosis is mainly associated with the handling and processing of poultry meat (Hanes, 2003).

It is important to control foodborne pathogens to ensure that food products are safe for consumption. Marination can enhance the safety and shelf life of meat products by improving the flavor and tenderness as well as reducing bacterial growth on the meat (Björkroth, 2005). Demand for marinated chicken meat is increasing because it results in products with strong flavour development, tenderization and consumer convenience. In addition, marination acts as an antimicrobial treatment to reduce foodborne pathogens on broiler meat (Chan et al., 2001). Weak organic acids such as lactic, acetic, propionic, citric and benzoic acid have been reported to exhibit antimicrobial activities against pathogens such as *Escherichia coli, Listeria monocytogenes* and *Salmonella* (Doležalová et al., 2010; González-Fandos et al., 2009; Over et al., 2009). These acids have been widely used as food preservatives as well as in the pre-harvest

and post-harvest phases in food processing. Organic acids are compounds which include saturated straight-chain carboxylic acids and their respective derivatives such as unsaturated, hydroxylic and phenolic forms (Ricke, 2003). Since organic acids in marinades play an important role in their antimicrobial activities on meat, we investigated the comparative effect of organic acids and inorganic acid on the survival of *Salmonella* on chicken skin and meat.

3.2 Materials and Methods

3.2.1. Preparation of chicken skin and meat samples

Chicken breasts with skin from freshly slaughtered chickens were purchased from a local wet market. Sub-samples of chicken skin and meat were aseptically cut from the breasts to a weight of 5.0 g ± 0.2 g each and placed in polyethylene bags for further use.

3.2.2 Preparation of Salmonella *inoculum*

Salmonella Typhimurium (ATCC 33062 and ATCC 14028) and *Salmonella* Enteritidis (ATCC 13076 and ATCC 49216) were used in the study and were obtained from American Type Culture Collection (Manassas, USA). Each of the *Salmonella* strains was streaked on Tryptic Soy Agar (TSA) (Oxoid, UK) from pure glycerol stock cultures and incubated for 24 hours at 37 ℃. The *Salmonella* strains were then streaked on Xylose Lysine Deoxycholate Agar (XLD) (Oxoid, UK) to verify their characteristics. *Salmonella* colonies growing on this medium will produce black colonies. A colony of each *Salmonella* strain was taken from the XLD and inoculated into 10 ml of Tryptic Soy Broth (TSB) (Oxoid, UK) and incubated for another 24 hours at 37 ℃. After incubation, the cultures were centrifuged at 5000 x *g*

(Hettich, USA) for 10 minutes. The supernatant was discarded and the pellet was re-suspended in 10 ml of 1 x PBS. A total of 110 ml of the suspension was prepared for all the inoculation.

3.2.3 Inoculation of skin and meat samples

A 5 ml aliquot of *Salmonella* inoculum was inoculated onto chicken skin and meat samples. The *Salmonella* cells were allowed to adhere to the skin and meat samples for 1 hour at room temperature. The skin and meat samples were then rinsed three times with 1 x PBS to ensure all unattached cells had been removed. Rinsed samples were then assigned to different treatments.

3.2.4 Comparative effect of organic acids and HCl on the survival of Salmonella *on chicken skin and meat*

Concentrated (5M) stock solutions of three organic acids (acetic acid, citric acid, lactic acid) and an inorganic acid (hydrochloric acid) were diluted in 1 x PBS to concentrations of 0.05 M, 0.1 M, 0.2 M, 0.3 M, 0.4 M and 0.5 M. Inoculated chicken skin and meat was placed into the diluted acids as described above and were incubated at 4 ℃ for 24 hours. The pH of each of the acid dilutions was measured before and after incubation. *Salmonella* was enumerated before and after acid treatment and compared to *Salmonella* in corresponding acidified PBS without chicken as described above. The viable count of *Salmonella* was plotted against the pH of each diluted acid and was the result of triplicate independent experiments.

3.2.5 Enumeration of Salmonella *on treated chicken skin and meat samples*

The thin agar layer (TAL) method was used for this study as it has a better bacterial recovery rate than XLD (Kang & Fung, 2000; Wu & Fung, 2001). The TAL was prepared according to Kang and Fung (2000). Tryptic Soy Agar was used as non-selective medium while XLD was used as selective medium. Both the selective and non-selective media were prepared according to the manufacturer's instructions. Approximately 25 ml of sterilized XLD agar was solidified in a petri dish (8.5 cm diameter) as a base and overlaid by 14 ml of sterilized TSA.

Treated samples were transferred into sterile stomacher bags and 50 ml of Buffered Peptone Water (BPW) (Oxoid, UK) was added to each sample respectively. Samples were homogenized for 2 minutes. Serial decimal dilutions were conducted and 0.1ml of appropriately diluted samples was inoculated directly onto the TAL medium using spread plate technique. The plates were counted after incubation for 24 hours at 37 ℃ and results recorded as colony forming units per ml (CFU/ml (or g)).

3.2.6 Statistical analyses

The reduction of *Salmonella* treated with different acids was represented by the slopes generated from the log curves with the equation, $y = m\ In\ (x) - c$, where m represents the slope (Δ viable count / Δ pH) of each acid. The plot of viable count (log CFU/ml) of one strain of *Salmonella* against pH was presented as an example of these slopes. Significant differences of reduction of viable count as pH decreased were determined using a one-way ANOVA. Post-hoc Tukey's test was used for pairwise comparison to determine the significant difference between samples at $p < 0.05$. All tests were conducted using SPSS software version 16.0 (PASW Statistics 20; SPSS Inc.).

3.3 Results and Discussion

3.3.1 Comparative effect of organic acids and HCl on survival of Salmonella *on chicken skin and meat*

The viable count of *Salmonella* decreased as the pH decreased for all acid treatments. A plot of the viable count of *Salmonella* Typhimurium ATCC 14028 against pH is presented as an example of these slopes in Figure 3. The plots of viable count of other three strains of *Salmonella* are showed in Appendix III. In PBS without the presence of chicken, for example, a count of ~9 log CFU/ml of *Salmonella* was reduced to ~7 log CFU/ml when the pH decreased from pH 7.3 to 5 in acetic acid. The reduction of the *Salmonella* viable count after exposure to four acids in PBS were represented by slopes generated from the equations of logarithmic curves, $y = m \ln(x) - c$; where y represents the viable count of *Salmonella*, x represents the pH, m represents the slope (Δ viable count/Δ pH) and c represents the constant of each acid. For example, when $y = 7.8985 \ln(x) - 4.7974$, the slope is 7.8985.

The degrees of reduction (slopes) of all four *Salmonella* strains attached to chicken skin and meat and in PBS without chicken after exposure to four acids are summarized in Table 1. Of the four acids studied, acetic acid resulted in the highest reduction of viable count of *Salmonella* in the range of 5.79 - 10.63 log CFU/ml(or g)/pH-unit which corresponds to a decrease in 1 pH unit ($p < 0.001$), followed by citric acid (4.08 - 7.80 log CFU/ml(or g)/pH-unit, $p < 0.05$), lactic acid (4.92 - 8.79 log CFU/ml(or g)/pH-unit, $p < 0.05$) and HCl (2.92 - 6.52 log CFU/ml(or g)/pH-unit, $p < 0.001$) (Table 1). The reason the inhibitory effect of acetic acid is higher than other organic acids may be because of the higher concentration of undissociated acid. For example, it has been reported that the percentage of undissociated acetic acid

(16.08 mM; 94.63%) was higher than citric acid (9.85 mM; 57.99%), lactic acid (14.90 mM; 87.69%) and HCl (1.54 mM; 9.09%) at a given pH (pH 3.48) (Narendranath et al., 2001). The amount of undissociated acid is strongly linked to the pKa value of the organic acid. At a given acidic pH there is more undissociated acetic acid present than would be found with an equal concentration of lactic acid due to the higher pKa value of acetic acid (4.74) as compared to, for example, lactic acid (3.86) (Lindgren & Dobrogosz, 1990). An early experiment by Levine and Feller (1940) demonstrated that acetic acid was more lethal to microorganisms than lactic acid and HCl because of the concentration of undissociated acid. The undissociated forms of organic acids can easily penetrate the lipid membrane of bacterial cell and dissociate into anions and protons once internalized into the neutral pH of the cell cytoplasm (Eklund, 1985; Ricke, 2003; Salmond et al., 1984).

The reduction in numbers of *Salmonella* varied based on the different chicken components and the different *Salmonella* strains (Table 1). The reduction of *S.* Typhimurium ATCC 14028 caused by acids in PBS without chicken did not differ ($p > 0.05$) to that attached to chicken skin, but were significantly lower ($p < 0.001$) as compared to those attached to chicken meat. The degree of reduction of another strain of *S.* Typhimurium (ATCC 33062) treated with acids in PBS without chicken was significantly lower ($p < 0.05$) than those attached to chicken skin and meat. The degrees of reduction of the two strains of *S.* Enteritidis were different ($p < 0.05$) when the bacteria were attached to chicken skin, chicken meat and the PBS with no chicken. Although strain variations occurred, regardless of whether *Salmonella* were attached to chicken or not, organic acids treatment had a better inhibitory effect than HCl.

In addition, acetic acid, citric acid and lactic acid effectively eliminated *Salmonella* on chicken meat at a range of pH 2.9 to pH 3.8, while the organic acids eliminated *Salmonella* attached to chicken skin at a range of pH 2.0 to 3.1. The pHs at which *Salmonella* were rendered undetectable by four acid treatments are presented in Appendix IV. *Salmonella* attached to chicken skin and meat were no longer viable at ~pH 2 - 4 when treated with organic acids, but survived at a lower pH (1.2) in HCl. Since elimination of *Salmonella* by organic acids occurred at a higher pH compared to HCl, we suggest that there may be other modes of action of organic acids in reducing *Salmonella* in addition to the effects of acidic pH. As indicated above the inhibitory actions of organic acids are associated with the amount of undissociated acid. The pKa values of acetic acid (4.74), lactic acid (3.86) and citric acid (3.14) are higher than HCl (-7.0) which results in a higher percentage of undissociated acid in the organic acids than HCl (Birk et al., 2010; Narendranath et al., 2001). The undissociated acid molecules can freely diffuse into the cytoplasm of microbial cells and dissociate into anions. This may cause accumulation of high levels of organic acid anions in the cytoplasm and interfere with essential metabolic reactions (Davidson, 2001; Ricke, 2003). In order to maintain the neutral pH in the cytoplasm and to restore homeostasis bacteria may induce energetically expensive stress responses which requires excess ATP consumption and may result in depletion of cellular energy (Davidson, 2001). It is also assumed that the ability of organic acids to inhibit growth is related to lipid permeability, perturbation of membrane function, accumulation of inhibitory levels of weak organic acid anions and disturbances to the metabolism and activity of enzyme reactions in the cytoplasm (Brul & Coote, 1999; Salmond et al., 1984; Theron & Lues, 2007). The antimicrobial modes of action of

organic acids are not yet fully understood and therefore further work is required to determine this.

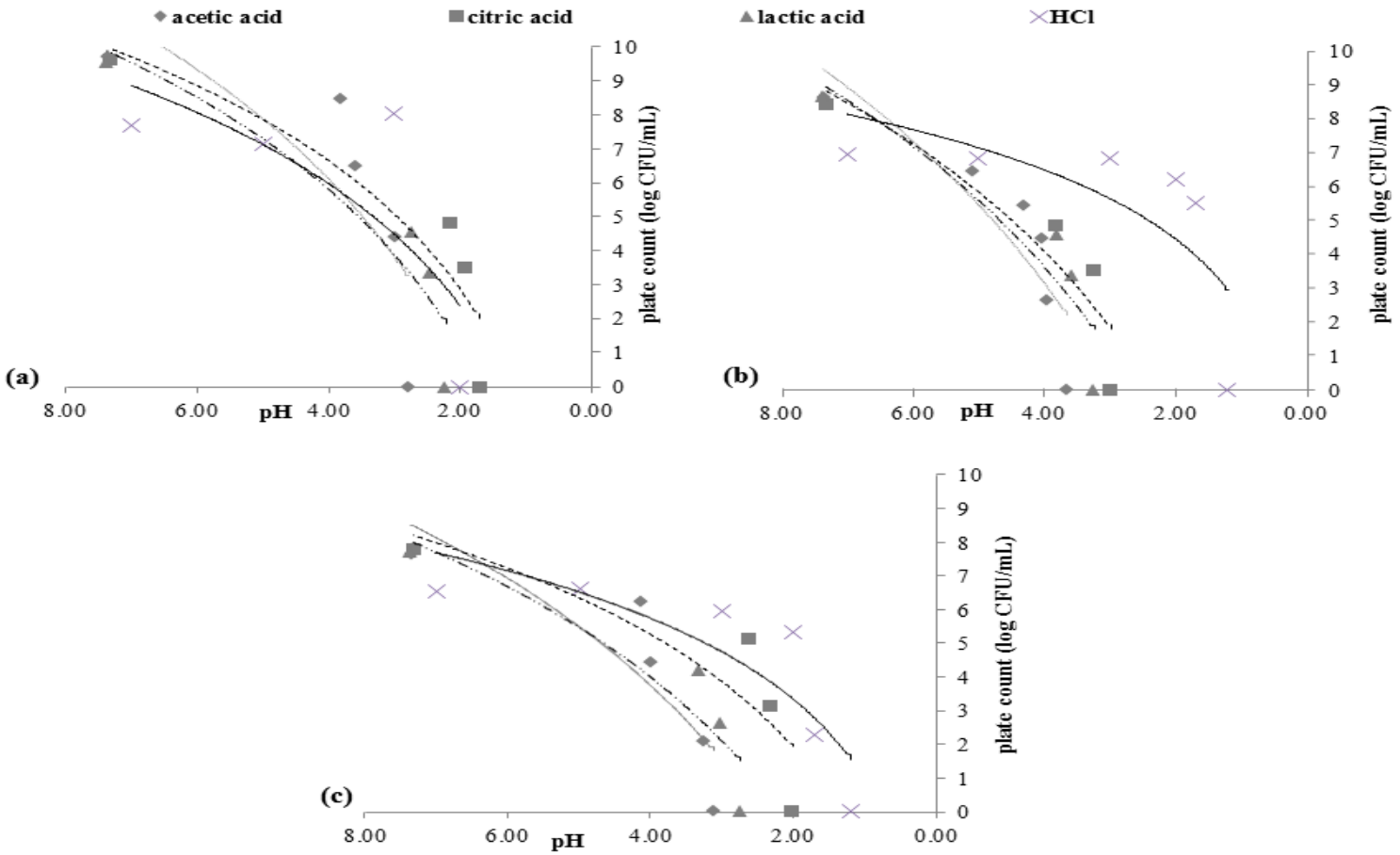

Figure 3. The effect of four acids on the survival of *S.* Typhimurium ATCC 14028 (a) in PBS alone; (b) attached to chicken meat; (c) attached to chicken skin. The reduction of *Salmonella* viable count of each acid as pH decreased was defined as the slope of these curves.

Table 1. Effect of treatment with four acids on the degree of reduction of *Salmonella* attached to chicken skin or meat as pH decreased.

Strains	**Chicken components**	**Degree of reduction (slope = Δ viable count / Δ pH) (log cfu/ml/pH-unit)**			
		acetic acid	citric acid	lactic acid	HCl
S. Typhimurium ATCC 14028	control	7.89 ± 0.21 ^{A,a}	5.45 ± 0.08 ^{A,b}	6.69 ± 0.42 ^{A,a,b}	5.16 ± 0.11 ^{A,b}
	skin	7.80 ± 0.28 ^{A,a}	4.85 ± 0.21 ^{A,b}	6.61 ± 0.23 ^{A,c}	3.47 ± 0.14 ^{A,d}
	meat	10.35 ± 0.54 ^{B,a}	7.80 ± 0.25 ^{B,b}	8.79 ± 0.30 ^{B,b}	2.92 ± 0.13 ^{B,c}
S. Typhimurium ATCC 33062	control	6.79 ± 0.22 ^{A,a}	5.91 ± 0.16 ^{A,b}	6.87 ± 0.09 ^{A,a}	6.17 ± 0.06 ^{A,b}
	skin	5.79 ± 0.41 ^{B,a}	4.28 ± 0.05 ^{B,b}	4.92 ± 0.13 ^{B,b}	3.19 ± 0.16 ^{B,c}
	meat	8.80 ± 0.05 ^{C,a}	5.69 ± 0.23 ^{B,b}	5.25 ± 0.41 ^{B,b}	3.65 ± 0.10 ^{B,c}
S. Enteritidis ATCC 13076	control	6.72 ± 0.39 ^{A,a,b}	5.69 ± 0.11 ^{A,a}	7.10 ± 0.18 ^{A,b}	6.52 ± 0.07 ^{A,b}
	skin	6.19 ± 0.35 ^{A,a}	4.49 ± 0.14 ^{B,b}	5.49 ± 0.09 ^{B,c}	3.38 ± 0.23 ^{B,d}
	meat	10.63 ± 0.45 ^{B,a}	7.04 ± 0.29 ^{C,b}	7.51 ± 0.32 ^{C,b}	3.58 ± 0.09 ^{B,c}
S. Enteritidis ATCC 49216	control	7.16 ± 0.26 ^{A,a}	5.59 ± 0.20 ^{A,b}	6.75 ± 0.44 ^{A,a}	5.30 ± 0.04 ^{A,b}
	skin	5.97 ± 0.16 ^{B,a}	4.08 ± 0.24 ^{B,b}	5.73 ± 0.42 ^{B,a}	3.15 ± 0.25 ^{B,c}
	meat	9.61 ± 0.29 ^{C,a}	7.44 ± 0.09 ^{C,b}	7.89 ± 0.31 ^{C,b}	2.97 ± 0.03 ^{C,c}

Data are presented as mean ± standard deviation where n = 3. ^{A,B,C} Different uppercase letters indicate significant difference in comparisons between treatments (skin, meat, PBS) for each acid at $p < 0.05$ i.e. within columns. ^{a,b,c} Different lowercase letters indicate significant differences between the acids for each treatment and bacterial strain at $p < 0.05$ i.e. within rows.

3.4 Conclusion

The buffering effect of chicken meat and skin, which was effective against HCl and protected *Salmonella* on chicken, did not provide sufficient protection in the case of organic acids. We suggest that the buffering effect is able to provide protection to *Salmonella* cells on chicken against the effect of acidic pH but not the other antimicrobial mode of action of organic acids. Our work shows that acetic acid can eliminate *Salmonella* on chicken meat at approximately pH 4 and that it has potential for practical application in commercial marination.

Chapter 4

Fat Contributes to the Buffering Capacity of Chicken Skin and Meat but Enhances the Vulnerability of attached *Salmonella* Cells to Acetic Acid Treatment

The work presented in this chapter represents the following publication accepted for peer review:

Tan, S.M., Lee, S.M., & Dykes, G.A. (Accepted) Fat contributes to the buffering capacity of chicken skin and meat but enhance the vulnerability of attached *Salmonella* cells to acetic acid treatment. *Food Research International,* doi: 10.1016/j.foodres.2014.10.007.

Declaration for Thesis Chapter 4

Declaration by candidate

In the case of Chapter 4, the nature and extent of my contribution to the work was the following:

Nature of contribution	**Extent of contribution (%)**
I was responsible for 100% of data analysis and 90% of drafting and writing.	90%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	**Nature of contribution**	**Extent of contribution (%)**
Sui Mae Lee	SL was responsible for 2% of writing and the review of the publication.	2%
Gary Dykes	GD was responsible for 8% of writing and the review of the publication.	8%

Candidature's Signature		**Date**

Declaration by co-authors

The undersigned hereby certify that:

(1) The above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors;

(2) They meet the criteria for authorship in that they have participated in the conception, execution or interpretation, of at least that part of the publication in their field of expertise;

(3) They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;

(4) There are no other authors of the publication according to these criteria;

(5) Potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and

(6) The original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	**School of Science, Monash University Malaysia**

Signature 1	[redacted]	**Date**
Signature 2	[redacted]	**Date**

4.1 Introduction

Poultry meat is a major source of protein worldwide. For example, it is the second most important meat in the European Union with a per capita consumption increasing from 22.4 kg in 2007 to 23.1 kg in 2012 (AVEC, 2013), while in the United States the per capita consumption of poultry is estimated to be 45 kg (USDA, 2014).

Salmonella is recognized as a major foodborne pathogen and is one of the most common encountered on poultry and poultry products (Capita et al., 2001; Jørgensen et al., 2002). For example, approximately 22.2 *Salmonella* infections per 100,000 populations were reported in the European Union in 2012. The two most commonly reported *Salmonella* serovars were *S. enterica* serovar Enteritidis and *S. enterica* serovar Typhimurium, representing 41.3% and 22.1% of all confirmed salmonellosis cases, respectively (EFSA, 2014). *Salmonella* are commonly recovered from a wide range of poultry products, including skin and meat, and surfaces associated with poultry processing (Firstenberg-Eden et al., 1978).

Chicken skin is an excellent substrate for the attachment of bacteria and extensive studies have been undertaken in an attempt to reduce or eliminate spoilage and pathogenic bacteria attached to, or entrapped in, skin on chicken carcasses (Alonso-Hernando et al., 2010; Doležalová et al., 2010; Hinton & Ingram, 2005; Kim et al., 1994; Riedel et al., 2009; Tamblyn & Conner, 1997; Wang et al., 1997; Xiong et al., 1998). As reported by Björkroth (2005), Ramnani et al. (2010) and Tan et al. (2014), chicken skin and meat has a buffering capacity which may neutralize the acidic pH and potential antimicrobial effects of treatments such as marinades.

The buffering capacity of a meat is its ability to modulate acidic or alkaline pH towards neutral and to withstand rapid pH fluctuations (Goli et al., 2007). Bate-Smith (1938) published the first analysis of the buffering capacity of different meats as established by titrating with dilute acids or bases. Svensson and Tornberg (1998) proposed titrating minced beef with a strong acid, such as hydrochloric acid, to better establish the buffering capacity of the meat as strong acids can be completely dissociated. These studies were based on determining the balances of chemical compounds at equilibrium at a given quantity of weak or strong acid solution. In these approaches it is assumed that the meat matrix acts as a base that will be protonated by the acid added in the pH range (usually that of marinades) used (Goli et al., 2007). Several authors have also presented values for the buffering capacity of meat by titrating weak or strong acids and bases at different pH ranges. Castellini and Somero (1981) reported that the buffering capacity of pork *adductor* and beef *temporalis* muscle are 50 and 52 mmol H^+/ (pH* kg meat), respectively, in a pH range of 6 to 7. The buffering capacity of beef *longissimus* muscle was reported as 49 mmol H^+/ (pH* kg meat) at pH 5 (Rao & Gault, 1989). Puolanne and Kivikari (2000) also reported that the buffering capacities for pork and beef *triceps brachii* and *longissimus* muscles are 45, 52, 48 and 51 mmol H^+/ (pH* kg meat), respectively, in a pH range of 5.5 to 7. In the same study, the buffering capacity for broiler breast muscle was reported as 58 mmol H^+/ (pH* kg meat) in the same pH range. The buffering capacity of broiler muscle was higher than pork and beef most probably due to the fact that light muscles in white meat have a higher content of histidine compounds than the red meat (Olsman & Slump, 1981). Due to the varying acids and dilution ratios used in these studies it is difficult to compare the results.

Bonifer and Froning (1996) reported that chicken skin is composed of 78.5% fat and 19.3% protein. The fat composition found in broiler skin by Piette et al. (2001) was 43.4%. The high

levels of fat from chicken skin are significantly different as compared to that from chicken meat which is in the range of 0.68 – 11.7% depending on the breed and muscle types (Mourao et al., 2008; Wattanachant et al., 2004). In a previous study (Tan et al., 2014) it was suggested that a different buffering effect by chicken skin as compared to that by chicken meat may impact the survival of *Salmonella* attached to these components when treated with acids. The aim of this study was: (1) to investigate whether the difference in fat composition of chicken skin and chicken meat plays a role in the previously observed differences in buffering capacity: and (2) to investigate if the presence of fat plays a role in the survival of *Salmonella* on chicken meat and chicken skin treated with acetic acid.

4.2 Materials and Methods

4.2.1 Fat extraction from chicken skin and meat

Fresh chicken breasts with skins were purchased from a local market. Sub-samples of chicken skin and meat were aseptically cut to a weight of 500 g ± 0.2 g each, vacuum-packed and stored frozen in -45 ℃. The frozen samples were thawed overnight at 4 ℃ before use.

Fat extraction was performed as described previously (Piette et al., 2001) with modifications. Frozen skin and meat samples were comminuted in a blender (Kenwood, China) equipped with a 9 mm knife set. Comminuted skin was heated in an 80 ℃ shaking water bath for 2 hours until the core temperature reached 80 ℃. Samples were kept at 80 ℃ for an additional 5 minutes, centrifuged (15 minutes at 10,000 x *g*; Hettich, USA) and left for 18 hours at 1 ℃ to allow the supernatant fat layer to solidify. Separated fats were removed from the gelled aqueous phase and the total amount of extracted fat was weighed. This was repeated with the meat samples in a parallel experiment. Extract yield was expressed as the percentage of

extracted fat and as the percentage of fat initially present in the skin that was recovered, according to the equation below:

% of extracted fat = (weight of extracted fat per 100 g skin or meat/ initial fat content of 100 g skin or meat sample) x 100

Initial fat content of skin and meat sample was determined in triplicate using Soxhlet solvent extraction procedure number 991.36 (AOAC, 2000). The extracted fat and the remnants of skin and meat left after fat extraction were stored at -20 ℃ for further use.

4.2.2 Buffering capacity determination

Chicken skin and meat samples were prepared as described in 4.2.1. Fat extracted from chicken skin and meat and the skin and meat material left after fat extraction (hereafter referred to as skin remnants and meat remnants) were weighed (5 g ± 0.2 g) for buffering capacity determination. Phosphate buffered saline (PBS; 2.7 mM KCl, 10 mM Na_2HPO_4, 17 mM KH_2PO_4, 137 mM NaCl; 1st-Base Singapore) was used as a control.

The buffering capacity determination was conducted according to Puolanne and Kivikari (2000) with modifications. Briefly, each sample was homogenized with sterile distilled water with a ratio of 5 g sample to 50 ml water (1:10). The homogenates were titrated using 1M HCl (Merck, Germany) and 1M NaOH (Merck, Germany). A 1 ml of titrant was added at 2 minutes interval. The homogenates were stirred during titration and the titrations were carried out at room temperature. The pH of homogenates was measured using a calibrated pH meter (Sartorius, Germany).

The titration curve for pH 4 to 8 was obtained by combining data from the acid and base titrations. Buffering capacity was calculated and expressed in mmol H^+/ (pH*kg meat) according to the equation below:

$BC_n = \Delta A / \Delta pH$,

where

BC_n = the average buffering capacity for the range between two successive observations,

ΔA = the increment of acid or base, and

ΔpH = the corresponding change in pH

Buffering capacity curves were constructed with BC_n values plotted against the midpoint of each respective pair of pH values. Fourth degree polynomial equations were fitted to the buffering capacity curves and the buffering capacity was calculated according to the equations.

4.2.3 Survival of Salmonella *on chicken components treated with acetic acid*

Fat was extracted from chicken skin and meat as described in section 4.2.1. A 5 ml aliquot of extracted fat from skin was solidified in a sterile petri dish (Jatikhas, Malaysia) at 4 ℃ to allow for easy attachment of *Salmonella.* Fresh chicken meat with skin was bought from local market, transported to the laboratory and used immediately after aseptically cutting it into 5 g ± 0.2 g sub-samples. Sub-samples of skin remnants and meat remnants were also weighed to 5 g ± 0.2 g for use in attachment studies.

Two strains of *Salmonella* Typhimurium (ATCC 33062 and ATCC 14028) and two strains of *Salmonella* Enteritidis (ATCC 13076 and ATCC 49216) were used in the study. Experiments were conducted on each strain independently and the inoculum for each *Salmonella* strain

was prepared according to Tan et al. (2014). Briefly, each of the *Salmonella* strains was streaked on Tryptic Soy Agar (TSA; Oxoid, UK) from frozen pure glycerol stock cultures and incubated for 24 hours at 37 ℃. A colony of each *Salmonella* strain was taken from the TSA and inoculated into 10ml of Tryptic Soy Broth (TSB; Oxoid, UK) and incubated for another 24 hours at 37 ℃. After incubation the cultures were centrifuged at 5000 x *g* (Hettich, USA) for 10 minutes. The supernatant was discarded and the pellets were re-suspended in 10 ml of 1 x PBS.

A 5 ml aliquot of *Salmonella* was inoculated onto 5 g of each of the samples (chicken skin, chicken meat, chicken fat, skin remnants and meat remnants). The *Salmonella* cells were allowed to attach to the sample surfaces for 1 hour at 25^{o}C with shaking at 100 rpm (N-biotek, Korea). Samples were then rinsed 3 times with 1 x PBS to ensure all unattached cells had been removed.

A concentrated (5M) stock solution of acetic acid (Merck, Germany) was diluted in 1 x PBS to concentration of 0.3M (pH 3.8). Inoculated chicken components were placed into the diluted acid and were incubated at 4 ℃ for 24 hours. These incubation conditions were used to mimic typical commercial or home marination. *Salmonella* was enumerated according to Tan et al. (2014). Briefly, inoculated chicken components were transferred into sterile stomacher bags and 50 ml of Buffered Peptone Water (BPW; Oxoid, UK) was added to each. Samples were homogenized for 2 minutes. Serial decimal dilutions were conducted and 0.1 ml of appropriate diluted samples was inoculated directly onto selective plates prepared using the thin agar layer technique. Xylose Lysine Deoxycholate Agar (XLD; Oxoid, UK) was used as selective medium and TSA as the non-selective overlay. The plates were counted after

incubation for 24 hours and 37 ℃ and results were recorded as colony forming units per g (CFU/g).

4.2.4 Scanning electron microscopy (SEM)

Salmonella attached to chicken skin and meat was examined under SEM as previously described by Noriega et al. (2010) with modifications. Chicken skin was removed from the underlying muscle using a sterile scalpel and forceps. Sub-samples of skin of approximately 5 mm thickness were cut and placed in a sterile petri dish (Jatikhas, Malaysia). Sub-samples of chicken breast meat of approximately 5 mm thickness were also and placed in another sterile petri dish. A 100 µL of *Salmonella* inoculum was deposited carefully onto the surfaces of the skin and meat. All samples were then left for 60 minutes at room temperature to allow *Salmonella* to attach. The samples with *Salmonella* attached were then chemically fixed with 2.5% glutaraldehyde (R&M Chemicals, USA) for 40 minutes and gently washed twice in PBS. Dehydration of the samples was conducted in a series of ethanol concentrations (20, 40, 60, 80 and 100% v/v ethanol in water; 20 minutes in each concentration). Samples were then transferred to a series of acetone concentrations (30, 50, 70, 90 and 100% v/v acetone in ethanol; 20 minutes in each concentration) and air dried in the laminar flow. Samples were gold-sputtered using a sputter coater (Q150RS; Quorum, UK) and viewed under a SEM (S-3400N; Hitachi, Japan).

4.2.5 Statistical analyses

All experiments were conducted as three independent trials. Differences in fat extraction between chicken skin and meat, between the buffering capacity values calculated for the chicken components and between *Salmonella* plate counts in the attachment and acid treatment assays were determined using one-way ANOVAs. A post-hoc Tukey's test was

used for pairwise comparison to determine the significant difference between samples at $p < 0.05$. A Pearson correlation test was conducted to determine if there was a correlation between buffering capacity and the viable count of *Salmonella* after treatment with acetic acid at $p < 0.05$. All tests were conducted using SPSS software version 16.0 (PASW Statistics 20; SPSS Inc).

4.3 Results and Discussion

4.3.1 Fat extraction

The initial fat content of the chicken skin used in this study was 51.51 g / 100 g and that in meat was 2.83 g / 100 g as determined using Soxhlet extraction (Table 2). This result is in agreement with a higher fat content in chicken skin as compared to chicken meat reported in the literature (Mourao et al., 2008; Wattanachant et al., 2004). The initial fat content of the chicken meat in this study was lower than that reported by Mourao et al. (2008) in Portuguese broiler meat (11.7%) but higher than that reported by Wattanachant et al. (2004) in Thai broilers (0.68%) and Thai indigenous chicken meat (0.37%). This suggests that the fat composition of chicken meat varies depending on geographic region, species and muscles type.

The percentage of fat extracted in our study was similar to the 76.5% reported by Piette et al. (2001) (Table 2). This may be because of the grind size of the chicken skin and temperature used were the same for the two studies. A temperature of 80 ℃ was chosen for our study (instead of the commonly used 50 ℃) as both were used in Piette et al. (2001) but the higher temperature is more effective in reducing microbial contamination. Due to the low amount of fat extracted from the chicken meat, only fat from the skin was used for further experiments in this study.

Table 2. Initial fat content and yield of extracted fat from chicken skin and meat

	Sample type	
Fat content	**Skin /100g**	**Meat /100g**
Initial fat content	51.51 ± 1.35 [a]	2.83 ± 0.63 [b]
Extracted fat	37.17 ± 0.89 [a]	0.27 ± 0.05 [b]
Percentage of fat extracted	72.16 ± 2.18 [a]	9.54 ± 0.89 [b]

[a,b] Different lowercase letters indicate significant difference in the comparisons between chicken skin and meat at $p < 0.05$.

4.3.2 Buffering capacity determination

The raw data for the buffering capacity of PBS, chicken skin, meat, extracted fat, skin remnants and meat remnants are presented in Appendix V. The buffering capacity of each chicken component at a given pH can be predicted using the polynomial equations obtained from the buffering capacity curves (Figure 4). For example, at pH 4 (the pH of 0.3M acetic acid used in section 4.3.3) the buffering capacity of PBS was the highest ($p < 0.05$), followed by meat, meat remnants, extracted fat, skin remnants and lastly skin (Table 3). At a slightly higher pH (pH 5), the buffering capacity of PBS was still the highest ($p < 0.05$) but was followed by meat, meat remnants, skin and lastly extracted fat and skin remnants (Table 3). The PBS control buffers acidic solutions to pH 7.4 and therefore at pH 4 and 5, used in the examples, PBS has a large buffering effect allowing it to buffer the low pH solutions to near neutral pH. Chicken meat has been previously found to have a buffering effect which neutralizes acidic pH and protects *Salmonella* cells on chicken against hydrochloric acid (Tan et al., 2014). It is not surprising therefore that chicken meat has the high buffering capacity values shown in Table 3. The meat remnants had a slightly lower buffering capacity

compared to the meat suggesting that the fat which was extracted in low amounts from the meat (Table 2) may have contributed to some of its buffering capacity. The buffering capacity of chicken skin calculated at pH 5 was a positive number but at pH 4 was negative indicating there is no buffering effect of skin at the latter pH. This may be because pH 4 is near the saturation point of the buffering capacity of the skin. Buffering capacity has limits and once this has reached saturation point the ability to withstand the pH fluctuations is poor (Goli et al., 2007; Kyl ä-Puhju et al., 2004). It is interesting to note that the buffering capacities of fat extracted from skin and remnants of the skin were no different from each other ($p > 0.05$). This indicates that both fat alone and skin remnants alone were not able to contribute a strong buffering effect individually. When the components occur together in the skin, however, a stronger buffering capacity as compared to fat and skin remnants alone is present. This suggests that fat and other components in skin, such as lactates and proteins, may contribute to the buffering capacity. The design of this study allowed provides general information about the buffering effect of chicken fat when present. It cannot be extrapolated to specific *in situ* cases in which, for example, skin has been removed from a carcass or part of it as this was not investigated.

From Table 3 it can be seen that the buffering capacities of all components (except skin) at pH 4 were higher ($p < 0.05$) than buffering capacities at pH 5. This is probably because as more acid is added to a buffer solution the ability to withstand pH fluctuations is larger (Kyl ä-Puhju et al., 2004). In the present study fourth degree polynomial equations were used to predict the values of the buffering capacity of each component at a given pH. Fourth degree polynomials were used as they had the least change in variance values as compared to other degree polynomials (for example, 0.000003042 for the fourth degree as compared to 0.00031234 for the third degree). It should be noted that the best polynomial of degree k+1

will always fit as well as the best polynomial of degree k because the set of degree k+1 includes all k degree polynomials (Hazewinkel, 2002). However, in some cases, over-fitting may occur which exaggerates the minor fluctuations of the data and affects the accuracy of predictive model. This is a different approach from that reported in previous studies by Goli et al. (2012), Hill et al. (1985), Puolanne and Kivikari (2000), Rao and Gault (1989) who used a spline smoothing procedure to fit the curves. Mean buffering capacity was calculated from the curves by taking the average of minimum and maximum buffering capacity points for a given pH range. For example, Puolanne and Kivikari (2000) constructed a buffering capacity curve for broiler breast meat using the spline smoothing procedure and reported that the mean buffering capacity of the meat was 58 mmol H^+/(pH*kg) in a pH range of 5.5 to 7. Similarly, chicken breast meat was reported to have a buffering capacity of 151 $\pm$ 1 mmol H^+/ (pH*kg) in a pH range of 3.5 to 5 (Goli et al., 2012). As spline smoothing does not fit a curve of a specific shape it may fail to reveal overlapping of peaks. It may therefore not be the best way to analyse data of this sort and we instead used polynomial equations with high R^2 values. The limitation of using polynomials is that the predictive accuracy and reliability of the curves may be limited outside a range of pH 4 to 8.

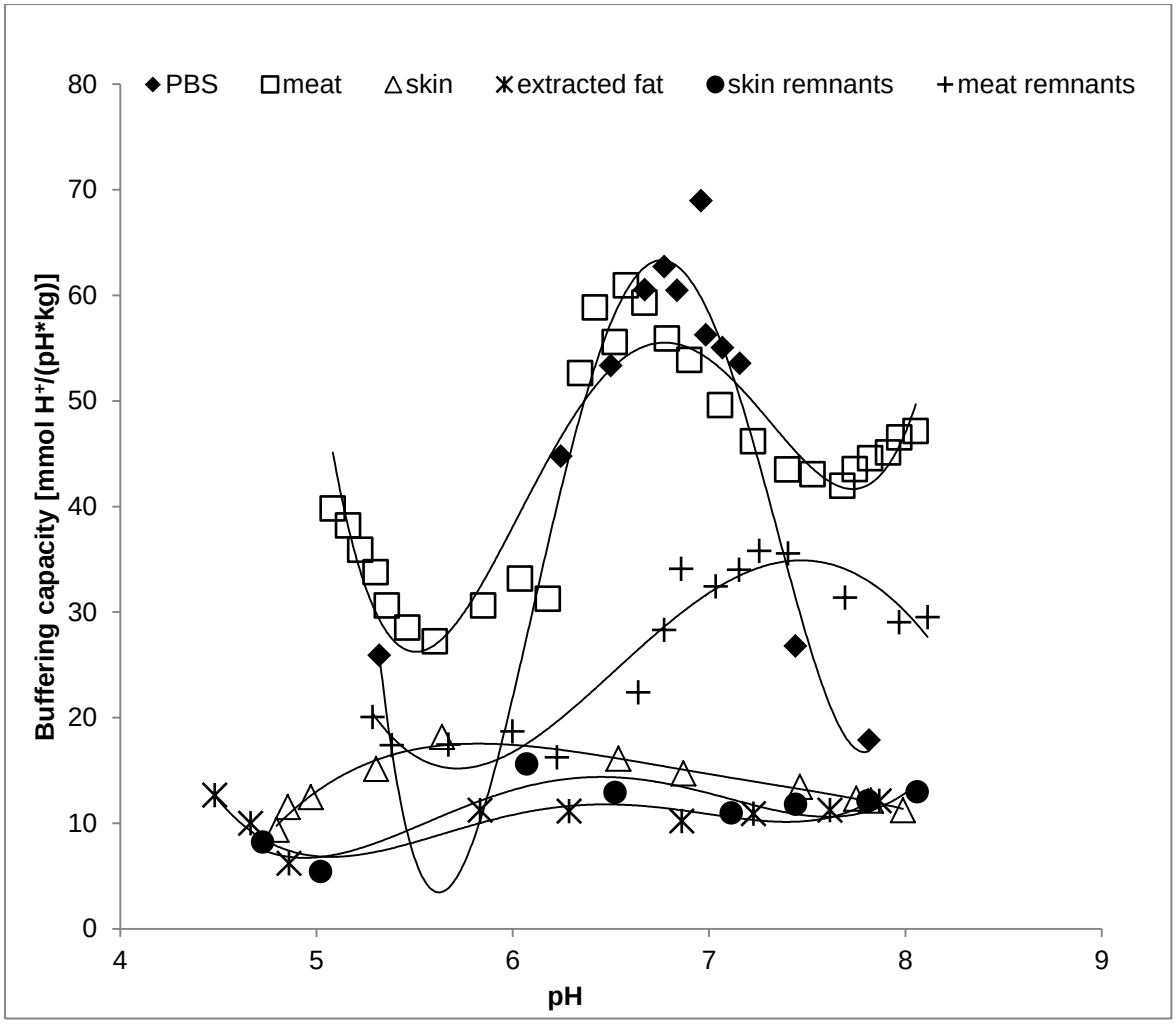

Figure 4. Buffering capacity curves of PBS, chicken skin, meat, extracted fat, skin remnants and meat remnants.

Table 3. Polynomial equations and examples of calculated buffering capacity of chicken components and a PBS control

Component	Polynomial equations obtained from BC curves	Buffering capacity [mmol H^+/(pH*kg)]	
		pH 4	**pH 5**
PBS	$y = 14.071x^4-388.22x^3+3960.6x^2-17707x+29318$, $R^2= 0.934$	615.7 ± 19.11 ^{A,a}	64.9 ± 6.11 ^{A,b}
Meat	$y = 13.55x^4-361.55x^3+3583.8x^2-15630x+25333$, $R^2= 0.824$	483.4 ± 10.04 ^{B,a}	53.0 ± 1.49 ^{A,b}
Skin	$y = -0.502x^4+14.417x^3-149.35x^2+696.6x-1191$, $R^2= 0.964$	-17.3 ± 3.13 ^{C,a}	13.0 ± 2.51 ^{B,b}
Extracted fat	$y = 1.683x^4-42.515x^3+398.1x^2-1635.7x+2495.4$, $R^2= 0.790$	32.1 ± 1.98 ^{D,a}	7.03 ± 1.86 ^{C,b}
Skin remnants	$y = 1.749x^4-44.293x^3+414.5x^2-1695.7x+2566.6$, $R^2= 0.810$	28.7 ± 2.79 ^{D,a}	6.85 ± 3.14 ^{C,b}
Meat remnants	$y = 1.134x^4-37.265x^3+439.5x^2-2219.3x+4089.4$, $R^2= 0.904$	149.6 ± 7.61 ^{E,a}	31.3 ± 3.71 ^{D,b}

Data are presented as mean ± standard deviation where n = 3. ^{A,B,C} Different uppercase letters indicate significant difference in comparisons between components (PBS, meat, skin etc.) at $p < 0.05$ i.e. within columns. ^{a,b,c} Different lowercase letters indicate significant difference between pH 4 and pH 5 at $p < 0.05$ i.e. within rows.

4.3.3 Survival of Salmonella *on chicken components after treatment with acetic acid*

The behaviours of all four strains of *Salmonella* attached to chicken components after acid treatment were not significantly different and therefore only the data for *S.* Typhimurium ATCC 33062 is presented as an example in Figure 5. The data for the other three *Salmonella* strains are presented in Appendix VI. From an initial inoculum (before attachment) of ~9log CFU/g, *S.* Typhimurium ATCC 33062 attached best ($p<0.05$) to chicken meat (~8log CFU/g), followed by meat remnants (~7.5log CFU/g, skin (~7log CFU/g), skin remnants (~4log CFU/g) and extracted fat (~1.5log CFU/g). *Salmonella* cells were detached from chicken components by homogenizing to most effectively harvest all bacteria present. The higher numbers of *Salmonella* on chicken meat and skin, as compared to other components, may be because the cells were both actively attached to, and also potentially trapped, in the irregular structure and crevices on skin or the network of collagen and reticular fibres in meat surfaces (Gill et al., 1984; Thomas & McMeekin, 1980). As can be seen in the micrograph in Figure 6A the surface of chicken skin is irregular and consists of crevices. The normal histology of non-processed chicken skin consists of an epidermis with keratin and lipids and an underlying dermal stratum consisting of connective tissue. However, the epidermal is largely removed during processing, leaving cellular debris attached to the dermis and many capillary-size holes (Thomas et al., 1987). A closer examination (Figure 6B and 6C) shows *Salmonella* cells attached to the rough surface and trapped in the crevices of the chicken skin. The micrograph in Figure 6D shows the homogenous structure of chicken meat which is composed of a network of collagen and fibres. Meat tissue consists of long, thin, parallel cells arranged into fibre bundles which surrounded by elastin fibres (Noriega et al., 2010). A closer examination (Figure 6E and 6F) shows *Salmonella* cells trapped between the swollen fibres. Thomas et al. (1987) suggest that the cells can be drawn in with water into the fibre tissues and migrate to a depth of about 25 mm. Bacteria that are trapped and attached firmly to these

structures are hard to remove by decontamination and simple rinsing (Noriega et al., 2010). The poor attachment on the surface of fat seen in the present study may be because the surface is not as rough as the meat, skin and their respective remnants. The adhesion of bacteria to a surface is influenced by surface composition, roughness, charge and hydrophobicity of both surface and cells (Treese et al., 2007). Bacterial cells attached to and colonize surfaces which are more elastic, porous and rougher surfaces as compared to dense and smooth surfaces (Katsikogianni & Missirlis, 2004; Wan Norhana et al., 2009).

A 0.3M concentration of acetic acid was used to treat the attached cells because it has been established to be bactericidal to *Salmonella* in a previous study (Tan et al., 2014). When treated with 0.3M acetic acid numbers of *Salmonella* attached to skin decreased the most by ~7 log CFU/g ($p < 0.05$), followed by those attached to meat and meat remnants (both by ~6 log CFU/g), extracted fat (by ~1.5 log CFU/g) and lastly skin remnants (by ~1 log CFU/g) (Figure 5). The substantial reduction in numbers of *Salmonella* attached to skin as compared to other components after acetic acid treatment may be related to the buffering capacity of the skin. The Pearson correlation test showed that there is a significant positive correlation between buffering capacity and the viable count of *Salmonella* after treatment with acetic acid ($r = 0.623$, $n = 15$, $p<0.05$). The pH of 0.3M acetic acid was 3.8 which is close to pH 4 at which skin has no buffering capacity as described in section 4.3.2. Meat on the other hand has a better buffering effect which is probably able to neutralize the acidic pH of acetic acid and protect *Salmonella* against acetic acid (Bjökroth, 2005; Ramnani et al., 2010; Tan et al., 2014). Interestingly, the results also indicate that after extraction of fat from skin the remnants are better in protecting attached *Salmonella* cells from acetic acid as compared to other components (Figure 5). We suggest that fat content present in skin may enhance the vulnerability of the attached cells to acetic acid by a mechanism yet to be determined.

However, we could not exclude the possibility that some components of meat and skin, such as amino acids, may contribute to the homeostasis of intracellular pH of the *Salmonella* and exert a protective effect against acetic acid. Further work need to be carried out to determine the exact cause of the vulnerability of attached cells to acetic acid.

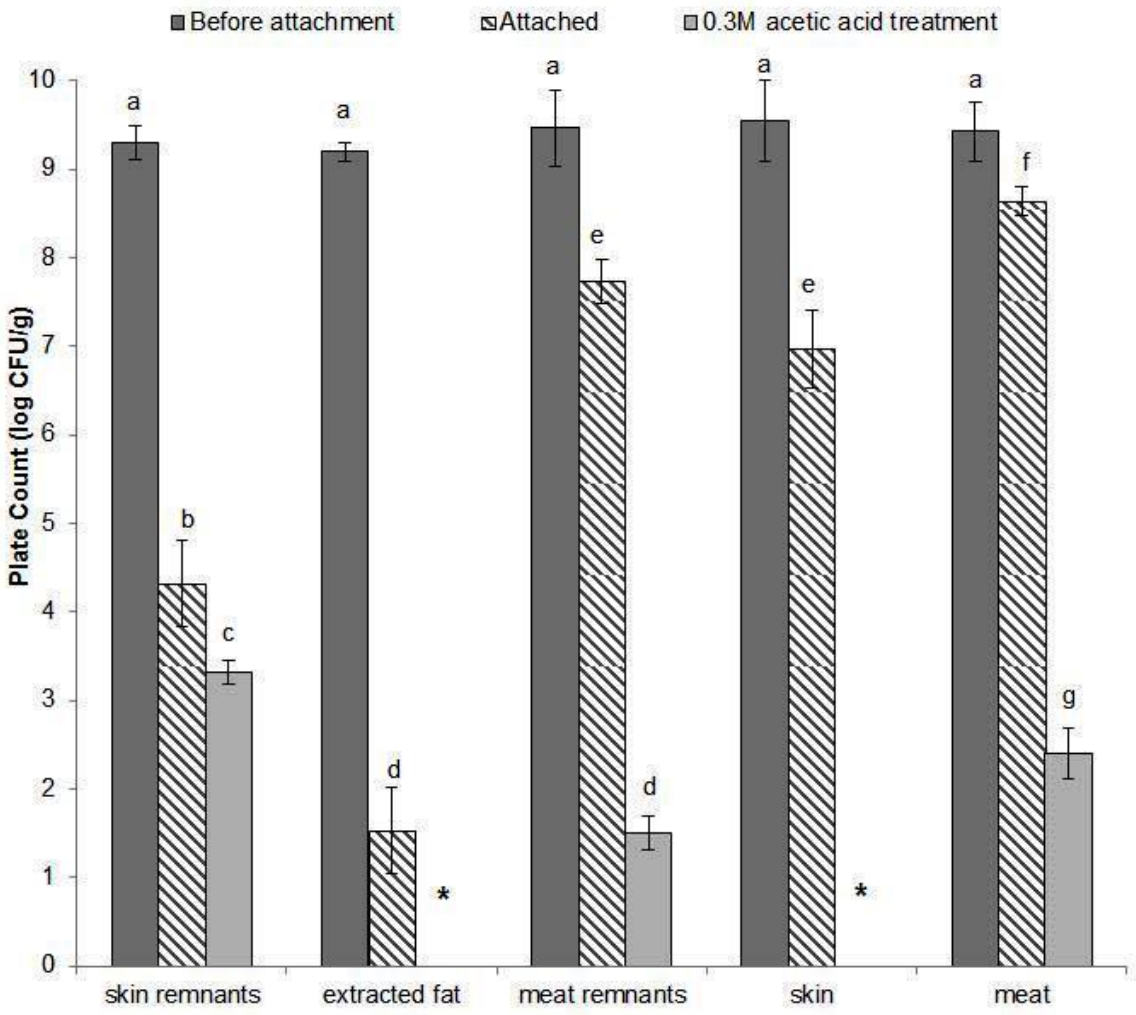

Figure 5. Viable count of *S.* Typhimurium ATCC 33062 before attachment, firmly attached to the surfaces of each component and after treatment with 0.3M acetic acid. Results are presented as mean ± SD where n = 3; lowercase letters (a,b,c) indicate significant difference between number of cells attached and after acetic acid treatment. * indicates no viable count was observed after the acetic acid treatment.

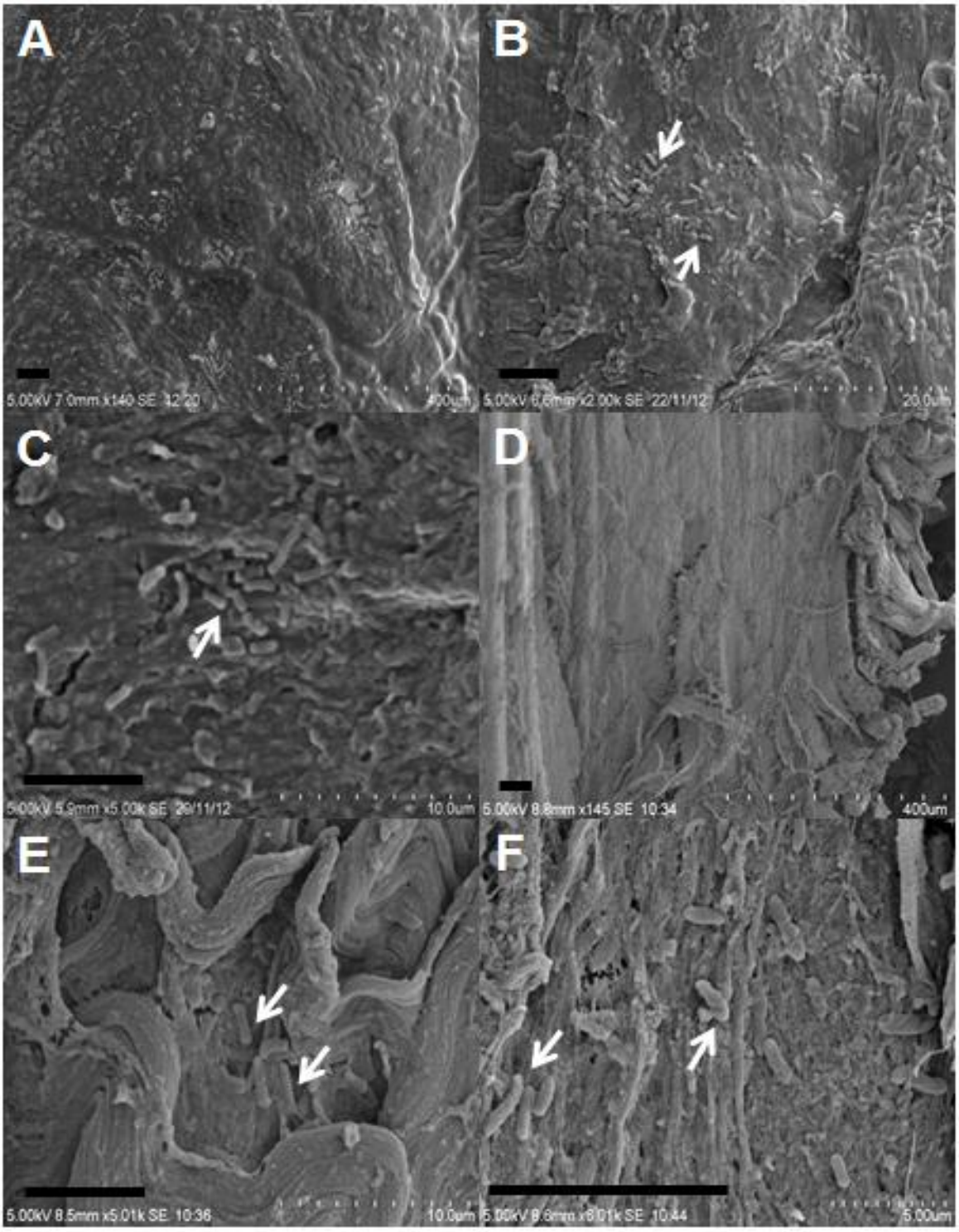

Figure 6. SEM images of *S.* Typhimurium ATCC 33062 attached to chicken skin and meat. A = chicken skin surface; B, C = cells attached to and trapped in the rough surface and crevices of skin (shown by arrows); D = chicken meat surface; E, F = cells attached to and trapped in the network of meat fibres (shown by arrows). Scale bars = 2.5 μm.

4.4 Conclusion

Chicken skin has a higher fat content as compared to chicken meat. The difference in fat composition between chicken skin and meat has an effect on the buffering capacity of these components. However, in addition to fat composition other components, such as proteins, may influence the buffering capacity of chicken skin and meat. Extracted fat alone and skin remnants alone was not able to contribute to a strong buffering capacity but when the components occur together a strong buffering capacity was observed. *Salmonella* cells attached better to chicken meat, skin and their respective remnants than fat probably because the surface of extracted fat is not as rough as these components. Skin remnants were better at protecting attached *Salmonella* from acetic acid than other components. We suggest the fat content present in the skin may increase the vulnerability of attached cells to acetic acid.

Chapter 5

The Antimicrobial Mode of Action of Acetic Acid against *Salmonella* on Chicken

The work presented in this chapter represents the following publication accepted for peer review:

Tan, S.M., Lee, S.M., & Dykes, G.A. (Accepted) Acetic acid induces pH independent cellular energy depletion in *Salmonella enterica. Foodborne Pathogens and Disease*.

Declaration for Thesis Chapter 5

Declaration by candidate

In the case of Chapter 5, the nature and extent of my contribution to the work was the following:

Nature of contribution	**Extent of contribution (%)**
I was responsible for 100% of data analysis and 90% of drafting and writing.	90%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	**Nature of contribution**	**Extent of contribution (%)**
Sui Mae Lee	SL was responsible for 2% of writing and the review of the publication.	2%
Gary Dykes	GD was responsible for 8% of writing and the review of the publication.	8%

Candidature's Signature		**Date**

Declaration by co-authors

The undersigned hereby certify that:

(1) The above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors;

(2) They meet the criteria for authorship in that they have participated in the conception, execution or interpretation, of at least that part of the publication in their field of expertise;

(3) They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;

(4) There are no other authors of the publication according to these criteria;

(5) Potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and

(6) The original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	**School of Science, Monash University Malaysia**

Signature 1	[redacted]	**Date**
Signature 2	[redacted]	**Date**

5.1 Introduction

Organic acids are saturated straight chain carboxylic acids and their derivatives including unsaturated, hydroxylic and phenolic compounds (Cherrington et al. 1991). The application of organic acids to food and food processing surfaces, and particularly meat surfaces, to control microorganisms is a common procedure as it is both simple and effective (Hinton & Corry, 1999). Several studies have demonstrated the bactericidal activity of acetic acid, lactic acid, citric acid, propionic acid and sorbic acid on food (Doležalová et al., 2010; González-Fandos et al., 2009; Narendranath et al., 2001; Over et al., 2009; Smaoui et al., 2011; Tamblyn & Conner, 1997).

Despite their widespread use the antimicrobial mode of action of organic acids is still not yet fully understood (Mani-López et al., 2012). A number of explanations have been forwarded for the antimicrobial activity of organic acids. Organic acids can exist either as charged or uncharged molecules, depending on the protonation state of their acidic group and as determined by the pKa of the acid and the pH of the environment (Brul & Coote, 1999). The antimicrobial effect of organic acids may be due to the lipid permeability of the undissociated molecules which can freely diffuse into the cytoplasm. As these molecules diffuse across the membrane and encounter a neutral environment in the cytoplasm dissociation of the acid into anions and free protons is favoured (Eklund, 1985; Salmond et al., 1984). The accumulation of acid anions in the cytoplasm is a possible mode of antimicrobial action (Cherrington et al., 1991). In addition, due to the pH shifting from the internal pH range of optimal enzymatic activity, protein and nucleic acid synthesis are adversely affected by the presence of organic acids at an elevated level (Cherrington et al., 1990). Russell (1992) also reported that the accumulation of the acid anion hinders the proton motive force and inhibits the microbes' ability to re-alkalinize the cytoplasm. The accumulation of high levels of organic acids anions

in the cytoplasm may interfere with essential metabolic reactions. In order to maintain the neutral pH and to restore homeostasis, bacterial cells induce an energetically expensive stress response which may result in depletion of cellular energy (Davidson, 2001; Ricke, 2003). Roth and Keenan (1971) reported that lactic acid can cause sublethal injury to *Escherichia coli* and indirect evidence indicated that the injury involved the disruption of the lipopolysaccharide (LPS) layer. A later study (Alakomi et al., 2000) reported that lactic acid permeabilized the membrane of Gram negative cells and released the LPS from outer membrane. Citric and malic acids were found to chelate (or intercalate with) the outer membrane of *Salmonella* (Helander & Mattila-Sandholm, 2000).

Acetic acid is one of the most commonly used organic acids and has generally recognized as safe (GRAS) status for use in food and meat products (FDA, 2013). This includes its use in marinades for chicken in the form of vinegar and as a disinfectant on chicken carcasses (Doležalová et al., 2010; Liao et al., 2003; Over et al., 2009). A previous study (Tan et al., 2014) suggested that the inhibitory activity of acetic acid against *Salmonella* on chicken was due to other factor than its acidic pH. Here we report on an investigation into the mode of action of acetic acid against *Salmonella* with respect to its ability to disrupt cellular membranes and deplete cellular energy.

5.2 Materials and Methods

5.2.1 Preparation of Salmonella *cell suspension and acid treatments*

Salmonella enterica serovar Typhimurium (ATCC 33062 isolated from chicken tissue and ATCC 14028 a laboratory strain of human origin) and *Salmonella enterica* serovar Enteritidis (ATCC 13076 of human origin and ATCC 49216 of animal origin) were used in the study and cell suspensions were prepared according to Tan et al. (2014). Briefly, each of the

Salmonella strains was streaked on Tryptic Soy Agar (TSA; Oxoid, UK) from pure glycerol stock cultures and incubated for 24 hours at 37 ℃. A colony of each *Salmonella* strain was inoculated into 10 ml of Tryptic Soy Broth (Oxoid, UK) and incubated for another 24 hours at 37 ℃. The broth cultures were centrifuged at 5000 x *g* (Hettich, USA) for 10 minutes at 4 ℃. The supernatant was discarded and the pellet was re-suspended in 10 ml of 1 x Phosphate Buffered Saline (PBS; 2.7 mM KCl, 10 mM Na_2HPO_4, 17 mM KH_2PO_4, 137 mM NaCl; 1st-Base, Singapore) and used immediately.

Three set of acid treatments were prepared for experiments. For the first set of treatments a 5M stock solution of acetic acid (Merck, Germany) was diluted in 1 x PBS(1st-Base, Singapore) to a concentration of 0.3M (pH 3.8) and 0.5M (pH 3.25). These concentrations were used to simulate the use of acetic acid in the form of vinegar in marinades (~pH 4) and were established to be bactericidal to *Salmonella* in a previous study (Tan et al., 2014). For the second set of treatments solutions of 0.3M and 0.5M acetic acid were prepared as above and the pH was adjusted to pH 6.4 using a few drops of concentrated sodium hydroxide (NaOH; Merck, Germany) to maintain the concentrations of the solutions. This was done to simulate the pH environment that *Salmonella* attached to chicken meat surfaces are exposed to as reported in a previous study (Tan et al., 2014). A third acid treatment consisted of 1M HCl adjusted to pH 3.8 and pH 3.25 using concentrated NaOH to allow comparison between the effect of organic and inorganic acid at the same pH. Ampicillin-treated cells (United States Biological, US) were used as a positive control and un-treated cells were used as a negative control. For experiments a 5 ml aliquot of each acid treatment was added to a 5 ml *Salmonella* inoculum and incubated at 4 ℃ for 24 hours.

5.2.2 Determination of leakage of intracellular components

The integrity of cellular membrane was determined initially and after 24 hours incubation at 4 ℃ treated with 0.3M (pH 3.8) acetic acid and 0.5M (pH 3.25) acetic acid according to Dykes (1999) with modifications. Aliquots of the cells were centrifuged for 10 minutes at 10,000 x *g*, 4 ℃. The absorbance of the cell-free supernatant fluid was read at 260 nm (represented the leakage of nucleic acids) and 280 nm (represented the leakage of proteins) with a UV-VIS Spectrophotometer (Shimadzu, Japan) using 1 x PBS as a reference and ampicillin (United States Biological, US) as a positive control.

5.2.3 Scanning electron microscopy

Glass slides (Thermo Scientific, US) were broken into approximately 5 mm x 5 mm sections and placed in a sterile petri dish (Jatikhas, Malaysia). *Salmonella* inoculum was prepared and treated with 0.5M (pH 3.25) acetic acid. Specifically, a 100 µl of untreated *Salmonella* inoculum and a 100 µl acetic acid-treated *Salmonella* inoculum were allowed to attach on the glass slides for 60 minutes at room temperature. All sample slides were rinsed in 1 x PBS, air dried and chemically fixed with 2.5% glutaraldehyde (R&M Chemicals, USA) for 40 minutes and gently washed twice again in PBS. Dehydration of the samples was conducted in a series of ethanol concentrations (20, 40, 60, 80 and 100% v/v ethanol in water; 20 minutes in each concentration) and air dried in the laminar flow. Samples were gold-sputtered using a sputter coater (Q150RS; Quorum, UK) and viewed under a SEM (S-3400N; Hitachi, Japan). The area, length and perimeter of acetic acid-treated and controls cells under SEM were analysed using ImageJ 1.22d (The National Institutes of Health, USA).

5.2.4 Measurement of the effect of acetic acid on the intracellular ADP/ATP ratio

All three sets of acid treatments as described above were used on *Salmonella* cells in this assay. ATP and ADP were measured using the ADP/ATP Ratio Bioluminescent Assay Kit

(ab65313; Abcam, UK) according to the manufacturer's instruction. Briefly, 100 µl of reaction mix (10 µl of ATP Monitoring Enzyme and 90 µl of Nucleotide Releasing Buffer) was added to the appropriate wells of a 96-well white plate (Greiner Bio-one, Germany) and left for 2 hours at room temperature to eliminate ATP contamination. Background luminescence (Data A) was then read using an Infinite® 200 PRO microplate reader (Tecan, Switzerland). The treated and untreated *Salmonella* cultures prepared as above were centrifuged at 10,000 x *g* for 10 minutes and resuspended in 1 x PBS to remove any interfering substances or acid from the cells. A 10 µl of cell suspension was transferred to a 96-well white plate and luminescence (Data B) was read. The level of ATP was calculated by subtracting the background luminescence from the ATP luminescence reading. To measure ADP levels, 1 µl of ADP Converting Enzyme was added to the wells and luminescence (Data C) was read again after 2 minutes. The ADP/ATP ratio was calculated using the equation provided by the manufacturer:

(Data C – Data B) / (Data B – Data A)

5.2.5 Statistical analyses

All experiments were conducted in triplicates with independent cultures. Differences in the leakage of intracellular components of the respective controls and acetic acid treatments were determined using one-way ANOVA. A post-hoc Tukey's test was used for pairwise comparison to determine the significant difference between samples at $p < 0.05$.The differences of the ADP/ATP ratios between acid treatments and the four *Salmonella* strains were analysed using a two-way ANOVA with a Tukey's multiple-comparison test. All tests were conducted using SPSS software version 16.0 (PASW Statistics 20; SPSS Inc.).

5.3 Results and Discussion

5.3.1 Determination of leakage of intracellular components

The results of the cellular leakage experiments for all four strains of *Salmonella* treated with 0.3M and 0.5M acetic acid were not significantly different and therefore the data for *S.* Typhimurium ATCC 33062 is presented as an example in Figure 7. The data for the other three *Salmonella* strains are presented in the Appendix VII. The cells treated with ampicillin (positive control) had substantial leakage of intracellular nucleic acids (260 nm) and proteins (280 nm). Ampicillin belongs to the β-lactams antibiotics family which acts upon the penicillin-binding protein (PBP), leading to the weakening of the cell wall and ultimately cell death (Anderson et al., 2012). The level of nucleic acid and protein after treatment of cells with 0.3M and 0.5M acetic acid was not different ($p > 0.05$) as compared to untreated (negative control) cells indicating these treatments did not result in leakage of intracellular components. By contrast lactic acid is able to cause lipopolysaccharide release in *Pseudomonas, E. coli* and *Salmonella* Typhimurium by disrupting the outer membrane of the cells (Alakomi et al., 2000). In addition, sorbic acid was found to cause membrane disruption in yeast as reported by Stratford and Anslow (1998) and Bracey et al. (1998). These studies hypothesized that the undissociated acid molecules of both lactic and sorbic acid are responsible for membrane disruption. The pKa values of lactic acid, sorbic acid and acetic acid are 3.86, 4.75 and 4.74, respectively (Narendranath et al., 2001). At a given acidic pH there is more undissociated acetic acid and sorbic acid present than in an equal concentration of lactic acid due to their higher pKa values. Our results showed that acetic acid at a high concentration of undissociated acids did not cause cellular membrane disruption. This indicates that different organic acids may inhibit bacteria by different mechanisms. For example, Maiorella et al. (1983) showed that acetic acid can interfere with yeast metabolism which increased in the ATP requirement for cell maintenance whereas lactic acid can inhibit

growth of yeast by disrupting cell membrane and releasing lipopolysaccharide from cells (Alakomi et al., 2000; Narendranath et al., 2001). Further work needs to be done to verify the differences in the antimicrobial mode of action of different organic acids. We concluded that acetic acid is unlikely to disrupt the cell membrane of *Salmonella* as no leakage of intracellular component was apparent.

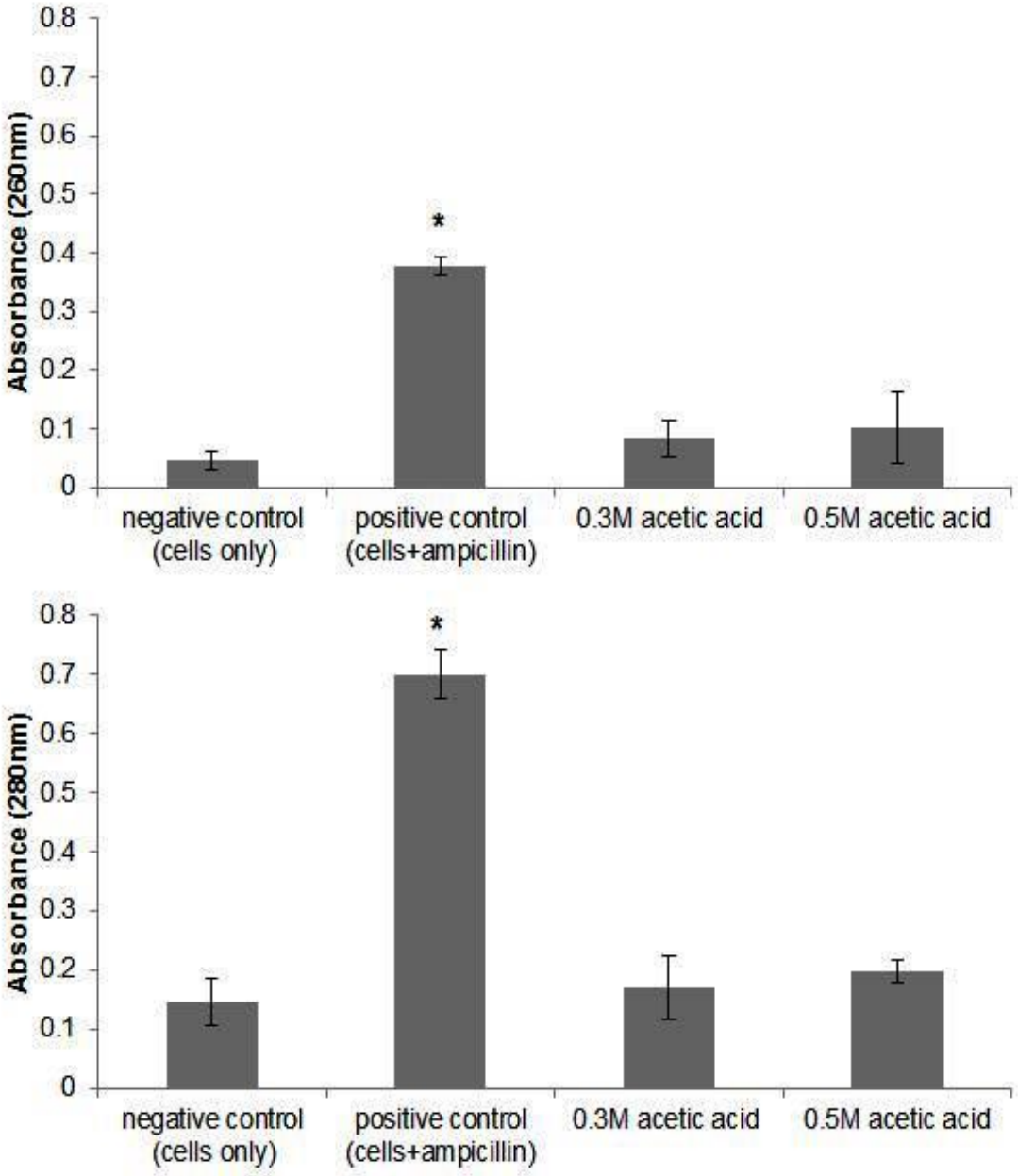

Figure 7. The leakage of intracellular components of *S.* Typhimurium ATCC 33062 at 260 nm (nucleic acid) and 280 nm (proteins) after treatment with 0.3M and 0.5M acetic acid. Results are presented as mean ± SD where n = 3. * indicates a significant difference between positive control and acetic acid treatments.

5.3.2 SEM of acetic acid treated Salmonella *cells*

The SEM micrographs in Figure 8 show the cellular morphology of acetic acid-treated and untreated *Salmonella* cells. Some cells shrinkage was observed due to the alcohol dehydration process during the SEM preparation techniques (Sutton et al., 1993). Figure 8B showed no acid-induced disruption of outer cell membrane or cell wall after treated with 0.5M acetic acid as compared to untreated cell (Figure 8A). This further confirms that acetic acid does not cause cell wall or cell membrane disruption in *Salmonella*. Our study is in agreement with that of Thompson and Hinton (1996) who reported that there was no formic and propionic acid-induced damage on cytoplasmic membrane of *Salmonella* Enteritidis in a transmission electron microscopy (TEM) study. The mean area, length and perimeter of untreated *Salmonella* cells and acetic acid-treated cells are presented in Table 4. All these measures increased significantly ($p < 0.05$) for cells treated with acetic acid. Individual cells with a length of up to 7.35 μm were apparent in acetic acid- treated but not in untreated samples (Figure 8C, 8D, 8E and 8F).The increase in the length of acid-treated cells may be because of the inhibition of DNA synthesis whilst other macromolecules, such as RNA and proteins, continue to synthesize resulting in increased cell length without cell division (Thompson & Hinton, 1996). Other foodborne pathogens such as *Listeria monocytogenes*, *E. coli* and *Bacillus cereus* have been reported to produce filaments after prolonged exposure to pH and other stresses (Bereksi et al., 2002; den Bensten et al., 2009; Jones et al., 2003; Jydegaard-Axelsen et al., 2005). This feature is of concern because filamentous cells can rapidly divide into multiple daughter cells if non-stressful conditions return. In addition, filament formation can result in an underestimation of viable bacterial count because filamentous cells form single colony on plated solid agar medium (Giotis et al., 2007; Jones et al., 2013). This may lead to a food safety risk for the consumers.

Table 4. Image analysis of acetic acid-treated *Salmonella*

	area (μm)	length (μm)	perimeter (μm)
untreated cells	1.199 ± 0.357^{a}	1.672 ± 0.548^{a}	4.500 ± 1.506^{a}
acetic acid-treated cells	2.017 ± 0.428^{b}	2.783 ± 0.451^{b}	6.937 ± 0.514^{b}

Data are presented as mean ± SD where n = 8. Different lowercase letters indicate significant difference in perimeter and length between treated cells and untreated cells at $p < 0.05$.

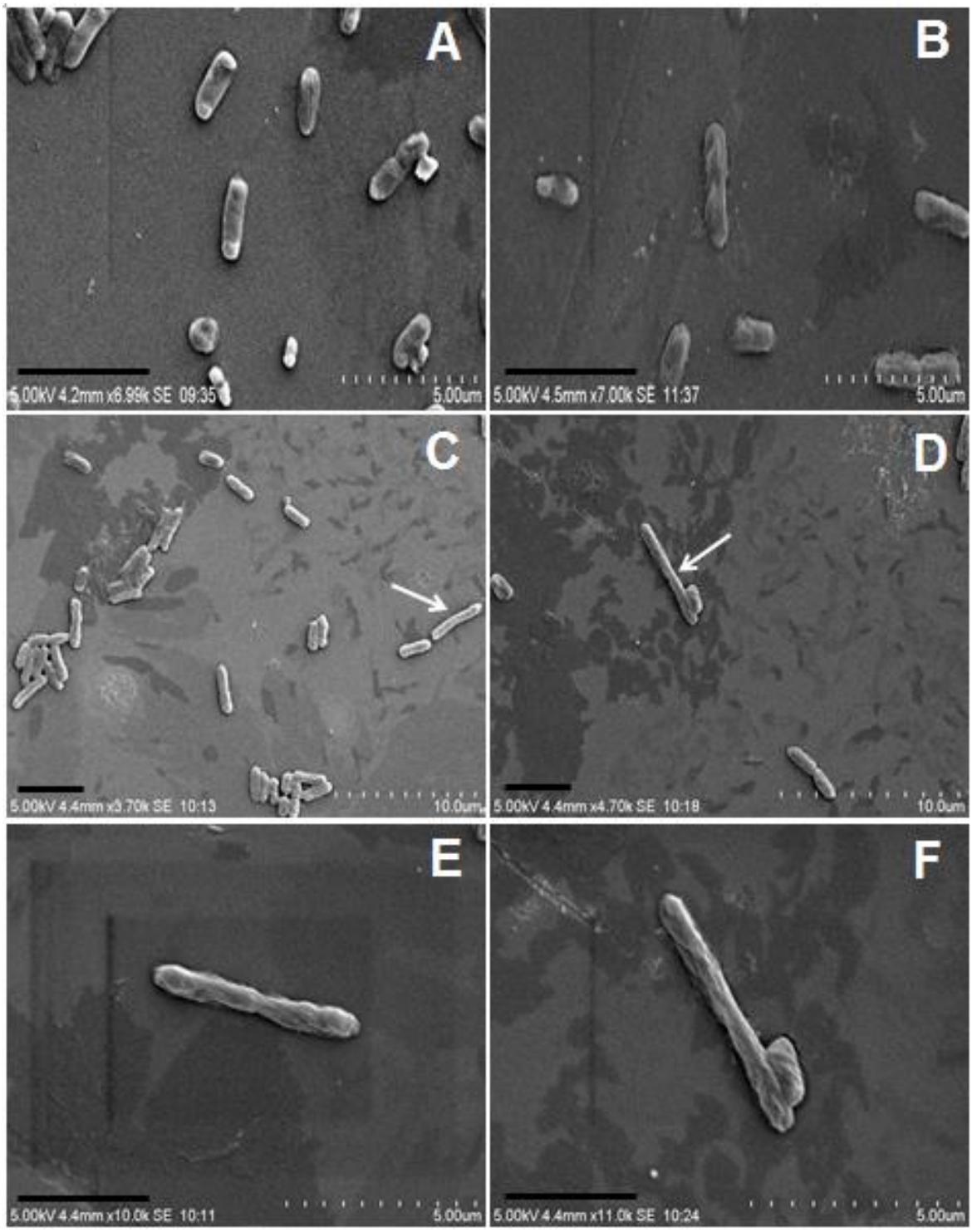

Figure 8. SEM images of *S.* Typhimurium ATCC 33062 on glass slides. A = untreated cells; B = acetic acid treated cells; C, D = acetic acid treatment resulted in some elongated cells (shown by arrows); E, F = increased magnification of the elongated cells (x 10000 magnification). Scale bars = 5.0 μm.

5.3.3 Measurement of the effect of acetic acid on the intracellular ADP/ATP ratio

As shown in Table 5, of all the treatments applied 0.5M acetic acid resulted in the highest ADP/ATP ratio ($p < 0.05$) followed by 0.3M acetic acid ($p < 0.05$), pH 6.4 adjusted 0.5M acetic acid ($p < 0.05$), pH 6.4 adjusted 0.3M acetic acid ($p < 0.05$) and lastly HCl treatments at pH 3.8 and pH 3.25 ($p < 0.05$). There was no difference in this ratio for HCl treatment cells at pH 3.8 and pH 3.25 and the negative control. This indicates that the HCl treatments at pH 3.8 and pH 3.25 were not causing energy exhaustion in *Salmonella* cells. *Salmonella* can survive from pH 3 - 9 if the pH value is purely due to the presence of HCl and NaOH (D'Aoust & Maurer, 2007; Tan et al., 2014). On the other hand, energy exhaustion was observed in cells treated with 0.3M acetic acid and 0.5M acetic acid at the same pH. This indicates that the energy exhaustion of cells is not merely cause by the acidic pH but also by the undissociated acetic acid molecules which easily diffuse into the cell cytoplasm and dissociate into anions. This in turn leads to an accumulation of high levels of toxic anions and interference with essential metabolic reactions (Ricke, 2003).

Table 5 also shows that 0.5M acetic acid at pH 6.4 induces a higher ADP/ATP ratio (for example, a ratio of 1.85 ± 0.42 for *Salmonella* Typhimurium ATCC 14028; $p < 0.05$) as compared to 0.3M acetic acid at pH 6.4 (0.75 ± 0.19; $p < 0.05$). This may be due to a higher concentration of undissociated acid molecules in the 0.5M acetic acid at pH 6.4. A higher concentration of undissociated acid molecules result in higher accumulation of acid anions in the cell cytoplasm and cause more energy depletion than a low concentration acid (Davidson, 2001). The 0.5M acetic acid with an initial pH 3.25 induced a higher ADP/ATP ratio (Table 5, $p<0.05$) than the 0.5M acetic acid at pH 6.4, and this indicates that at the same concentration the acetic acid with a lower pH has a better bactericidal activity as compared to that at a near neutral pH. As reported by Davidson (2001) pH is considered a primary determinant of

effectiveness of organic acids because it affects the concentration of undissociated acid formed. In addition, the uncharged, undissociated acid molecules which are primarily responsible for the antimicrobial acitivity are most prevalent at low pH and therefore organic acids have optimal inhibitory activity at a low pH (Aslim et al., 2005; Nazer et al., 2005). The overall ADP/ATP ratio of *S.* Typhimurium ATCC 33062 was higher than for the other three strains ($p < 0.05$; Table 5), whereas *S.* Typhimurium ATCC 14028 had a lower overall ADP/ATP ratio ($p < 0.05$) as compared to other strains. This indicates that *S.* Typhimurium ATCC 33062 is more sensitive and *S.* Typhimurium ATCC 14028 is more resistant to acetic acid treatments than other strains. We do not know what caused the differences between strains and this needs to be further investigated. Although strain variations were apparent in the study, 0.3M and 0.5M acetic acid at their respective initial pH had a better inhibitory effect as compared to acetic acid at pH 6.4 and HCl treatments. We conclude that the undissociated acid molecules can cause energy depletion in *Salmonella* in the absence of a pH influence. However, the antimicrobial effect of acetic acid was better in the presence of both acidic pH and undissociated acid molecules than only one of them. This suggests that marinades or other treatments with acetic acid should be effective against *Salmonell*a to some extent on chicken meat even though the buffering effect meat neutralizes the acidic pH. It also implies that acetic acid could be applied as an antimicrobial against *Salmonella* on food products for which a neutral pH is an intrinsic property and necessary for its organoleptic properties.

Table 5. ADP/ATP ratio of *Salmonella* at different acid treatments

Treatments	ADP/ATP ratio			
	S. Typhimurium ATCC 33062	*S.* Typhimurium ATCC 14028	*S.* Enteritidis ATCC 13076	*S.* Enteritidis ATCC 49216
Ampicillin (positive control)	6.61 ± 0.99 ^{A,a}	4.96 ± 0.32 ^{A,b}	4.42 ± 0.58 ^{A,bc}	5.25 ± 1.15 ^{A,ac}
Untreated cells (negative control)	0.48 ± 0.37 ^{B,a}	-0.45 ± 0.17 ^{B,b}	-0.55 ± 0.29 ^{B,bc}	-0.39 ± 0.27 ^{B,ac}
0.3M acetic acid	2.66 ± 1.05 ^{C,a}	2.16 ± 0.30 ^{C,b}	4.19 ± 0.98 ^{C,bc}	5.02 ± 0.89 ^{C,ac}
0.5M acetic acid	5.56 ± 1.27 ^{A,a}	2.33 ± 0.24 ^{A,b}	5.67 ± 1.62 ^{A,bc}	6.31 ± 1.27 ^{A,ac}
pH 6.4 0.3M acetic acid	2.53 ± 0.75 ^{D,a}	0.75 ± 0.19 ^{D,b}	0.48 ± 0.32 ^{D,bc}	0.52 ± 0.25 ^{D,ac}
pH 6.4 0.5M acetic acid	2.63 ± 0.52 ^{E,a}	1.85 ± 0.42 ^{E,b}	1.67 ± 0.71 ^{E,bc}	1.64 ± 0.65 ^{E,ac}
pH 3.8 HCl	0.27 ± 0.15 ^{B,a}	-0.42 ± 0.28 ^{B,b}	-0.84 ± 0.18 ^{B,bc}	-0.10 ± 0.15 ^{B,ac}
pH 3.25 HCl	0.29 ± 0.13 ^{B,a}	-0.07 ± 0.11 ^{B,b}	-0.47 ± 0.42 ^{B,bc}	-0.43 ± 0.32 ^{B,ac}

Data are presented as mean ± SD where n = 3. Different uppercase letters indicate significant differences between acid treatments (within column) at a $p < 0.05$ level. Different lowercase letters indicate significant differences between bacterial strains (within rows) at a $p < 0.05$ level.

5.4 Conclusion

The antimicrobial mode of action of acetic acid includes factors other than its acidic pH. Acetic acid did not cause leakage of intracellular components and the SEM study further confirmed that membrane disruption is not the antimicrobial mode of action of acetic acid. Some elongated *Salmonella* cells observed in the micrographs show a possibility of acetic acid may inhibit DNA synthesis in the bacterial cells. At a neutral pH, acetic acid caused energy depletion in cells probably due to the undissociated acid molecules. The antimicrobial effect of acetic acid was better under acidic conditions where the role of low pH and undissociated acid molecules may act together. We concluded that the inhibitory effect of acetic acid is not solely attributable to the low pH but also the undissociated acid molecules.

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Chapter 6

General Discussion and Conclusions

6.1 Major findings and contributions of this study

The primary aims of this project were to investigate: (i) the role that the buffering capacity of chicken skin and meat play in protecting *Salmonella enterica* against pH stress and organic acids; (ii) the role that the fat composition of chicken skin and meat play in their buffering capacity and in the survival of *Salmonella* attached to chicken skin and meat treated with acetic acid and (iii) the nature of the antimicrobial mode of action of acetic acid against *Salmonella* on chicken meat. Based on the results obtained from this project a number of conclusions can be drawn.

First, chicken skin and meat has a buffering effect, with chicken meat showing a better effect than chicken skin (Chapter 2). The plausible reason for the difference in buffering capacity of chicken skin and meat was the different fat composition of skin and meat. Results showed that after fat extraction, the buffering capacity of skin and meat was higher than their respective remnants (Chapter 4). In addition, the buffering capacity of extracted fat alone was no different from the skin remnants. This suggests that the buffering capacity of chicken skin and meat is based on their different chemical composition. For example, we showed that the buffering capacity of skin was affected by the fat composition while that of meat might be affected by the collagen networks which constituted approximately 80% of the chicken meat (Weston et al., 2002). Further work should focus on the chemical composition of chicken skin and meat and the influence of this composition on the buffering capacity.

Second, the buffering effect of chicken skin and meat protected *Salmonella* on chicken against pH stress caused by HCl (Chapter 2). However, the buffering effect did not protect *Salmonella* on chicken against acetic, lactic and citric acid (Chapter 3).

This is because the buffering effect of chicken skin and meat could not protect the bacterial cells from the inhibitory effect caused by undissociated acid molecules of weak organic acids. The reduction in numbers of *Salmonella* attached to chicken skin when treated with acetic acid maybe related to the absence of a buffering effect of chicken skin at an acidic pH (pH 4) (Chapter 4). It was surprising that skin remnants, which had a lower buffering capacity than skin and meat, were shown to better protect *Salmonella* attached to it from acetic acid as compared to other chicken skin and meat components. It is therefore reasonable to suggest that the fat composition in chicken skin may enhance the vulnerability of attached cells to acetic acid. The mechanism of this feature was not investigated in the project but it is an interesting finding worthy of future research.

Third, *Salmonella* attached to chicken skin and meat was rendered undetectable by acetic acid and HCl at pH 3.8 and pH 2, respectively (Chapter 3). This suggests that the antimicrobial mode of action of acetic acid is due to factors other than its acidic pH. The ability of acetic acid to eliminate *Salmonella* on chicken meat at approximately pH 4 also shows the potential for practical application in commercial marination.

Fourth, acetic acid did not cause leakage of intracellular components from *Salmonella* cells or cause membrane disruption, as confirmed in the SEM study. In addition, elongated or filamentous cells observed in the micrographs suggest a possibility that acetic acid may inhibit DNA synthesis in the bacterial cells (Chapter 5). The inhibition of DNA synthesis could be an alternative antimicrobial mode of action of acetic acid against *Salmonella*. The formation of filamentous cells might have an

implication for food safety due to their ability to revert to normal virulent cells when exposed to ideal growth conditions (Jones et al., 2013). At a neutral pH acetic acid caused energy depletion in *Salmonella* cells and this is most probably due to the undissociated acid molecules. The results of this study also showed the inhibitory effect of acetic acid was better at an acidic pH (Chapter 5). It was therefore concluded that the antimicrobial effect of acetic acid occurred both in the presence of acidic pH and undissociated acid molecules and these factors caused energy depletion in the *Salmonella* cells.

Overall, these findings contribute to the literature on the effect of pH and organic acids as components of marinades with respect to *Salmonella* attached to chicken skin and meat. Based on the conclusions above it can be suggested that marinades or other treatments with acetic acid should be effective against *Salmonell*a to some extent on chicken skin and meat, even though the buffering effect of meat neutralizes the acidic pH.

6.2 Future directions

This project provides some of the insight and understanding to the buffering capacity of chicken skin and meat and its effect on the survival of *Salmonella* attached to them, and in particular exposed to inorganic and organic acid stress. Listed below are potential areas of future research that could be conducted in order to gain a better understanding of the meat environment and the effect *Salmonella* and other pathogens attached to meat and which may help in minimizing food safety risk.

6.2.1 Chemical composition of chicken skin and meat and the effect of this on buffering capacity

Chicken skin and meat had different fat composition and the fat composition was found to influence the buffering capacity of skin and meat (Chapter 4). In future research, a detailed assessment of the composition parameters of chicken skin and meat such as water activity, protein content and carbohydrate content should be carried out to see if these different components would affect the buffering capacity.

6.2.2 The role of fat in skin in enhancing the vulnerability of attached bacterial cells to organic acid treatment

Salmonella attached to skin remnants, which had a low buffering capacity, were better protected than when attached to the other components (Chapter 4). It is therefore suggested that the fat in skin may enhance the vulnerability of attached cells to acetic acid. The mechanism behind this was not investigated in this project. In future research, the mechanism should be determined since the number of viable *Salmonella* surviving acetic acid treatment on skin remnants was substantial (~3.5 log CFU/g). The association between the fat content in chicken skin, the foodborne pathogens attached to skin and the effect of the organic acid treatment should be studied to give a broader sense of how these various components interact with each other.

6.2.3 Alternative antimicrobial mode of action of acetic acid in inhibiting DNA synthesis in bacterial cells

Some elongated *Salmonella* cells were observed in the scanning electron micrograph (Chapter 5) indicating the possibility that acetic acid inhibits DNA synthesis in the bacterial cells. As reported by Thompson and Hinton (1996) the elongation of acid-

treated cells is due to the impeding of DNA synthesis whilst the continuation of synthesis of other macromolecules, such as RNA and proteins, results in the cell size increase without undergoing cell division. It is therefore appropriate to investigate alternative antimicrobial modes of action of acetic acid against foodborne pathogens.

6.2.4 Further confirmation of the energy depletion of Salmonella

Results from Chapter 5 showed energy depletion occurred in acetic acid-treated *Salmonella* cells. It is suggested that the energy depletion is caused by undissociated acetic acid molecules. However, the quantity of undissociated acid molecules in the assay was not determined. Further confirmation, for example using fluorescent labeling to measure undissociated acid molecules in the energy depletion process, should be performed (Coconnier-Poulter et al., 2005). Similar studies on other pathogens on poultry such as *Campylobacter* and *Listeria* should be conducted with other organic acids treatments to assess the safety implications of this finding in the food and poultry processing industry.

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Xiong, H., Li, Y.B., Slavik, M.F., & Walker, J.T. (1998). Spraying chicken skin with selected chemicals to reduce attached *Salmonella* Typhimurium. *Journal of Food Protection, 61*, 272-275.

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Appendix I

Linear regression equations for the investigation of buffering effect of chicken skin, meat and PBS alone

The following appendix contains the graphs of linear regression generated from three sets of transformed pH data for the investigation of buffering effect of chicken components (Chapter 2).

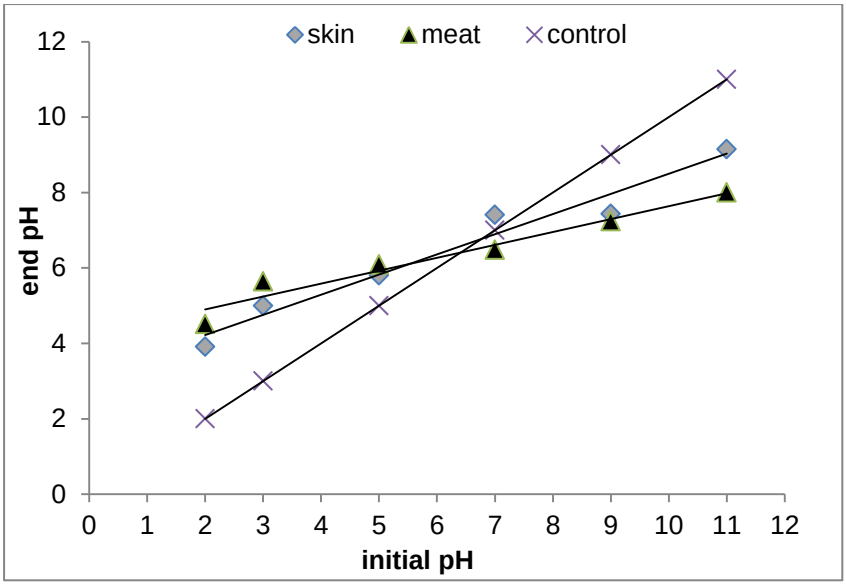


Figure A1.1 Linear regression trend-line and equations generated from the first data set (data set 1) for investigation of buffering effect of chicken skin and meat. The linear equations of (a) chicken skin: $y = 0.037x + 0.563$, $R^2 = 0.935$; (b) chicken meat: $y = 0.024x + 0.646$, $R^2 = 0.918$; (c) PBS without chicken: $y = x$, $R^2 = 1$.

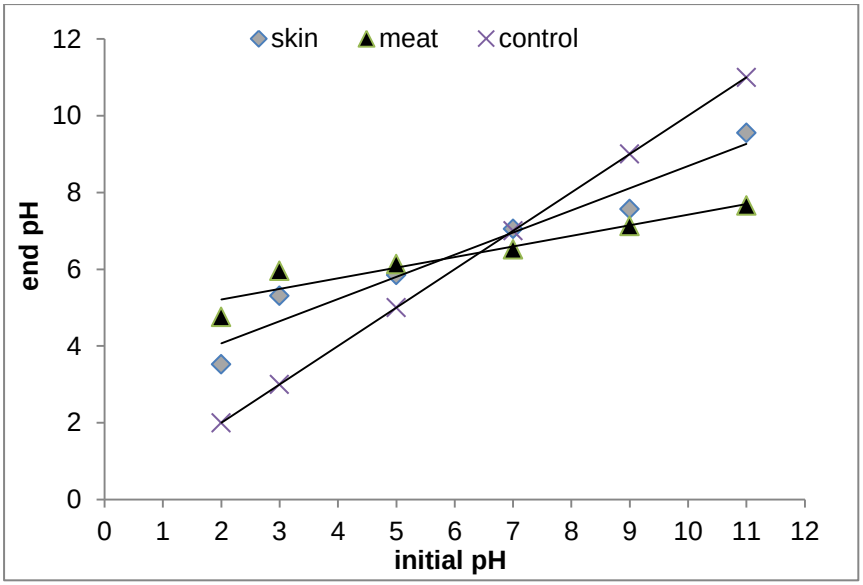


Figure A1.2 Linear regression trend-line and equations generated from the second data set (data set 2) for investigation of buffering effect of chicken skin and meat. The linear equations of (a) chicken skin: $y = 0.041x + 0.541$, $R^2 = 0.897$; (b) chicken meat: $y = 0.019x + 0.680$, $R^2 = 0.874$; (c) PBS without chicken: $y = x$, $R^2 = 1$.

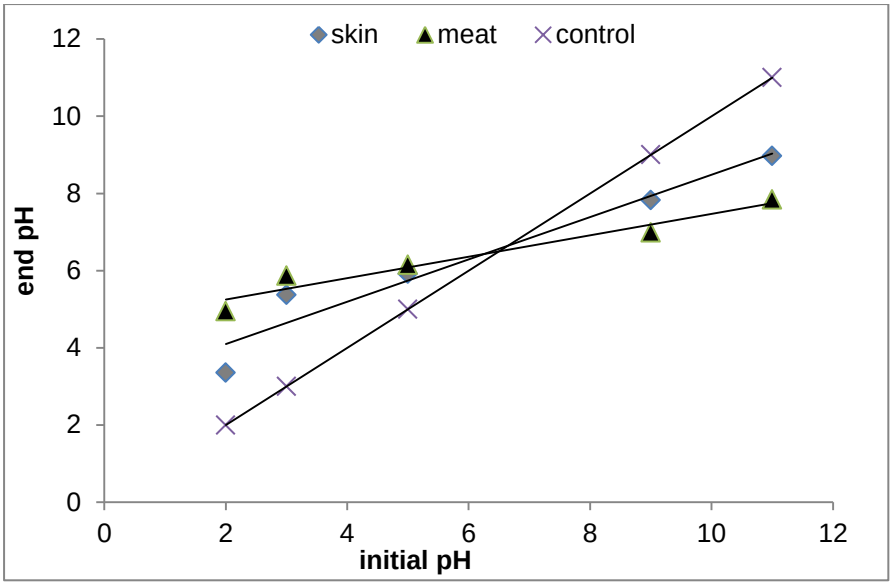


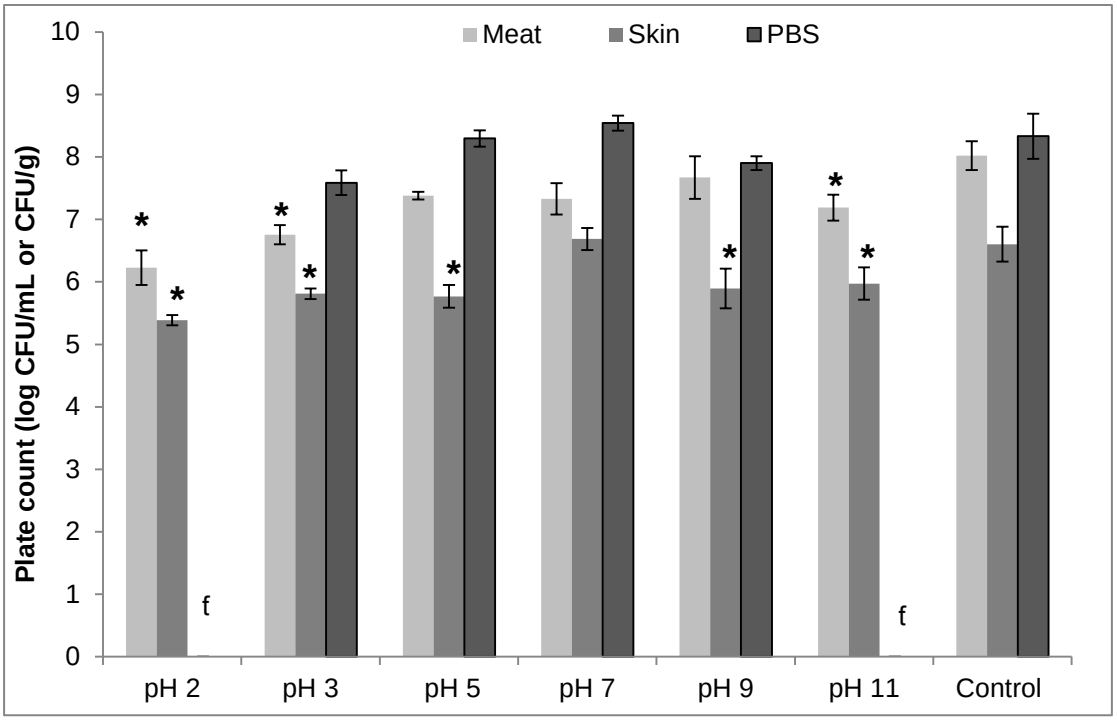
Figure A1.3 Linear regression trend-line and equations generated from the third data set (data set 3) for investigation of buffering effect of chicken skin and meat. The linear equations of (a) chicken skin: $y = 0.040x + 0.540$, $R^2 = 0.858$; (b) chicken meat: $y = 0.019x + 0.684$, $R^2 = 0.915$; (c) PBS without chicken: $y = x$, $R^2 = 1$.

Appendix II

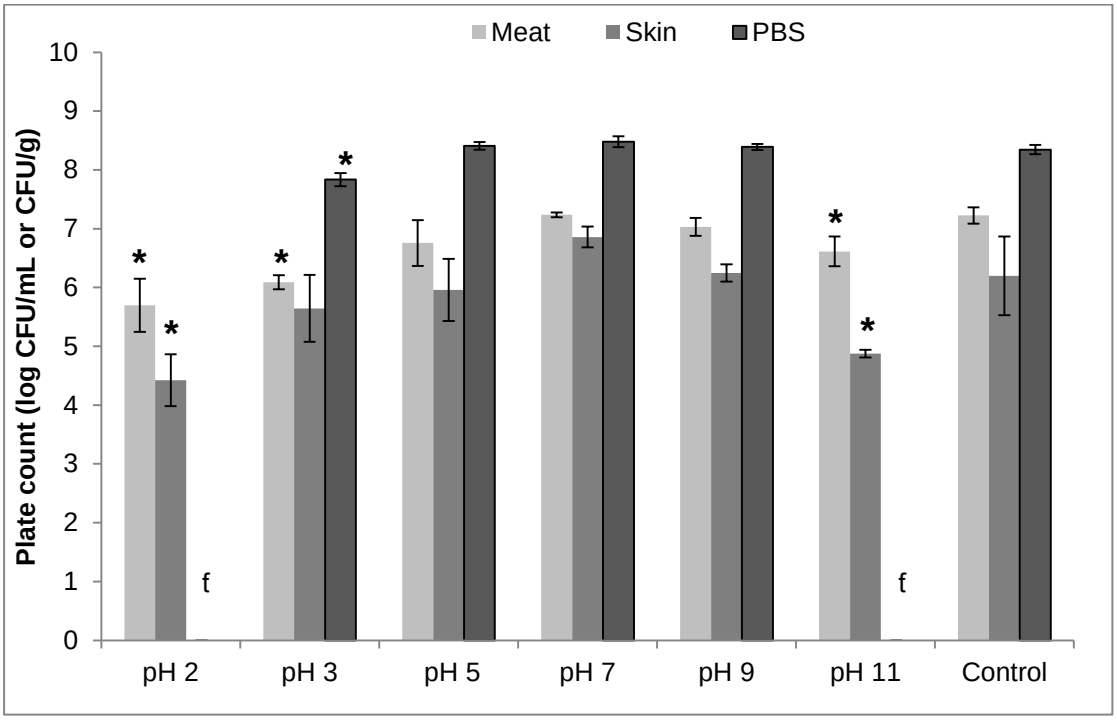
The effect of pH on the survival of *S.* Typhimurium ATCC 33062, *S.* Enteritidis ATCC 13076 and *S.* Enteritidis ATCC 49216

The following appendix contains the results obtained from the effect of pH on the survival of *Salmonella* attached to chicken skin and meat (Chapter 2).

(A)


Meat
Skin
PBS
Plate count (log CFU/mL or CFU/g)
10
9
8
7
6
5
4
3
2
1
0
*
*
f
*
*
*
*
*
f
pH 2
pH 3
pH 5
pH 7
pH 9
pH 11
Control

(B)


Meat
Skin
PBS
Plate count (log CFU/mL or CFU/g)
10
9
8
7
6
5
4
3
2
1
0
*
*
f
*
*
*
*
f
pH 2
pH 3
pH 5
pH 7
pH 9
pH 11
Control

(C)

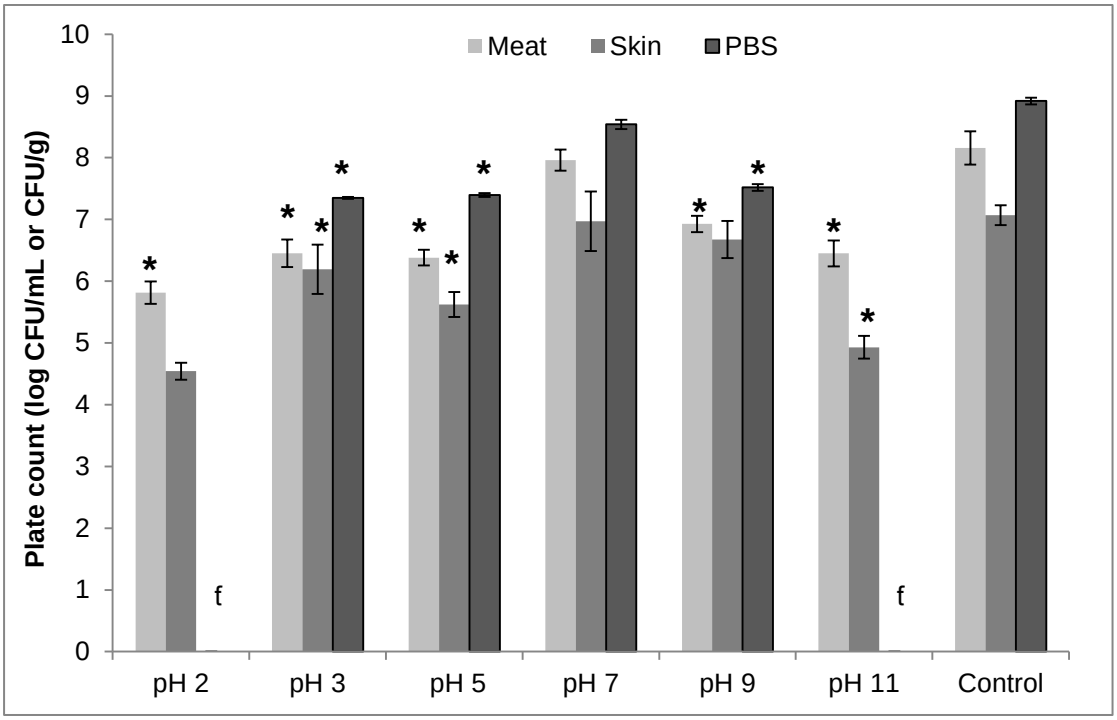


Figure A2. The effects of pH on the survival of **(A)** *S.* Typhimurium ATCC 33062; **(B)** *S.* Enteritidis ATCC 13076; **(C)** *S.* Enteritidis ATCC 49216, attached to chicken meat and skin, and in PBS alone. The control is *Salmonella* attached to chicken meat and skin and in PBS alone, with no treatment applied. Results are presented as mean ± SD where n = 3. * indicates a significant difference between pH treatments and the control; **f** indicates no viable count was observed for the pH treatments.

Appendix III

Plots of viable counts against pH of *S.* Typhimurium ATCC 33062, *S.* Enteritidis ATCC 13076 and *S.* Enteritidis ATCC 49216

The following appendix contains the results obtained from the effect of organic acids on the survival of *Salmonella* attached to chicken skin and meat (Chapter 3).

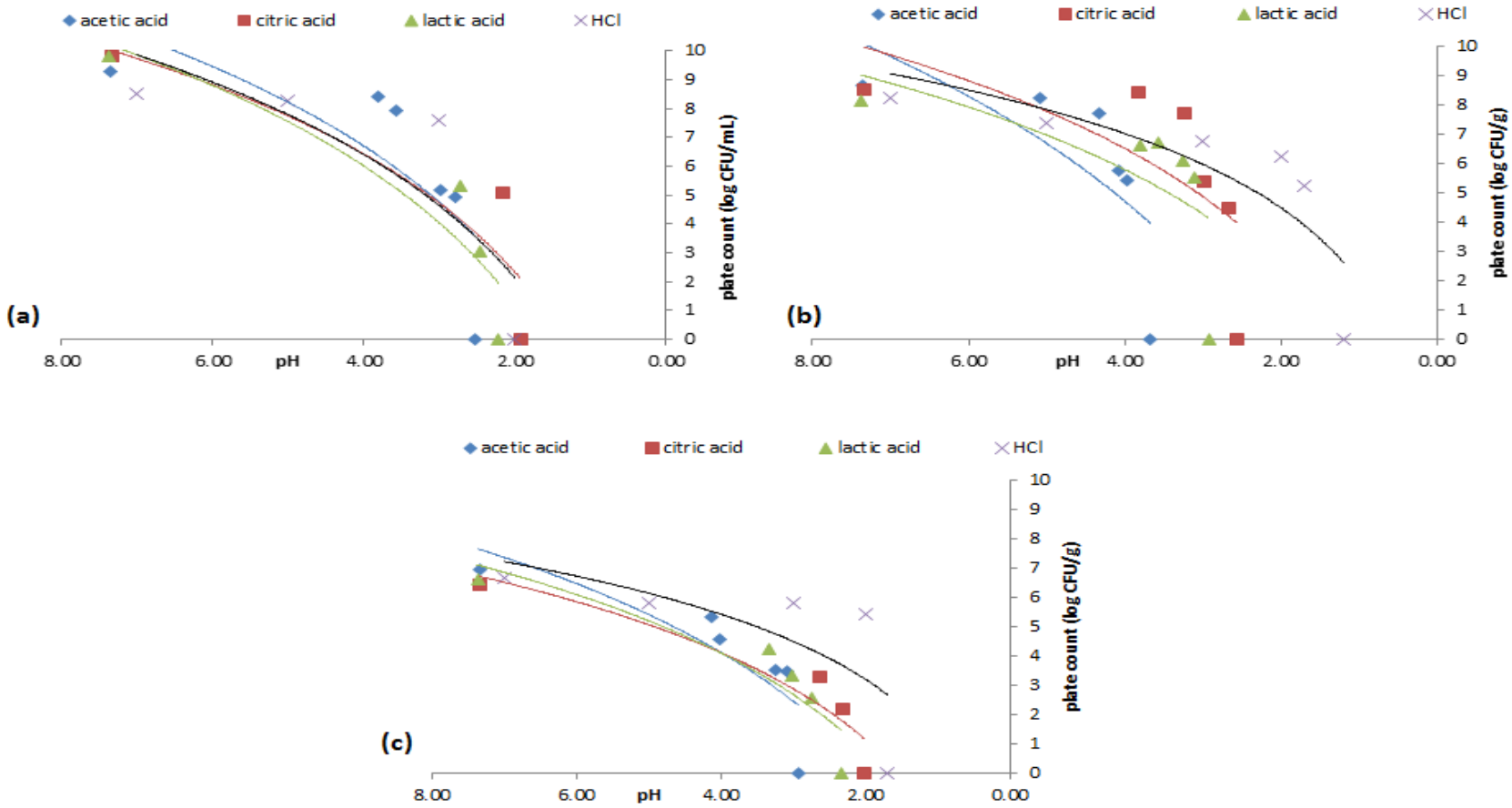


Figure A3.1 The effects of four acids on the survival of *S.* Typhimurium ATCC 33062 (a) in PBS alone; (b) attached to chicken meat; (c) attached to chicken skin.

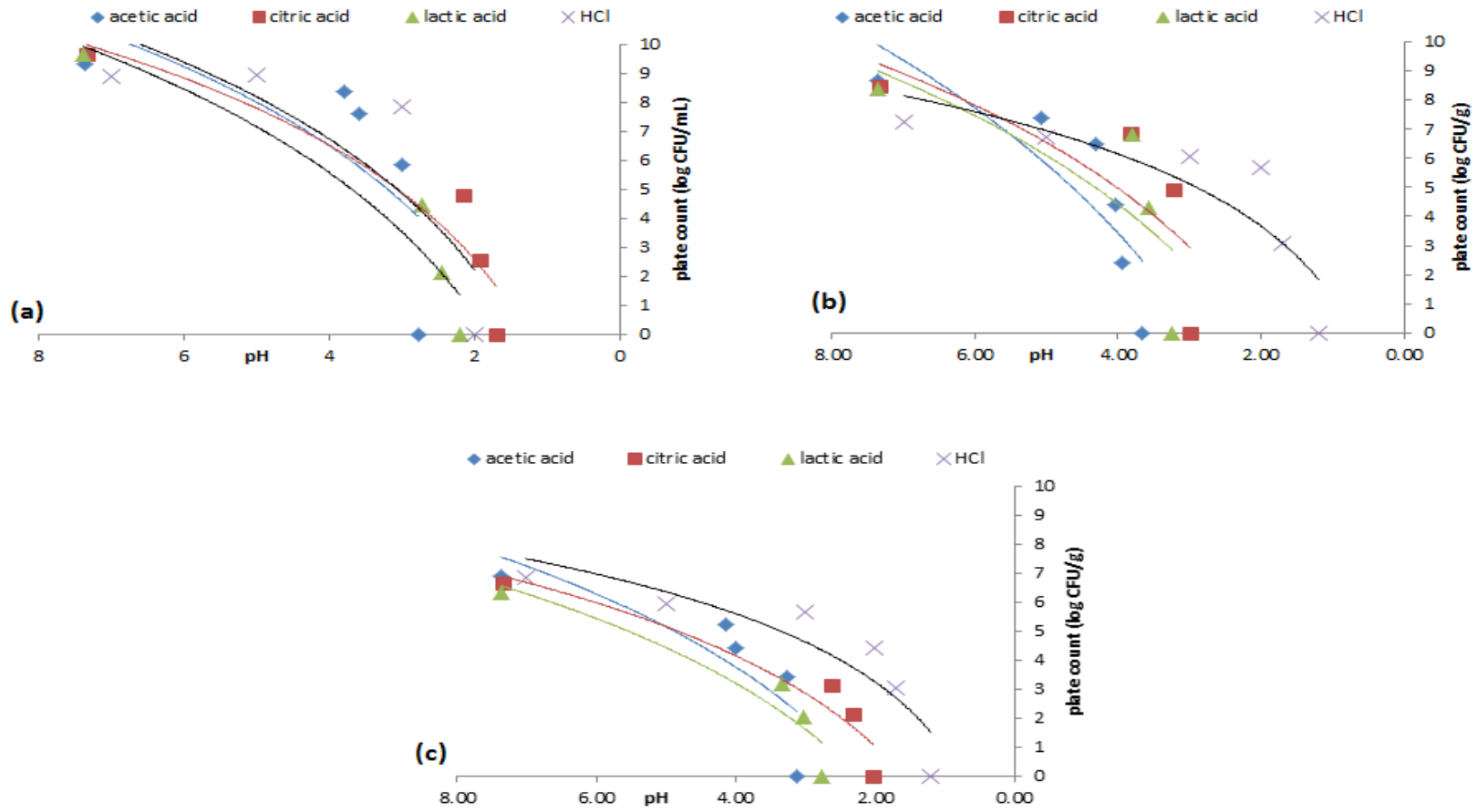


Figure A3.2 The effects of four acids on the survival of *S.* Enteritidis ATCC 13076 (a) in PBS alone; (b) attached to chicken meat; (c) attached to chicken skin.

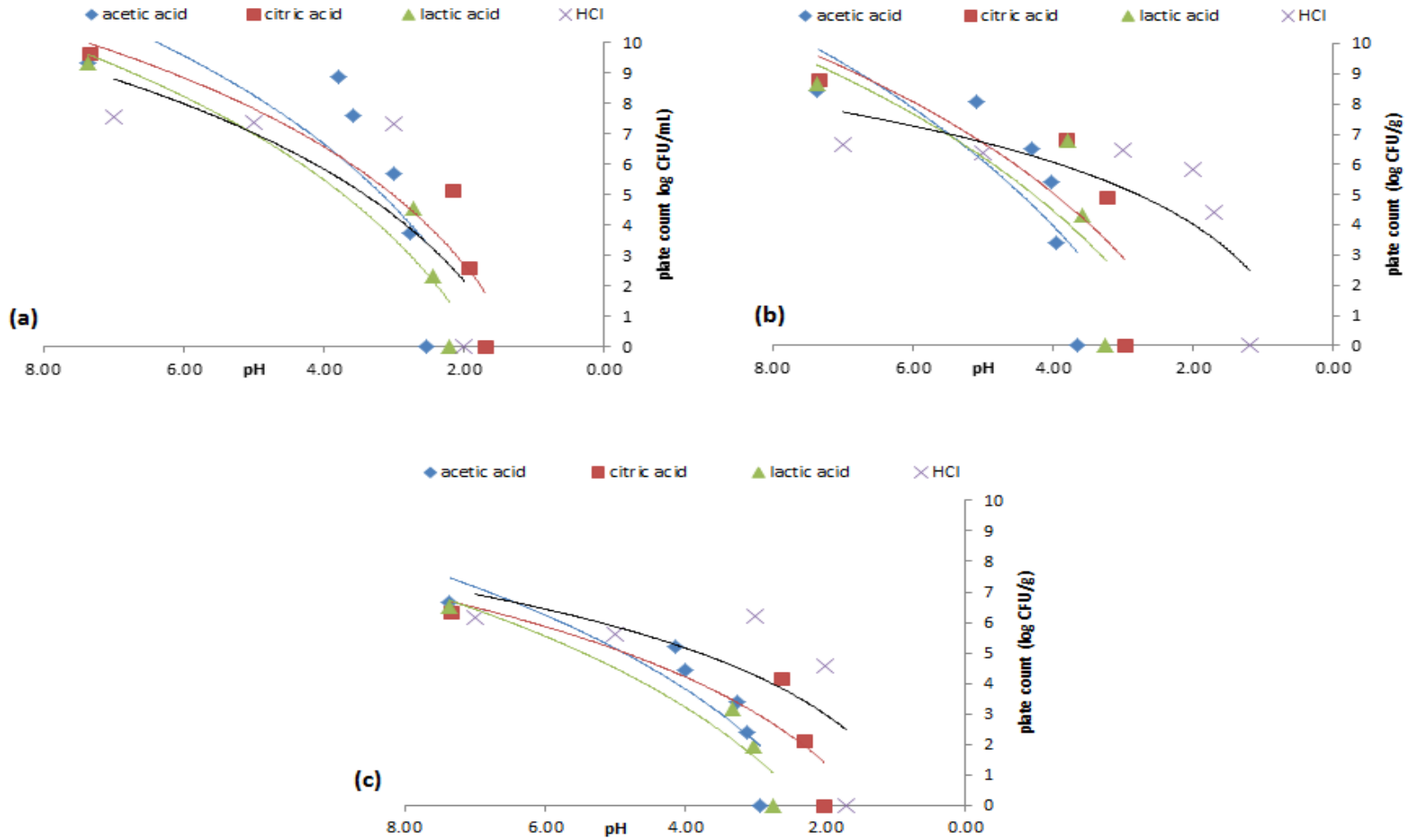


Figure A3.3 The effects of four acids on the survival of *S*. Enteritidis ATCC 49216 (a) in PBS alone; (b) attached to chicken meat; (c) attached to chicken skin.

Appendix IV

The pH at which four strains of *Salmonella* were rendered undetectable by organic acids and HCl treatments

The following appendix contains the results obtained from the effect of organic acids on the survival of *Salmonella* attached to chicken skin and meat (Chapter 3).

Table A1. Effect of treatment with four acids at the pH at which all *Salmonella* were rendered undetectable

Strains	**Chicken components**	**pH at which cells were rendered undetectable***			
		acetic acid	citric acid	lactic acid	HCl
S. Typhimurium	PBS	2.8 ± 0.02	1.7 ± 0.02	2.2 ± 0.02	2.0 ± 0.05
ATCC 14028	skin	3.1 ± 0.03	2.0 ± 0.02	2.8 ± 0.02	1.2 ± 0.05
	meat	3.8 ± 0.02	2.9 ± 0.02	3.2 ± 0.02	1.2 ± 0.05
S. Typhimurium	PBS	2.5 ± 0.02	1.9 ± 0.01	2.2 ± 0.02	2.0 ± 0.05
ATCC 33062	skin	2.9 ± 0.02	2.0 ± 0.03	2.4 ± 0.05	1.7 ± 0.03
	meat	3.7 ± 0.02	2.6 ± 0.02	2.9 ± 0.02	1.2 ± 0.07
S. Enteritidis	PBS	2.8 ± 0.02	1.7 ± 0.02	2.2 ± 0.04	2.0 ± 0.02
ATCC 13076	skin	3.1 ± 0.03	2.0 ± 0.03	2.8 ± 0.02	1.2 ± 0.02
	meat	3.8 ± 0.04	3.0 ± 0.02	3.3 ± 0.03	1.2 ± 0.03
S. Enteritidis	PBS	2.6 ± 0.02	1.7 ± 0.02	2.2 ± 0.02	2.0 ± 0.02
ATCC 49216	skin	2.9 ± 0.03	2.0 ± 0.03	2.8 ± 0.02	1.7 ± 0.03
	meat	3.7 ± 0.04	2.9 ± 0.02	3.3 ± 0.02	1.2 ± 0.04

* Difference between the pH where cells were rendered undetectable were all significant at $p<0.05$.

Appendix V

Raw data for the buffering capacity of PBS, chicken skin, meat, extracted fat, skin remnants and meat remnants

The following appendix contains the results obtained from the role of fat plays on the survival of *Salmonella* attached to chicken skin and meat (Chapter 4).

Table A2. Raw data for the buffering capacity of PBS, chicken skin, meat, extracted fat, skin remnants and meat remnants

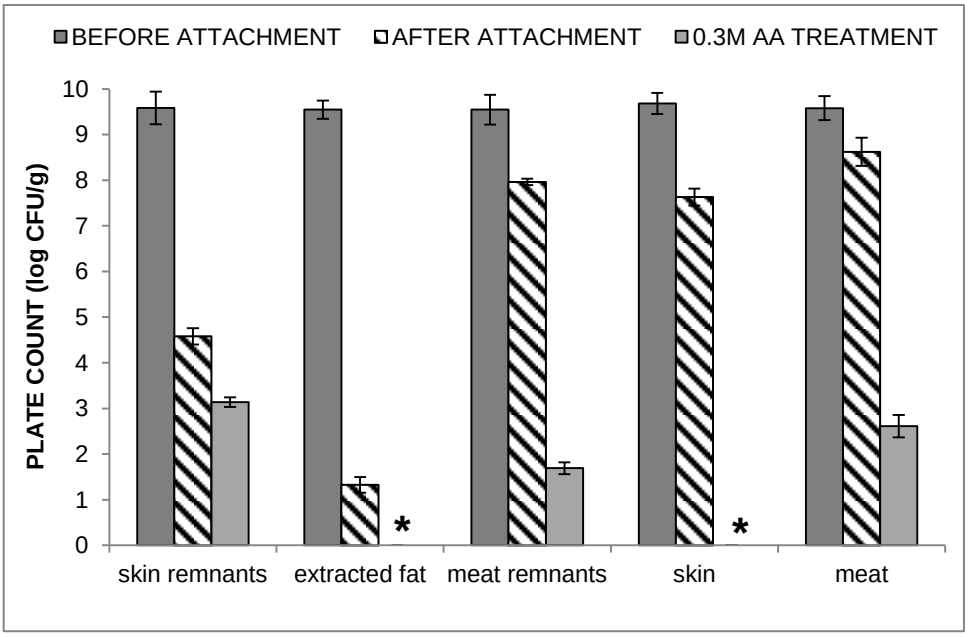
	pH 1	pH 2	pH 3	0.1M HCl/ NaOH added (mmol)		pH 1	pH 2	pH 3	0.1M HCl/ NaOH added (mmol)
PBS	8.38	8.36	8.38	0.2	**skin**	8.80	8.81	8.82	0.2
	7.63	7.61	7.60	0.1		7.61	7.60	7.63	0.1
	7.06	7.02	7.03	0.0		6.95	6.95	6.96	0.0
	6.89	6.88	6.89	0.1		5.11	5.12	5.10	0.1
	6.72	6.72	6.71	0.2		4.44	4.43	4.39	0.2
	6.67	6.63	6.66	0.3	**meat**	8.81	8.79	8.82	0.9
	6.42	6.41	6.42	0.4		8.55	8.52	8.53	0.8
	6.29	6.28	6.25	0.5		8.20	8.21	8.21	0.7
	6.09	6.05	6.06	0.6		7.86	7.84	7.83	0.6
	5.75	5.72	5.56	0.7		7.55	7.52	7.54	0.5
	5.24	5.23	5.22	0.8		7.32	7.31	7.35	0.4
meat	8.97	8.99	9.00	0.8		7.09	7.08	7.10	0.3
remnants	8.39	8.35	8.36	0.7		6.91	6.93	6.90	0.2
	8.09	8.03	8.04	0.6		6.79	6.78	6.77	0.1
	7.89	7.85	7.88	0.5		6.59	6.55	6.54	0.0
	7.65	7.64	7.65	0.4		6.44	6.43	6.42	0.0
	7.30	7.31	7.29	0.3		6.02	6.01	6.02	0.1
	7.12	7.10	7.12	0.2		5.74	5.71	5.72	0.2
	6.86	6.88	6.89	0.1		5.36	5.37	5.36	0.3
	5.92	5.90	5.93	0.0		4.87	4.88	4.88	0.4
	5.46	5.44	5.48	0.1		4.59	4.55	4.58	0.5
	4.81	4.83	4.80	0.2		4.38	4.36	4.35	0.6
	4.23	4.25	4.22	0.3		4.27	4.26	4.25	0.7
	4.04	4.02	4.03	0.4		4.11	4.10	4.08	0.8
skin	9.13	9.10	9.05	0.5	**extracted**	9.06	9.05	9.03	0.4
remnants	8.48	8.44	8.47	0.4	**fat**	8.33	8.32	8.34	0.3
	7.29	7.30	7.27	0.3		7.19	7.20	7.19	0.2
	6.39	6.37	6.40	0.2		6.28	6.26	6.25	0.1
	5.85	5.87	5.89	0.0		5.67	5.61	5.64	0.0
	4.10	4.09	4.11	0.1		4.05	4.06	4.04	0.1

Appendix VI

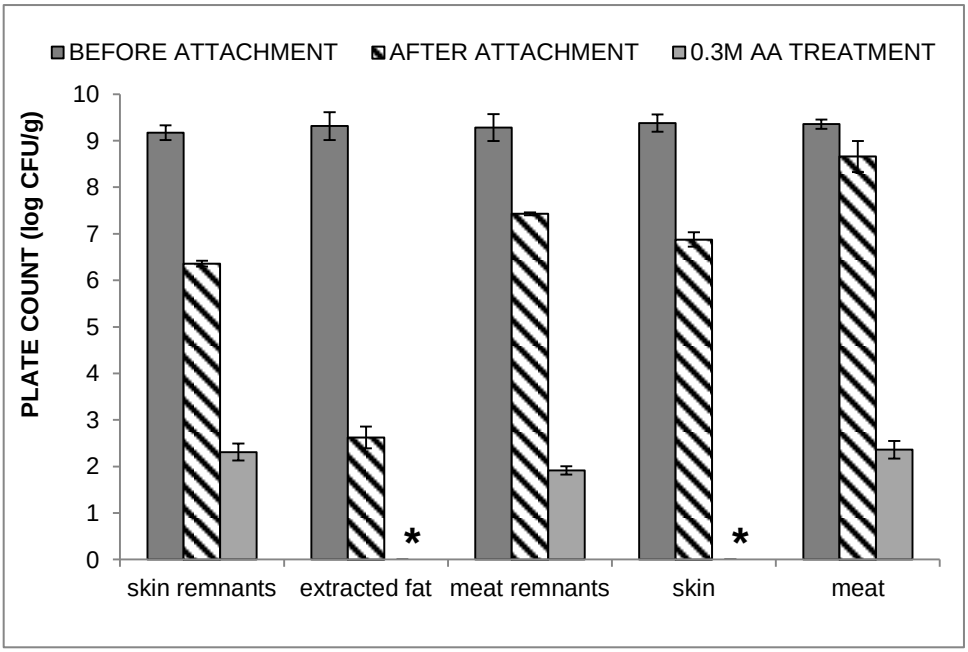
Viable count of *S*. Typhimurium ATCC 14028, *S*. Enteritidis ATCC 13076 and *S*. Enteritidis ATCC 49216 on chicken components after treatment with acetic acid

The following appendix contains the results obtained from the role of fat plays on the survival of *Salmonella* attached to chicken skin and meat (Chapter 4).

(A)


BEFORE ATTACHMENT
AFTER ATTACHMENT
0.3M AA TREATMENT
PLATE COUNT (log CFU/g)
10
9
8
7
6
5
4
3
2
1
0
*
*
skin remnants
extracted fat
meat remnants
skin
meat

(B)


BEFORE ATTACHMENT
AFTER ATTACHMENT
0.3M AA TREATMENT
PLATE COUNT (log CFU/g)
10
9
8
7
6
5
4
3
2
1
0
*
*
skin remnants
extracted fat
meat remnants
skin
meat

(C)

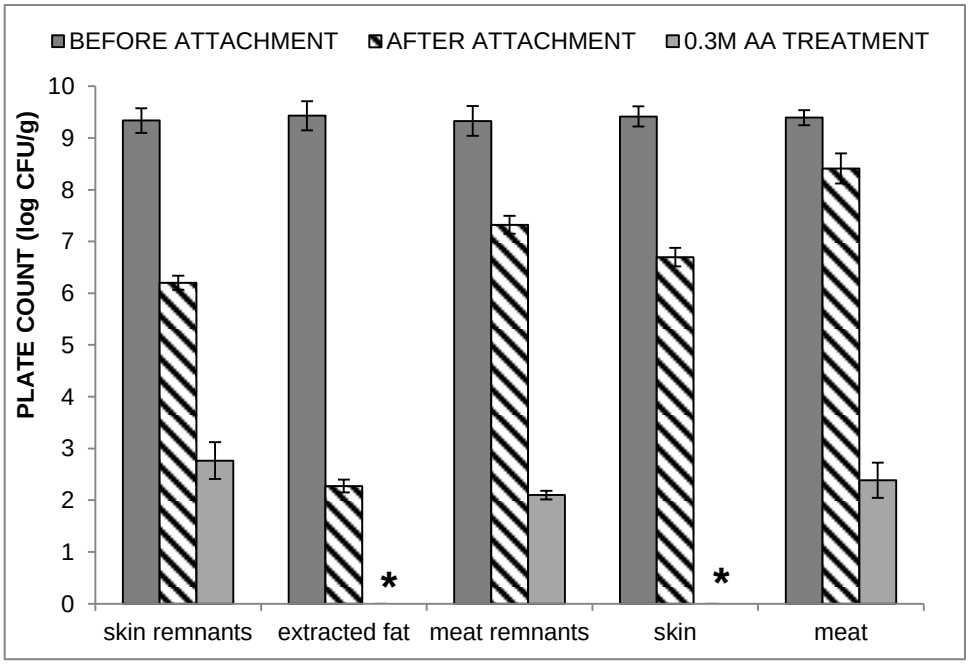


Figure A4. Viable count of **(A)** *S.* Typhimurium ATCC 14028; **(B)** *S.* Enteritidis ATCC 13076; **(C)** *S.* Enteritidis ATCC 49216, before attachment, firmly attached to the surfaces of chicken components and after treated with 0.3M acetic acid. Results are presented as mean ± SD where n = 3. * indicates no viable count was observed after acetic acid treatment.

Appendix VII

Determination of the leakage of intracellular components of *S*. Typhimurium ATCC 14028, *S*. Enteritidis ATCC 13076 and *S*. Enteritidis ATCC 49216 treated with acetic acid

The following appendix contains the results obtained from the antimicrobial mode of action of acetic acid (Chapter 5).

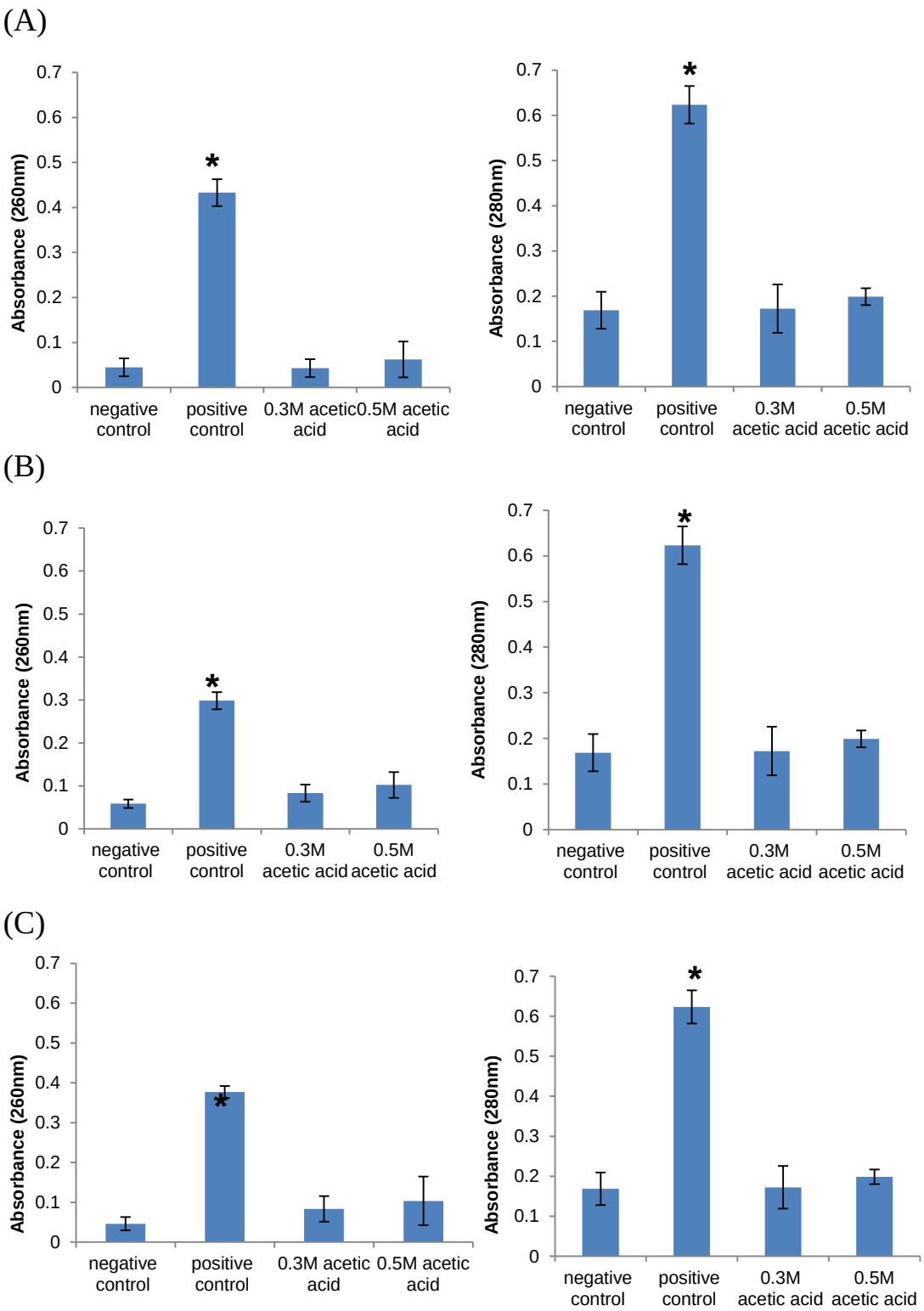


Figure A5. The leakage of intracellular components from **(A)** *S*. Typhimurium ATCC 14028; **(B)** *S*. Enteritidis ATCC 13076; **(C)** *S*. Enteritidis ATCC 49216 after treatment with 0.3M and 0.5M acetic acid. Results are presented as mean ± SD where n = 3. * indicates a significant between positive control and treatments.

Appendix VIII

Publications related to this thesis