ANTIMICROBIAL PROPERTIES OF SODIUM METASILICATE AGAINST FOODBORNE PATHOGENS

By

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To my revered parents, loving wife and lovely son

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The objectives of this research were to determine the antimicrobial effects of sodium metasilicate (SMS) against *Salmonella* Typhimurium, *Campylobacter jejuni* and *Listeria monocytogenes* in suspension, in meat system and to elucidate the mechanism of antimicrobial action of SMS.

In suspension, there was immediate inactivation of *S.* Typhimurium with no detectable survivors following exposure to SMS concentrations as low as 0.5%, whereas exposure of *C. jejuni* to 0.5% SMS for 1 min resulted in more than 4 log reductions ($P < 0.05$). No significant reduction in *L. monocytogenes* ($P > 0.05$) was observed with 1 min exposure to 1.0 and 2.0% SMS. However 1.0, 2.0 and 3.0% SMS reduced *L. monocytogenes* by more than 5 logs after 30 min.

Skinless and boneless chicken breast meat (skinless) treated with 1 and 2% SMS in marinade resulted in 0.83 to 0.91 and 1.04 to 1.16 log cfu/g reductions of *S.* Typhimurium ($P < 0.05$), respectively, with no significant reduction in *C. jejuni* counts. In a second study, skin-on boneless chicken breast meat treated with 1 and 2% SMS (by weight of meat) resulted in 1.11 to 1.43 and 2.48 to 4.11 log cfu/g reductions ($P < 0.05$)

of *S.* Typhimurium, respectively, and 1.12 to 1.26 and 3.27 to 3.79 log cfu/g reductions of *C. jejuni*, respectively. Psychrotrophic counts for breasts (skin-on) treated with 2% SMS were lower ($P < 0.05$) and the pH values were higher ($P < 0.05$) for all SMS treatments for both *S.* Typhimurium and *C. jejuni* experiments. Sodium metasilicate at 300 and 600 ppm concentration was not effective in reducing *L. monocytogenes* ($P > 0.05$) in turkey ham, and pH values were similar ($P > 0.05$) for all treatments.

Sodium metasilicate was ineffective against *Salmonella* after pH neutralization in chicken breasts (skinless). As for mechanism of action, loss of cell viability and membrane integrity was evidenced by uptake and staining of *S.* Typhimurium and *L. monocytogenes* cells with propidium iodide following treatment with SMS. Transmission electron microscopic examination of SMS treated *S.* Typhimurium and *L. monocytogenes* cells showed disruption in cytoplasmic membrane and leakage of cytoplasmic contents.

CHAPTER 1
INTRODUCTION

Foodborne infections are a major public health concern even in most developed nations like the United States. It is estimated that every year foodborne diseases cause approximately 9.4 million illnesses, 55,961 hospitalizations and 1,351 deaths in the US with nontyphoidal *Salmonella* spp. (11%), *Clostridium perfringens* (10%), and *Campylobacter* spp. (9%) as the leading bacterial causes (Scallan et al., 2011). There were 1,097 foodborne disease outbreaks reported during 2007, which resulted in 21,244 cases of illness and 18 deaths (CDC, 2010a). *Salmonella* was the leading cause among bacterial pathogens, which was responsible for 5 out of the 18 reported deaths. Poultry meat was cited to be the most common cause of illness among the 235 outbreaks attributed to a single food commodity, which was followed by beef and leafy vegetables (CDC, 2010a).

Salmonella and *Campylobacter* are among the leading bacterial pathogens responsible for the majority of foodborne illnesses reported in US. In 2009, the number of reported foodborne infections and incidence for *Salmonella* and *Campylobacter* were (7,039; 15.19) and (6,033; 13.02), respectively, out of a total of 17,468 laboratory-confirmed cases of infection (CDC, 2010b). *L. monocytogenes* is another major foodborne pathogen with public health significance because of its high case fatality rate (CDC, 2010b). Numerous outbreaks have been linked to consumption of ready-to-eat (RTE) meat products contaminated with *L. monocytogenes*. Due to its public health significance, USDA FSIS maintains a zero-tolerance policy for *L. monocytogenes* in ready-to-eat meat and poultry products.

As per capita consumption of poultry and poultry products has increased annually, the risk associated with foodborne illnesses from *Salmonella* and *Campylobacter* contaminated poultry products will also increase. *Salmonella* and *Campylobacter* are pathogens most commonly associated with raw poultry and it is estimated that every year poultry-borne salmonellosis and campylobacteriosis cost $426 to $814 million in the US (Bryan and Doyle, 1995), whereas *L. monocytogenes* is a problem with RTE poultry products. In order to improve the public health by ensuring food safety, there is great need to decrease the incidence of these pathogens in poultry products reaching the consumer table. Various post-harvest antimicrobial interventions have been approved and are being applied in poultry processing. These interventions include acidified sodium chlorite (Kemp et al., 2001; USDA FSIS, 2011), organic acids (Over et al., 2009; Birk et al., 2010), trisodium phosphate (Kim et al., 1994; Lillard, 1994; Bourassa et al., 2004) and natural antimicrobials (Skandamis et al., 2002; Burt, 2004) to restrict the growth of foodborne pathogens. There is a need for additional interventions to use along with the existing preventive measures to further restrict the occurrence of *Salmonella* and *Campylobacter* at various steps in poultry processing.

Sodium metasilicate is a USDA approved antimicrobial for use in marinades for poultry products. Its use can provide an additional intervention in final steps of poultry processing to ensure the final removal or restriction of the growth of Gram-negative foodborne pathogens such as *Salmonella* and *Campylobacter.* Limited research data are available that document the effectiveness of SMS in inactivating *Salmonella* and *Campylobacter* in poultry meat. The objectives of this research were to determine the antimicrobial properties of SMS against both gram-negative and Gram-positive

foodborne pathogens, especially *Salmonella* Typhimurium, *Campylobacter jejuni* and *Listeria monocytogenes* in suspension, raw poultry and ready-to-eat poultry products; to determine the antimicrobial effect of SMS on psychrotrophic organisms in chicken breasts for extension of shelf life, to ascertain the effects of various treatments on the pH and to study the antimicrobial mechanism of action of SMS against Gram-negative and Gram-positive foodborne pathogens.

CHAPTER 2
LITERATURE REVIEW

Pathogenic and Spoilage Microflora Associated with Fresh Poultry and Ready-to-Eat Poultry Products

The microbiology of poultry is very complex and has been widely investigated (Capita et al., 2001a; International Commission on Microbiological Specification for Foods, 2005; Foley et al., 2008; Young et al., 2009). Immediately after slaughtering and prior to storage poultry meat contains both spoilage and pathogenic microorganisms including Gram-negative and Gram-positive bacteria, yeasts and molds (Corry, 2007). The number of microorganisms, growth and predominance leading to spoilage of poultry are dependent on many factors such as the initial load of the microorganisms, processing methods and the storage conditions (e.g. storage temperature) for the meat. The steps in poultry slaughtering and processing that are major sources of microbial contamination on the final dressed bird carcass include: a) the bird itself: the feathers, skin and any fecal matter present on the bird and accidental rupturing of its intestinal contents during processing, b) the processing environment, processing equipment, knives, clothing, air and water and finally, c) personal hygiene, especially the hands of the persons handling the raw carcasses (Corry, 2007). Therefore, it is these factors determine the ultimate microbiological quality of fresh poultry.

The microflora found on fresh poultry consists primarily of spoilage microorganisms. However, poultry meat is also known to harbor many pathogens that are capable of causing human diseases. *Salmonella* and *Campylobacter* spp. are the most prevalent and by far the most important human pathogens associated with fresh poultry products (DuPont, 2007).However, a number of other pathogens have been isolated from raw poultry (e.g. *L. monocytogenes*, *Clostridium perfringens,*

Staphylococcus aureus), which are also responsible for foodborne illnesses throughout the world (Bryan and Kilpatrick, 1971; Gibbs et al., 1978; International Commission on Microbiological Specification for Foods, 2005). The consumption or handling of raw or undercooked poultry meat has been linked to many foodborne outbreaks particularly due to *Campylobacter jejuni* (Altekruse et al., 1999). *Salmonella* infections usually occur as outbreaks involving large populations whereas *Campylobacter* infections are reported as sporadic cases (International Commission on Microbiological Specification for Foods, 2005).

Spoilage Microflora of Fresh Poultry

Immediately after slaughtering, poultry meat contains various Gram-negative and Gram-positive spoilage bacteria (*Micrococcus*, *Pseudomonas, Acinetobacter, Enterobacteriaceae, Brochothrix thermosphacta, Flavobacterium, Shewanella),* yeasts and molds (Davies and Board, 1998; Hinton et al., 2004a; Corry, 2007). Growth and proliferation of these bacteria depend upon storage conditions, of which the temperature of storage and gaseous environment (aerobic vs. anaerobic) are most important. The initial microflora of freshly processed chicken carcasses consists primarily of mesophilic microorganisms. However, as refrigerated storage time increases the psychrotrophic bacteria predominate. Such psychrotrophic bacteria are especially prevalent on spoiled poultry. The predominant psychrotrophs on chicken carcasses after 10-11 d of storage at 1°C were *Pseudomonas* (90%), *Acinetobacter* (7%) and Enterobacteriaceae (3%) (Barnes and Thornley, 1966). *Pseudomonas* spp. are the predominant psychrotrophic bacteria associated with the spoilage of refrigerated poultry under aerobic conditions. Meat usually spoils when psychrotroph counts reach more than 10^7 – 10^8cfu/cm^2, which usually occurs after 6-8 d refrigerated storage (Nagel et al., 1960; Dainty and Mackey,

1992). Of the various *Pseudomonas* spp., the most commonly found on poultry are *P. fragi, P. lundensis, P. fluorescens* and other fluorescent pseudomonads closely related to *P. fluorescens* (Dainty and Mackey, 1992). The initial numbers of *Pseudomonas* spp. present in poultry meat directly relates to its aerobic shelf life at refrigeration or chill temperatures (Russell et al., 1996). *Acinetobacter* and *Aeromonas* spp. were the primary isolates recovered from carcasses sampled from the processing line in a commercial processing facility at various poultry processing sites that included pre-scalding, picking and chilling. *Pseudomonas* spp. were the predominant bacteria recovered from the carcasses stored under refrigeration (Hinton et al., 2004a).

Under anaerobic conditions or, when the oxygen concentration is depleted in meat the cause of spoilage is more related to Gram-positive and less oxygen-dependent bacteria such as lactobacilli, *Leuconostocs* and *Brochothrix thermosphacta* (Mead et al., 1986; Davies and Board, 1998; Corry, 2007). *B. thermosphacta* a Gram-positive non-sporulating, non-motile, rod-shaped bacteria and a facultative anaerobe, is one of the predominant spoilage organisms in chilled poultry and poultry products stored under modified atmospheres. *B. thermosphacta* causes spoilage because of its facultative anaerobic nature, which is often aided by increased carbon dioxide levels in vacuum packed products. In addition, *Shewanella putrefaciens,* which are strong sulfide producers can become a major component of the spoilage microflora under anaerobic conditions (Gill et al., 1990). Vacuum packaged broilers in an oxygen impermeable film with added carbon dioxide (Gill et al., 1990) had high initial populations of lactobacilli, which were subsequently outgrown by *Enterobacter* spp. with the development of a putrid spoilage odor.

Although yeasts and molds are often isolated on raw and spoiled poultry, they are not as important as psychrotrophic bacteria in causing spoilage of fresh poultry. The predominant species of yeasts and molds found on fresh poultry include *Candida, Debaryomyces, Kluyveromyces* and *Yarrowia* (Viljoen et al., 1988; Hinton et al., 2002).

Salmonella in Poultry Products

*Salmonella s*pp. are Gram-negative, non-spore forming, facultatively anaerobic, rod shaped bacteria belonging to the family *Enterobacteriaceae.* Most salmonellae are motile by peritrichious flagella except *Salmonella* serovar Gallinarum and *Salmonella* serovar Pullorum. Based on their somatic (O), flagellar (H), and capsular (Vi) antigenic patterns, the genus *Salmonella* has two species, *S. enterica* and *S. bongori,* which contain multiple serovars. There are a total of 2,541 serotypes, of which *S. enterica* and *S. bongori* contain 2,519 and 22 serotypes, respectively. Salmonellae are found worldwide and present in the intestinal tract of farm animals, birds, reptiles and humans. Poultry meat is a major reservoir for this bacterium in many countries, partly due to high-density farming operations which allow colonized birds to quickly spread salmonellae to other birds within a population. Also, the intestinal colonization by salmonellae increases the risk for contamination during slaughtering and processing processes. The National Antimicrobial Resistance Monitoring System (2007) conducted a five-year survey, which revealed presence of *Salmonella* in 11.5% of the chicken breast samples tested during this study in the US. Out of more than 2,300 serotpyes of *Salmonella enterica,* the most commonly found in poultry borne human gastroenteritis were *S.* Typhimurium, *S.* Enteritidis and *S.*Heidelberg (Foley et al., 2008; CDC, 2006). The recent data (CDC, 2010b) highlight the continuous dominance of *S.* Typhimurium, *S.* Enteritidis serotypes in causing foodborne illness. Usually the source of these

Salmonella serotypes is the contamination from intestinal tract during slaughtering and processing. Live birds can also harbor these pathogens in their skin and feathers and ultimately result in contamination of carcasses with *Salmonella*.

Prevalence of *Salmonella*

Salmonella was present in 10.2% of the broiler chicken carcasses tested (code A carcass rinse) at various federally inspected slaughter establishments (large, small and very small) in the US for a period of three years (1998-2000) whereas the prevalence of *Salmonella* in ground chicken samples was 14.4% (Rose et al., 2002). The results of the study also indicated that prevalence of *Salmonella* in most of the product categories appeared to be lower after the Final Rule on Pathogen Reduction; Hazard Analysis and Critical Control Point (PR/HACCP) implementation as compared to FSIS baseline studies and surveys conducted before the implementation of PR/HACCP (Rose et al., 2002). The Food Safety Inspection Service (FSIS) conducted a six year study to determine occurrence of *S.* Enteritidis in broiler chickens slaughtered at federally inspected establishments. The study revealed that out of the 51,327 broiler rinses tested, 280 (0.5%) *S*. Enteritidis isolates were recovered (Altekruse et al., 2006). During this survey, 90 establishments tested positive and the number of establishments testing positive increased from 17 (9%) of 197 in 2000 to 47 (25%) of 187 in 2005. During the period of the study, the number of states with *S*. Enteritidis in broiler rinses was also reported to increase from 14 to 24.

Bailey et al. (2002) documented the recovery of *Salmonella* in two separate trials as 7% and 36% in post-processing carcasses, which were tested as a carcass rinse method in the processing plants. The predominant serotypes isolated from the carcasses in trials 1 and 2 were *S.* Ohio and *S.* Senftenberg, respectively. Sampling of

broiler chicken carcasses at re-hang and post-chill sections during processing in 20 US poultry processing plants revealed the presence of *Salmonella* on 71.8% and 20.0% of the broiler carcasses at the re-hang station and after post-chill, respectively. These results indicated that the interventions employed during poultry processing were effective in lowering the levels of *Salmonella*. A total of 33 *Salmonella* serotypes were identified with the predominant serotypes being *S.* Kentucky, *S.* Heidelberg and S. Typhimurium (Berrang et al., 2009).

In a Danish study, (Dufrenne et al., 2001) most of the retail fresh chicken (89%) and frozen chicken (68%) samples had fewer than 10 *Salmonella* organisms per carcass when tested with MPN method. *Salmonella* numbers of more than 1,100 per chicken were encountered in only 2% of the samples.

***Salmonella* in pastured and free range poultry**

Free range poultry, pastured poultry or organic poultry have found great support among consumers. Producers are witnessing a growing demand for premium-priced pasture-raised poultry and eggs because of the high quality and unique flavor of such products. Many consumers assume that broiler chickens grown under traditional commercial conditions will have more *Salmonella* than pasture raised poultry, which usually are less crowded and are fed special diets. Siemon et al. (2007) compared the prevalence and antimicrobial resistance of *Salmonella* between pasture and conventionally reared poultry. No significant difference in *Salmonella* prevalence in feces of pasture and conventional poultry farms was observed. However, significant difference was observed on an individual specimen level where flocks reared conventionally had higher prevalence than in pasture. Of all the pasture isolates tested,

5% were resistant to ceftriaxone. None of the isolates from conventional flocks showed resistance to ceftriaxone.

The prevalence of *Salmonella* in retail organic chicken sold in the state of Maryland was determined by Cui et al. (2005). The results of the study indicated higher prevalence of *Salmonella* in organic chicken samples (61%) as compared to conventionally produced chicken (44%). The predominant *Salmonella* serotypes in organic and conventional chicken samples were *S.* Kentucky and *S.* Typhimurium, respectively. Both of these serotypes were more resistant to antibiotics than other serotypes isolated and tested during the study. In a similar study, *Salmonella* was detected in 22% of conventional and 20.8% of organic retail chicken carcass samples in Louisiana (Lestari et al., 2009). *S.* Kentucky, *S.* Hadar, and *S.* Enteritidis were the predominant serotypes isolated.

Melendez et al. (2010) recorded the prevalence of *S. enterica* in pasture raised poultry. The researchers reported that 59 out of 200 (29.5%) samples from pasture farms and retail carcasses tested positive for *Salmonella*, of which 50% were from retail carcasses and 25% from farm samples. A total of 6 serotpyes were isolated and the most predominant serotypes were *S.* Kentucky and *S.* Enteritidis. All isolates tested for antibiotics susceptibility were resistant to at least two antimicrobials, novobiocin and sulfisoxazole.

Campylobacter **in Poultry Products**

Campylobacter species are Gram-negative bacteria belonging to family *Campylobacteriaceae,* which are non-spore forming, curved, varying in size from 0.2 to 0.9 µm wide to 0.5–5.0 µm in length and catalase and oxidase positive. They are highly motile by single polar flagellum and exhibit corkscrew-like motion. *C. jejuni* cannot

multiply at refrigeration temperatures (40°F or below) used for storing raw poultry but can survive at refrigeration temperatures up to weeks. Of the 17 reported *Campylobacter* species, the most important are *C. jejuni, C. coli, C. fetus* and *C. lari.* However, only the thermophilic Campylobacters are usually associated with poultry which grow optimally at 42°C especially *C. jejuni.* It has been reported that in most countries, 50% to 80% of the chicken carcasses sold at retail levels were contaminated with *C. jejuni* (Hernandez, 1993; International Commission on Microbiological Specification for Foods, 2005). In poultry, the most common site for *Campylobacter* colonization, especially *C. jejuni* and *C. coli,* is the cecum where they can aggregate up to 10^6–10^7cfu/g of the cecal content. Berrang et al. (2000) reported high level of *Campylobacter* spp. populations of 5.4, 3.8, 4.7, 7.3 and 7.2 log cfu/g from feathers, skin, crop, ceca and colon of poultry, respectively. The high levels of *Campylobacter* found in crop and ceca can prove to be a significant factor in carcass contamination during processing particularly during evisceration. Defeathering is also one of the major sources of carcass contamination with *Campylobacter,* which can be related to the escape of contaminated feces from the cloaca during defeathering (Berrang et al., 2000).

Prevalence of *Campylobacter* spp.

The National Antimicrobial Resistance Monitoring System (2007) annual retail meat report revealed presence of *Campylobacter* in 49.9% of tested chicken breast samples. Based on high prevalence of Campylobacters in poultry it is not surprising that poultry products are the most often implicated foods in the cause of campylobacteriosis (Bryan and Doyle, 1995; Altekruse et al., 1999). *Campylobacter* was found in all areas of a poultry processing plant including pre-scald, post scald, post pick, post

evisceration, pre chill and post chill with numbers varying from 1.5 to 4.7 log cfu/mL (Berrang and Dickens, 2000). In a similar study, Son et al. (2007) examined chicken carcasses at pre-scald, pre-chill and post-chill sites from a commercial processing plant for the prevalence of *Campylobacter* spp. using a whole carcass rinse method. *Campylobacter* was isolated from 78.5% of the carcasses from all three collection sites. The percent prevalence of *Campylobacter* at pre-scald, pre-chill and post-chill carcasses was 92%, 100% and 52%, respectively. *C. coli* and *C. jejuni* were the two main *Campylobacter* spp. identified using the BAX® PCR assay with higher prevalence of *C. jejuni* (87.6%) than *C. coli* (12.4%).

Berrang et al. (2007) studied the prevalence and numbers of *Campylobacter* on broiler carcasses in 20 federally inspected US poultry processing plants at re-hang and post-chill sections of processing. The level of contamination was high in re-hang carcasses as compared to post-chill carcasses, which indicated that the interventions during poultry processing were effective in lowering the levels of *Campylobacter.* *Campylobacter* counts at re-hang stations ranged from 0.78 to 4.49 log cfu/mL of carcass rinse whereas the level of contamination decreased to 0.0 to 1.19 log cfu/mL of post-chill carcass rinse. In a similar study high counts of *Campylobacter (*5.40 log cfu/mL) were seen in pre-scald carcasses in a commercial processing facility followed by low levels in picked, eviscerated and chilled (less than 1.00 log cfu/mL) carcasses in decreasing order, which again indicates the susceptibility of *Campylobacter* to various processing techniques (Hinton et al., 2004b).

The prevalence of *Campylobacter* spp. in retail chicken carcasses sold in the Greater Washington D.C. area was 70.7% (Zhao et al., 2001). Of all the *Campylobacter*

isolates of chicken origin, nearly half (55.3%) were identified as *C. jejuni* based on multiplex PCR assay. A High prevalence rate of 83.3% for *Campylobacter* spp. in chicken carcasses sold in United Kingdom retail market was also observed with predominance of *C. jejuni* (77.3%) among various *Campylobacter* spp. (Kramer et al., 2000). A trend of high prevalence of *C. jejuni* in chicken during the warmer months was observed with recovery rates as high as 97% in market broilers in months of June and July (Willis and Murray, 1997).

***Campylobacter* in pastured and free range poultry**

Limited research is available concerning the prevalence and levels of contamination of free range poultry with *Campylobacter* spp. as compared to the conventionally raised commercial broiler chickens. Esteban et al. (2008) conducted a survey on the presence of major foodborne bacterial pathogens in 60 flocks of free-range chickens from 34 farms in the Basque Country (Northern Spain). *Campylobacter* was the most prevalent species, isolated in 70.6% of the farms among other pathogens such as *Salmonella,* and *Listeria.* In a recent study on the prevalence of *Campylobacter* in free range reared chickens, the birds were tested for the presence of *Campylobacter* before (anal swab samples) and after slaughter (Colles et al., 2010). C*ampylobacter* was present in both live and slaughtered birds with prevalence rates of 96% and 100%, respectively. In live birds, of various *Campylobacter* isolates 80.6% were *C. jejuni* and in chicken carcasses 62.0% were *C. jejuni* out of 155 *Campylobacter* isolates obtained. Other studies have reported significantly greater prevalence of thermophilic Campylobacters in free range and organic poultry as compared to conventionally reared poultry (Giessen et al., 1996; Heuer et al., 2001)

Listeria monocytogenes in Poultry Products

L. monocytogenes a psychrotrophic Gram-positive bacillus is an opportunistic pathogen. Although it is more commonly seen in RTE products, it has been isolated from raw poultry (Lawrence and Gilmour, 1994; Waldroup, 1996). Pregnant women, newborns, and immunocompromised persons are at the highest risk for contacting listeriosis due to *L. monocytogenes.* It is estimated that every year 2,500 cases of illness and 500 deaths occur due to listeriosis in the US (Lungu and Johnson, 2005; Juck et al., 2010). Approximately 13 different serotypes of *L. monocytogenes* have been described, and serotypes 1/2a, 1/2b, and 4b are the most commonly encountered in illnesses caused by *L. monocytogenes* (Kathariou, 2002; Gorski et al., 2006). Serotype 1/2a strains are most often isolated from foods and processing environment (Gorski et al., 2006). The presence of *L. monocytogenes* in RTE products usually involves post-processing contamination and is of high significance in terms of food safety. *L. monocytogenes* is a major concern for its presence in RTE foods. Outbreaks have been reported with consumption of RTE poultry products in the US (CDC, 2002), which highlights the public health significance of this pathogen in the RTE poultry products. Due to its high public health significance, the FSIS maintains a zero-tolerance policy for *L. monocytogenes* in RTE meat and poultry products.

Prevalence of *L. monocytogenes* in raw poultry

Barbalho et al. (2005) examined the presence of *Listeria* contamination in a commercial poultry processing plant by sampling carcasses at varying stages of processing*. Listeria* presence was also tested on the hands and gloves of food handlers and the chilling water used in the process. *Listeria innocua* was the most common species isolated, which was present at all sampling sites except the water samples. *L.*

monocytogenes was only detected on carcasses at packaging (14.3% of the samples) and on the hands and gloves (11.8% of the samples) of the employees. The employees whose hands and gloves were found to be contaminated with *Listeria* spp. were working in more than one area of the plant, which could contribute to cross contamination of the poultry carcasses.

Lawrence and Gilmour (1994) reported an overall incidence of 20% of *L. monocytogenes* from a poultry processing plant and poultry products (raw and cooked chicken carcass). None of the cooked carcasses contained *L. monocytogenes,* while 59% of the raw chicken carcasses were contaminated with *L. monocytogenes.* Bailey et al. (1989) recorded the presence of *L. monocytogenes* in 23% of broiler carcasses sampled in the Southeastern US. The predominant serotypes were 1/2 b and 1/2 c. A high prevalence rate of 62% *wa*s recorded for *L. monocytogenes* in retail broilers sampled at three different broiler abattoirs (Miettinen et al., 2001). The percent prevalence in chicken breasts, legs, drumstick and wings was 52, 68, 67, and 50, respectively. Serotype 1/2 c was the predominant serotype in raw broiler meat. Bohaychuk et al. (2006) reported the presence of *L. monocytogenes* in 34% of chicken leg samples collected from retail outlets in Edmonton, Canada where the predominant serotypes isolated were 1/2a, 1/2b and 1/2c.

Prevalence of *L. monocytogenes* in ready-to-eat poultry products

The incidence of *L. monocytogenes* in hot dogs, frankfurters and wieners sold in the US retail supermarkets was surveyed by Wang and Muriana (1994). *L. monocytogenes* was isolated from 7.5% of the 93 packages sampled and also *L monocytogenes* was the predominant *Listeria* species found among various retail franks tested. The only other *Listeria* spp. isolated was *L. innocua*. Most of the samples tested

were chicken and/or turkey meat type frankfurters. The numbers of *L. monocytogenes ranged from* 4.3 to 27.6 per package based on the most probable number (MPN) analysis. Hudson et al. (1992) analyzed RTE meat products including chicken and turkey products sold at retail outlets for the presence of *L. monocytogenes*. All turkey samples were negative for *L. monocytogenes* whereas 12.5% of the RTE chicken product samples were positive for *L. monocytogenes*. Bohaychuk et al. (2006) reported the presence of *L. monocytogenes* in 3% of processed turkey breast and 3% of chicken wiener samples collected from retail outlets in Edmonton, Canada where the serotypes isolated were 1/2a and 1/2b.

Gombas et al. (2003) surveyed *L. monocytogenes* in RTE foods in the US with an objective to develop data on the risk assessment of listeriosis. Different categories of RTE foods such as luncheon meats, deli salads, various cheeses, and smoked seafood were collected from retail markets at Maryland and northern California FoodNet sites. The overall prevalence was 1.82% from the 31,705 samples tested, with prevalence rates ranging from 0.17 to 4.7% among different categories. The highest prevalence rate was found in seafood categories followed by luncheon meats. The level of contamination of *L. monocytogenes* in the positive samples ranged from 0.3 MPN/g to 1.5×10^5cfu/g.

Current Methods in Post-harvest Processing and Further Processing of Poultry for Control of Foodborne Pathogens and Spoilage Microorganisms Using Food-grade Chemicals

The public health significance of *Salmonella* and *Campylobacter* spp. in meat and poultry products, has led USDA FSIS to develop guidelines to control the contamination of these pathogens in raw poultry products. The FSIS has updated the performance standards for *Salmonella* and introduced the performance standards for *Campylobacter*

for the first time in chicken carcasses since its first inception of PR/HACCP rule in 1996. USDA FSIS also maintains a zero tolerance policy for presence of *L. monocytogenes* in RTE foods in order to address the problem of food-borne listeriosis.

The recent FSIS compliance guidelines for controlling *Salmonella* and *Campylobacter* address various pre-harvest and post-harvest measures for the control of *Salmonella* and C*ampylobacter* spp. in poultry production. Currently, various food-grade chemicals such as organic acids, trisodium phosphate and chlorine based compounds, have been approved by USDA FSIS as safe and suitable for use in the poultry production at various stages of processing (USDA FSIS, 2011). These compounds are used as antimicrobial interventions to control the spoilage and pathogenic microorganisms in the final product.

Chlorine and Acidified Sodium Chlorite

Chlorine and various chlorine based compounds are the most commonly used compounds in poultry processing plants to reduce the level of bacterial contamination particularly *Salmonella* and *Campylobacter* from poultry carcasses (Northcutt et al., 2005; Oyarzabal, 2005; Hinton et al., 2007). Chlorine based compounds such as chlorine gas, sodium hypochlorite, calcium hypochlorite and electrolytically generated hypochlorus acid have been approved for use as a spray on whole or eviscerated poultry carcasses, in the water used for processing (except for product formulation) and in poultry chiller water at a level not exceeding 50 ppm as free available chlorine (USDA FSIS, 2011). Chlorine dioxide is a potent oxidizing agent that is approved for use in water designated only for poultry processing with levels not exceeding 3 ppm of residual chlorine dioxide (USDA FSIS, 2011).

Hinton et al. (2007) found that treating the broiler carcasses with chlorinated and electrolyzed oxidizing (EO) water as a spray resulted in significantly fewer psychrotrophic bacteria as compared to carcasses sprayed only with tap water. Electrolyzed oxidizing water was more effective in reducing the spoilage microorganisms as compared to chlorine, which was more effective than tap water. Fabrizio et al. (2002) showed that EO water treatment of artificially contaminated poultry carcasses resulted in significant reduction (1.06 log) of *S.* Typhimurium following extended refrigerated storage. Chlorination of water was effective in reducing the pathogen load from the chill water but this effect is reduced when chlorinated water is used to treat poultry carcasses. Northcutt et al. (2005) reported that chlorine level and water temperature had no effect in enhancing the removal of *Salmonella* and *Campylobacter* from broiler carcasses following spray washing broiler carcasses with 50 ppm chlorinated water at temperatures of 21.1, 43.3, or 54.4°C. Another study also reported no effect of chlorination of chill water on the prevalence of salmonellae in raw chicken carcasses and giblets (James et al., 1992). Yang et al. (2001) found that immediate chlorination (with 10 ppm of chlorine) of chiller water inoculated with *C. jejuni* and *S.* Typhimurium reduced *C. jejuni* and *S.* Typhimurium by 3.3 and 0.7 log cfu/mL, respectively. These pathogens were not detected following chlorination at levels of 30 and 50 ppm of chlorine. Chlorination of chilled water did not effectively reduce the bacteria attached on chicken skins. Ellis et al. (2006) observed significant reduction in *S.* Typhimurium populations (approximately 1 log unit) in samples treated with chlorine dioxide (ClO_2) (fast- and slow-release sachets) as compared to the controls. However, the ClO_2 treatment resulted in adverse effects on color of the chicken in areas close to

the ClO_2 sachet. Hong et al. (2008) revealed 1.37-1.44 log cfu/g reduction of S. Typhimurium following treatment of artificially inoculated chicken breast and drumstick with 100 ppm of ClO_2. Although the concentration of ClO_2 used in this study was more than the approved levels for the US, the data revealed significant improvement in the microbial safety of chicken during storage.

Acidified sodium chlorite (ASC) is a secondary direct food additive approved for use in poultry carcasses and products (USDA FSIS, 2011). It is produced by mixing an aqueous solution of sodium chlorite with any generally recognized as safe (GRAS) acid to achieve a pH of 2.2 to 3.0 and then further diluting this solution with a pH elevating agent (such as sodium bicarbonate, sodium carbonate) to achieve a final pH of 5.0 to 7.5. In poultry processing, it can be applied as a component of a carcass spray or dip solution prior to carcass immersion in a pre-chiller or chiller tank or in a pre-chiller solution for application to the intact carcass or as a component of a spray or dip solution for application to poultry carcass parts. It has been also approved for its use as a component of a post-chill carcass spray or dip solution when applied to poultry meat, organs, or related parts. The final concentration should be 500 to 1,200 ppm, when used as a spray or dip solution, and 50 to 150 ppm, when used in pre-chiller or chiller solutions.

del Río et al. (2007) reported 1.65 to 2.42 log cfu/g and 0.60 to 1.05 log cfu/g reductions of *S.* Enteritidis and *L. monocytogenes,* respectively, following the dipping treatment of chicken legs in ASC solution (1,200 ppm) and refrigerated storage for 5 days. Kemp et al. (2001) found that fecally contaminated poultry carcasses when subjected to continuous online processing (COP), which is the combined use of an

inside-outside-bird-washer and an online ASC spray system, significantly reduced the *Salmonella* incidence (10.0%) as compared to standard offline reprocessing (*Salmonella,* 31.6% incidence). COP treated carcasses also had a significant reduction in *Campylobacter* spp.

Sexton et al. (2007) reported significant reduction in the natural contamination levels of *Salmonella* and *Campylobacter* on chicken carcasses processed in South Australia following ASC treatment. Post chill application of ASC on broiler carcasses resulted in significant reduction in *Campylobacter* spp. prevalence and *counts* to less than 0.2 log cfu/mL of carcass rinse (Oyarzabal et al., 2004).

Organic Acids

Organic acids such as acetic acid, lactic acid, citric acid and their salts are commonly used antimicrobial preservatives in foods. They are generally recognized as safe (GRAS) and have been approved for use in meat and poultry processing (USDA FSIS, 2011). They are effective against spoilage as well as pathogenic microorganisms (Dickens and Whittemore, 1997; Over et al., 2009, Birk et al., 2010). Most of these organic acids are weak acids and they are more effective in their undissociated form. Okrend et al. (1986) found that the use of 0.1% solution of acetic acid in poultry scalding tanks restricted the growth of *Salmonella* by decreasing thermal tolerance of *S.* Newport, *S.* Typhimurium and *C. jejuni*. Over et al. (2009) tested acetic, citric acid, malic acid, and tartaric acid at 150.0 mM concentration on chicken breasts inoculated with *S.* Typhimurium and *L. monocytogenes*. Citric acid, malic acid, and tartaric acid at 150 mM concentration were effective in reducing *L. monocytogenes* and *S.* Typhimurium by more than 2 and 4-6 log cfu/g, respectively.

In another study using 0.6% acetic acid with air injection resulted in the significant reduction in *Salmonella* incidence in broiler carcasses (Dickens and Whittemore, 1994). A combination of 0.5% acetic acid with 0.05% potassium sorbate or 0.05% sodium benzoate reduced *C. jejuni* populations by more than 5 log cfu/mL within 1 min in suspension whereas 1.4 log cfu/g reduction of *C. jejuni* was observed for chicken wings when treated with 2% acetic acid (Zhao and Doyle, 2006).

Peroxyacetic acid also known as peracetic acid (PAA) is a weaker acid than acetic acid and is generated upon reaction of acetic acid with hydrogen peroxide. It can be helpful in reducing the level of contamination with pathogens and extension of product shelf-life (Bauermeister et al., 2008). PAA has been approved as an antimicrobial for use in poultry chillers and as acidifier in scalding tanks at levels not exceeding 220 ppm (USDA FSIS, 2011). Bauermeister et al. (2008) found that PAA when added to poultry chiller water was effective in decreasing both *S.* Typhimurium and *C. jejuni* levels on poultry carcasses with levels as low as 0.02%.

In meat and poultry processing, lactic acid is mainly used in beef decontamination and can be used in solutions up to 4% depending upon the type of meat product with levels not to exceed 0.5% of finished product formulation (USDA FSIS, 2011). It is also approved for use in RTE poultry products and has been found effective in reducing the level of *L. monocytogenes* on artificially inoculated frankfurters (Byelashov et al., 2010). Lactic acid spray had a significant bactericidal effect on *C. jejuni* on artificially contaminated poultry carcasses (Cudjoe and Kapperud, 1991). Ten minutes spray of 2% lactic acid solution resulted in total elimination of *C. jejuni* within 24 h whereas no effect on viability of *C. jejuni* was observed when carcasses were sprayed with 1% or

2% lactic acid 24 h after inoculation. Lecompte et al. (2009) reported that 5% lactic acid application on chicken skins' surface for 1 min inoculated with *S.* Enteritidis and *C. jejuni* resulted in 2.38 log reduction for *S*. Enteritidis after 7 d of storage, without affecting any organoleptic properties to the product. However, the decontamination treatment did not result in a significant reduction in *C. jejuni* cells.

Organic acids marinades (particularly the use of sodium lactate, potassium lactate, sodium citrate) are gaining popularity especially in RTE meat and poultry products because of their ability to reduce *L. monocytogenes* in these products (Miller and Acuff, 1994; Alvarado and Mckee, 2007).

Quaternary Ammonium Compounds

Among various quaternary ammonium compounds, only cetylpyridinium chloride (CPC) has been approved to treat the surface of raw poultry carcasses prior to or after chilling (USDA FSIS, 2011). Cetylpyridinium chloride has been found to be effective against different foodborne pathogens such as *S.* Typhimurium, *C. jejuni* and *L. monocytogenes* (Breen et al., 1997; Singh et al., 2005; Riedel et al., 2009). It can be applied as a spray with the concentration of CPC in the solution applied to the carcasses not exceeding 0.8% by weight. However, this treatment should be followed by a potable water rinse of the carcass if the application is not followed by immersion in a chiller (USDA FSIS, 2011).

Breen et al. (1997) tested different combinations of concentration and time of treatment of CPC on chicken skin artificially inoculated with *Salmonella* to determine its antimicrobial effects in reducing *Salmonella* levels. They found nearly 5 log reduction of S. Typhimurium with concentration of CPC as low as 0.4% and treatment time of 3 min on chicken skin. Xiong et al. (1998) reported 1.5 to 1.9 log reduction of *S.* Typhimurium

after spraying of chicken skin with 0.1% and 0.5% CPC solutions. Significantly greater reduction in *Salmonella* was seen with 0.5% CPC than 0.1% CPC.

CPC was also effective in reducing *C. jejuni* levels on chicken skin below detection after 1 min dip in 0.5% CPC solution (Riedel et al., 2009). As an anti-*Listeria* compound in RTE products, Singh et al. (2005) found no significant reductions in *L. monocytogenes* population on frankfurters inoculated with a 1% CPC solution treatment. However, 1.7 log cfu/g reduction was observed with 1% CPC solution treatment at 25°C, 20 psi and 30 s of exposure.

Trisodium Phosphate

Phosphates are widely used as food additives in meats and other food products of animal origin and serve many purposes including alkalization, acidification, emulsification, stabilizer, moisturizer, sanitization and etc. There is a long list of phosphates that are used in meat and poultry products, some of the commonly used polyphosphates are sodium acid pyrophosphate, sodium tripolyphosphate, sodium hexametaphosphate and trisodium phosphate (TSP). Although primarily they carry out the aforementioned functions, they do have variable antimicrobial properties (Shelef and Seiter, 1993). In US, the maximum permissible level of phosphates allowed in the foods particularly meat products is 0.5% of the finished product (USDA FSIS, 2011). The most effective antimicrobial phosphate compound which is currently used in poultry processing is TSP, although it is not a direct food additive but approved as carcass dips or spray and has been proven to be an effective antimicrobial agent when used at high (8-12%) concentrations (Kim et al., 1994; Kim and Slavik, 1994; del Río et al., 2007). USDA FSIS allows use of TSP on raw pre-chill poultry carcasses and giblets when used

as 8-12% solution applied by spraying or dipping giblets for up to 30 sec and carcasses (within a temperature range of 65°F to 85 °F) for 15 sec (USDA FSIS, 2011).

TSP is a highly alkaline compound and most of the studies focused on TSP antimicrobial effect were on *Salmonella* and *C. jejuni*. Lillard (1994) reported 2 log reductions of *S*. Typhimurium following immersion of poultry carcasses for 15 min in 10% TSP solution. Xiong et al. (1998) also reported 2.1 and 2.2 log reduction of *Salmonella* following spraying of chicken skin with 5% and 10% TSP solutions, respectively. Bourassa et al. (2004) reported a decrease in *Salmonella* recovery after pre-chill TSP treatment (10% solution) and refrigerated storage (2°C) of poultry carcasses. del Río et al. (2005) reported TSP (12% solution for 15 min) to be effective against common spoilage microorganisms such as *P. fluorescens* and *B. thermosphacta* on chicken skin during refrigerated storage.

Riedel et al. (2009) reported significant reduction in *C. jejuni* populations (1.74 log reduction) following 1 min dipping of chicken skin inoculated with *C. jejuni* in 10% TSP solution. Increasing the duration of treatment up to 15 min had no additional effect on reduction in *C. jejuni* populations. Other studies have also reported 1 to 2 log reduction of *C. jejuni* with 10% TSP treatments in poultry carcasses (Whyte et al., 2001; Arritt et al., 2002). TSP was effective against *L. monocytogenes* in chicken meat after several days of refrigerated storage (Capita et al., 2001b). Treatments with 8, 10, or 12% TSP solutions significantly reduced *L. monocytogenes* populations by more than 3 logs on chicken skin.

Natural Spices or Other Natural Ingredients

Spices are regularly used in various foods worldwide. Although they are mainly added to foods as flavoring and seasoning agents, many spices have significant

antimicrobial activity. The antimicrobial property is due to the specific chemicals or essential oils. Essential oils are the plant extracts or more precisely distilled aromatic oily liquids obtained from plant material such as herbs, roots, bark, seeds, leaves, etc. Their actual antimicrobial action is due to their active components and ingredients specific to that particular essential oil (e.g., eugenol in cloves, allicin in garlic, cinnamic aldehyde in cinnamon, allylisothiocyanate in mustard, eugenol and thymol in sage and carvacrol and thymol in oregano). Most of these essential oils have GRAS status for their intended use, under the FDA, CFR 21 section 182.20. Extensive research has been conducted and some is still in process concerning their antimicrobial activities in a variety of foods. Essential oils have been evaluated against most of the commonly encountered foodborne pathogens *in vitro* as well as in different food systems including poultry products (Pandit and Shelef, 1994; Hao et al., 1998; Friedman et al., 2002; Fisher and Phillips, 2006; Ruiz et al., 2009).

Researchers have reported that some essential oils contain high levels of phenolic compounds such as thymol and oregano, which possess stronger antibacterial activity against foodborne pathogens than other plant extracts or essential oil (Davidson, 1997; Lambert et al., 2001; Burt, 2004). Essential oils and their components are highly lipid soluble, which enables them to invade the bacterial cell membrane easily and disturb the internal cellular structures. These essential oils appear to be more effective against Gram-positive bacteria than Gram-negative bacteria (Vaara, 1992; Burt, 2004; Fisher and Phillips, 2006). One possible reason could be the presence of the outer membrane in Gram-negative bacteria, which restricts the diffusion of hydrophobic compounds through their lipopolysaccharide layer (Ratledge and Wilkinson, 1988; Vaara, 1992).

Oral et al. (2009) reported that addition of sachets or pads containing oregano essential oil in overwrap packed fresh chicken drumsticks extended the product shelf life by approximately 2 d. In a similar study, Chouliara et al. (2007) reported 3-4 days extension of shelf life of fresh chicken breast meat when treated with oregano essential oil and packaged under modified atmosphere. Skandamis et al. (2002) examined the effect of oregano essential oil on survival/death of *S.* Typhimurium in beef fillets stored at 5°C under different conditions of storage. Addition of 0·8% v/w oregano essential oil resulted in 1–2 log cfu/g reduction of *S.* Typhimurium.

Addition of allyl isothiocyanate (AITC) under controlled release combined with modified atmosphere packaging on the fresh chicken resulted in 0.77 and 1.3 log cfu/g reductions for *L. monocytogenes* and *S*. Typhimurium, respectively (Shin et al., 2010). Exposure of chicken breasts to 100, 300 and 600 µL of AITC at 4°C significantly affected the growth and survival of *Campylobacter* and psychrotrophic organisms (de Mello, 2002). Most of these essential oils are very effective *in vitro* against major food-borne pathogens but their efficacy is variable in the meat environment because of complex food matrices components such as high fat and protein in different meats, which restricts the inhibitory effects of AITC against pathogens in meat products (Pandit and Shelef, 1994; Burt, 2004; Piskernik et al., 2011). *C. jejuni* is also susceptible to various essential oils or extracts of spices and plants in pure culture or different foods (Friedman et al., 2002; Fisher and Phillips, 2006; Piskernik et al., 2011).

Sodium Metasilicate

Sodium metasilicate (SMS) is a white anhydrous fast dissolving, fine granular chemical with pH of 1% solution ranging from 12.5 to 13.0. Depending upon the number of water molecules associated with SMS chemical structure it is commercially available

in three different chemical forms: SMS anhydrous, SMS pentahydrate, and SMS nonahydrate with 0, 5 and 9 water molecules in its crystal structures, respectively. It is the pentahydrate form that is approved for its use in the food and meat industry and is considered generally recognized as safe (GRAS) chemical.

Sodium metasilicate is USDA FSIS approved as a processing aid and can be used as an antimicrobial component of marinades for meat and poultry products up to 2% by weight of the marinades and as a carcass rinse or spray on raw beef carcasses, subprimals and trimmings up to 6% of a solution (USDA FSIS, 2011). It has also been approved for use in RTE meat products where it can be used up to a 6% solution applied on the surface of the product at a rate not exceeding 300 ppm of the finished product (USDA FSIS, 2011). Recently, USDA FSIS approved a blend of SMS and sodium carbonate for use in RTE poultry products with levels up to 15% of a solution of SMS and sodium carbonate (SMS not to exceed 6%) applied as a surface application at a rate not to exceed 700 ppm by weight of the finished poultry product (USDA FSIS, 2011).

The antimicrobial activity of SMS has had limited *in vitro* investigation in meat systems, and has not been investigated at all in poultry products. Sodium metasilicate was examined for its *in vitro* antimicrobial activities in water at room temperature against *Escherichia coli* O157:H7 and generic *E. coli*. Exposure of *E. coli* O157:H7 to SMS (5 to 10 s) at a concentration as low as 0.6% resulted in complete inhibition with no recoverable *E. coli* O157:H7 (Weber et al., 2004).Carlson et al. (2008) observed 2.6 and 1.9 log cfu/cm^2 reduction of *Salmonella* and *E. coli* O157:H7, respectively, following the treatment of inoculated beef hides with a spray solution of 4% SMS (pH 12.94) at 23ºC.

Pohlman et al. (2009) reported significant reduction of *Salmonella* (more than 1.5 log) in beef trimmings prior to grinding, inoculated with *S.* Typhimurium and treated with 4% SMS solution with little impact on sensory odor and color characteristics. Huang (2010) studied the effectiveness of SMS as a marinade in chicken fillets against psychrotrophic microorganisms and found that the total psychrotrophic counts in chicken fillets treated with 4% SMS were significantly lower than the control fillets following refrigerated storage for 9 days. Although very little is known about the effectiveness of SMS as antimicrobial in fresh poultry and poultry products against common poultry-borne pathogens such as *Salmonella* and *Camplyobacter* spp, the research previously discussed suggested that SMS should have some effectiveness in controlling the growth of poultry spoilage microflora and poultry-borne pathogens such as *Salmonella* and *Camplyobacter* spp.

Mechanism of Action of High pH on Inactivation/Inhibition of Foodborne Pathogens

Alkaline chemicals such as TSP, sodium hydroxide (NaOH) and SMS have been found to be effective as either bactericidal or bacteriostatic against common foodborne pathogens such as *E. coli* O157:H7, *Salmonella* spp., *Campylobacter* spp. and *L. monocytogenes* both in suspension as well as in meat system (Teo et al., 1996; Taormina and Beuchat, 2002; Sampathkumar et al., 2003; Weber et al., 2004; Zhao and Doyle, 2006; del Río et al., 2007; Carlson et al., 2008; Pohlman et al., 2009). Limited studies have been undertaken to determine the mode of action of high pH on Gram-negative and Gram-positive pathogens (Mendonca et al., 1994; Sampathkumar et al., 2003) as a result of alkaline conditions produced by the use of the previously mentioned chemicals.

Previous studies have attempted to elucidate the high pH effect on foodborne pathogens by postulating that the action of alkaline conditions result in loss of membrane integrity as a result of disruption of cytoplasmic membrane, changes in cell morphology and leakage of cytoplasmic contents leading to cell death (Mendonca et al., 1994; Sampathkumar et al., 2003)

Mendonca et al. (1994) were the pioneers in studying the mechanism of action of high pH against common food-borne pathogens. They exposed *S.* Enteritidis, *E. coli* O157:H7 and *L. monocytogenes* to high pH conditions generated with sodium hydroxide and sodium bicarbonate buffers for different time periods. High pH (12.0) treatments resulted in approximately 8 log reductions in *S.* Enteritidis and *E. coli* O157:H7 and 1 log reduction in *L. monocytogenes* upon exposure to pH 12.0 for 10 min. They reported the reductions in *Salmonella* and *E. coli* as a result of cell death due to rupturing of cell membrane. This leakage of cytoplasmic contents particularly nucleic acids was determined by absorbance of cell filtrates at 260 nm, which was confirmed by agar gel electrophoresis of filtrates. Scanning electron micrographs of *S.* Enteritidis and *E. coli* O157:H7 exposed to pH 12 revealed collapsed and wrinkled cells.

Transmission electron microscopy of cells treated with high pH (12.0) revealed the separation of outer membranes of *S.* Enteritidis from the cell wall at various points. However, these changes were not observed in *L. monocytogenes* which indicated that the latter may be less susceptible to high pH effects, which may be attributed to the differences in cell wall structure of Gram-negative and Gram-positive bacteria, as the latter possess a thick peptidoglycan layer as compared to Gram-negative bacteria such as *Salmonella* (Murray et al., 1965; Sampathkumar et al., 2003).

In a similar study, Sampathkumar et al. (2003) determined that treating *S.* Enteritidis with TSP (pH 11.0) resulted in rapid cell death with no detectable survivors after 1 h. Electron microscopy revealed that there was disruption of cytoplasmic and outer membranes of *S.* Enteritidis as a result of the alkaline pH which led to permeabilization of cell membrane and leakage of intracellular contents and eventual cell death. The leakage of the cytoplasmic contents resulted in considerable release of DNA, proteins, and lipopolysaccharides as revealed by measurement of absorbance of cell filtrates at 260 nm, agarose gel electrophoresis, Bradford assay and tricine-sodium dodecyl sulfate gel electrophoresis of the filtrates.

The permeability of the cytoplasmic membrane of *S.* Enteritidis as a result of alkaline environment was also confirmed by the uptake of fluorescent propidium iodide (PI) dye (Sampathkumar et al., 2003), which is a membrane-impermeant dye and excluded by live cells (Kim et al., 2009). No scientific literature is documented on the bactericidal or bacteriostatic mechanism of action of SMS, which is also a high pH chemical. This investigation is also designed to elucidate the mode of antimicrobial action of SMS against both Gram-negative and Gram-positive foodborne pathogens.

Flow Cytometry Application in Determination of Live and Dead Bacterial Pathogens

Flow cytometry has been used in food microbiological applications in the enumeration of and detection of bacteria including the foodborne pathogens *L. monocytogenes*, *E. coli* O157:H7 and *Salmonella* in different food products (Donnelly and Baigent, 1986; Laplace-Builhé et al., 1993; McClelland and Pinder, 1994; Gunasekera et al., 2000; Comas-Riu and Rius, 2009). The ease of use and rapidity of the results have made flow cytometry a widely used microbiological tool. Also the

combination of flow cytometry with fluorescent stains can be used to assess variety of cell functions such as cell viability based on membrane integrity and other physiological functions (Davey and Kell, 1996; Gruden et al., 2004; Paparella et al., 2008).

Flow cytometry has been used in detection of live and dead cells both in eukaryotic cells and prokaryotic microorganisms (Jacobsen et al., 1997; Kim et al., 2009). The most commonly and widely used fluorescent stains in flow cytometry to differentiate live and dead bacterial cells are the propidium iodide (PI) and SYTO 9, both of which bind and stain the nucleic acids. Propidium iodide is a red fluorescent stain that can only enter and stain the cells presumed to be dead with damaged or permeabilized cytoplasmic membranes while SYTO 9, which emits green fluorescence is able to stain both live and dead cells.

Propidium iodide and SYTO 9 are commercially available and are the main components of the popular LIVE/DEAD BacLight kit (Invitrogen). These two nucleic acid-binding stains have been used to discriminate both live and dead Gram-negative and Gram-positive bacterial pathogens such as *E. coli*, *Salmonella* and *L. monocytogenes* (Berney et al., 2007; Kim et al 2009). In addition to PI and SYTO 9 dyes, there are other fluorescent dyes such as carboxyfluorescein diacetate (cFDA), chemchrome B and rhodamine 123 (rh 123) which have been employed to investigate the viability status of various pathogens (Jacobsen et al., 1997; López-Amorós et al., 1997; Paparella et al., 2008). Flow cytometry based evaluation of live and dead cells using these stains help in providing quick and efficient results and also help in quantification of live and dead cells. The combination of viability staining and flow

cytometry has been investigated for food-borne pathogens like *Salmonella* and *L. monocytogenes*.

In addition to discriminating live and dead cells, flow cytometry can be a valuable tool in detecting viable but non culturable cell (VBNC) states of pathogens when the appropriate fluorescent stains are employed. The VBNC state of pathogens has been seen in many Gram-negative food borne pathogens, but cannot be detected by conventional plating methods (Khan et al., 2010). Flow cytometry can also be utilized to determine the mode of action of various processing techniques such as supercritical carbon dioxide (SC-CO_2) treatment for inactivation of pathogens such as *Salmonella* (Kim et al., 2009). A disadvantage of the technique is that the use of SYTO 9 and PI is not fully reliable for discriminating live and dead bacterial populations, because SYTO 9 sometimes is excluded from live bacteria with intact membranes (Berney et al., 2007; Khan et al., 2010).

Swarts et al. (1998) studied the effect of the bacteriocin leucocin B-TA11a on *L. monocytogenes* and used flow cytometry to determine live and dead cells by using Dead/Live *Bac*light Bacterial Viability Kit™. They found that *Bac*light kit was suitable for viability determination of *L. monocytogenes* exposed to the bacteriocin leucocin B-TA11a, as it could distinguish viable *L. monocytogenes* from stressed cells in mixed populations. Kim et al. (2009) used flow cytometry to determine the viability of SC-CO_2 treated *S.* Typhimurium using double staining with SYTO 9 and PI. They also used double staining by combining SYTO 9 with ethidium bromide (EB) to interpret the physiological cell status of SC-CO_2 treated *S.* Typhimurium cells. They found that SC-

CO_2 treatment of *S.* Typhimurium resulted in loss of membrane integrity and affected the efflux pump activity of the treated cells.

Paparella et al. (2008) applied flow cytometry to evaluate the antimicrobial activity of oregano, thyme and cinnamon essential oils against *L. monocytogenes* using double staining with cFDA and PI. Flow cytometric evaluation revealed three different subpopulations of *L. monocytogenes* following treatment with essential oils i.e., viable, dead and injured cells. Cells treated with cinnamon essential oil had minimally damaged membranes whereas treatment with oregano and thyme essential oil resulted in membrane disintegration. Hardly any literature has been documented on the use of flow cytometry in differentiation of live and dead bacterial pathogens treated with sodium metasilicate or other high pH chemical such as sodium hydroxide or trisodium phosphate. So this study will be the first of its kind to observe the effects of SMS on Gram negative and positive pathogens and their differentiation of live and dead cells based on flow cytometry evaluation using various staining techniques.

CHAPTER 3
SODIUM METASILICATE AFFECTS GROWTH OF *SALMONELLA* TYPHIMURIUM IN CHICKEN BREASTS STORED AT FOUR DEGREES CELSIUS FOR SEVEN DAYS

Salmonellosis remains to be the number one cause of bacterial causes of foodborne infections in the US. (Scallan et al., 2011). Poultry has been cited to be the most common food implicated in foodborne illnesses (CDC, 2010a). *Salmonella* Typhimurium and *S.* Enteritidis are the most commonly found poultry borne *Salmonella* serotypes implicated in foodborne salmonellosis (CDC, 2006; Foley et al., 2008). In order to safeguard public health by ensuring food safety, there is a great need to decrease the prevalence of *Salmonella* in poultry products reaching the consumer table. Till date various antimicrobial interventions such as acidified sodium chlorite (Kemp et al., 2001; USDA FSIS, 2011), organic acids (Over et al., 2009, Birk et al., 2010), and trisodium phosphate (Kim et al., 1994; Lillard, 1994; Bourassa et al., 2004) have been studied and used in poultry processing to control the problem of *Salmonella* in poultry carcasses. But, no single method has been proven to be fully effective and resulted in total elimination of *Salmonella* in fresh poultry and poultry products. This demands additional interventions to use along with existing preventive measures to further restrict the occurrence of *Salmonella* at various steps in poultry processing. Cutting, packaging and further processing of poultry are among various potential sources in poultry processing where the poultry meat can become contaminated. Most of the antimicrobial interventions in poultry processing end with the refrigerated storage of carcasses after the chilling process. It is assumed that the residual effect of earlier treatments is sufficient to control the post processing contamination.

Sodium metasilicate (SMS) is a white anhydrous fast dissolving chemical with the pH of a 1% solution ranges from 12.5 to 13.0. Sodium metasilicate is USDA, FSIS

approved as a processing aid and can be used as an antimicrobial component of marinades for meat and poultry products up to 2% by weight of the marinades (USDA FSIS, 2011). Its application can provide an additional intervention in poultry processing to ensure the final removal or restriction of the growth of *Salmonella* and thereby provide additional protection. Sodium metasilicate has been effective against gram negative foodborne pathogens such as *Salmonella* and *Escherichia coli* O157:H7 both *in vitro* and in meat systems (Weber et al., 2004; Carlson et al., 2008; Pohlman et al., 2009), but it has not been evaluated for its efficacy against *Salmonella* in poultry meat. The objectives of this study were to determine the antimicrobial properties of SMS against *S.* Typhimurium and psychrotrophic microorganisms in fresh chicken breasts inoculated with *S.* Typhimurium and to ascertain the effects of the SMS treatments on the pH at approved levels as well as the usage level that would exert maximum antimicrobial effect during refrigerated storage of chicken breasts.

Materials and Methods

This study was designed and carried out in two different experiments during which SMS (Danisco, USA Inc.) was used and tested for its efficacy as a food antimicrobial when applied as a marinade (Experiment 1 – skinless and boneless chicken breasts) and carcass application (Experiment 2 – skin-on chicken breasts) to treat chicken breasts inoculated with *S.* Typhimurium. The study was carried out at the Meat Microbiology Laboratory, University of Florida, Gainesville, FL. Except for sample type, and application method, the same procedures were followed in both experiments.

Bacterial Strain and Inoculum Preparation

A reference strain of *S.* Typhimurium (ATCC 14028) was obtained from ABC Research Corporation in Gainesville, FL, and used as the inoculum in this study to

evaluate the antimicrobial properties of SMS. Stock cultures were prepared by transferring two colonies of reference strain to a test tube containing 10 mL of tryptic soy broth (TSB, 157152, MP Biomedicals, LLC, Solon, Ohio) supplemented with 0.5% yeast extract (212750, Becton, Dickinson and Company, Sparks, MD) followed by incubation at 35°C for 24 h. After incubation, the cultures were centrifuged in sterile 15 mL centrifuge tubes (05-539-5, Fisher Scientific, Pittsburgh, PA) at 5,000 g for 10 min at 4°C (Sorvall RC-5, Dupont Instruments, Newton, CT). The supernatant was discarded and the pellets were resuspended in 10 mL of sterile 0.1% peptone water (CM009, Oxoid Ltd., Basingstoke, Hampshire, England), centrifuged again and pellets were suspended in 1 mL of TSB supplemented with 30% glycerol in sterile 2 mL cryogenic vial (430488, Corning Inc., Corning, NY), stored at -80°C, and used as the stock culture for the inoculation studies. The working cultures were prepared by thawing the frozen culture at room temperature for 5 to 10 min, and a loopful was streaked onto tryptic soy agar (TSA) (MP Biomedicals, LLC, Solon, OH) followed by incubation at 35°C for 24 h. One colony was selected and transferred using a sterile loop to 10 mL of TSB supplemented with 0.5% yeast extract and incubated at 35°C for 20 h. Cells were pelleted by centrifugation at 5,000 g for 10 min at 4°C, harvested, washed and re-suspended in sterile 0.1% peptone water. Preliminary recovery studies were carried out with different volumes of inoculum used to determine the final concentration of inoculum between 5 to 6 log cfu/g in chicken beast samples. It was found that using 100 µL of 10^8 cfu/mL of inoculum onto 25 g of chicken breast meat resulted in desired final concentration of inoculum.

Sample Preparation, Inoculation and Treatment

Experiment 1. Uncooked skinless and boneless chicken breast meat samples with expiration date of at least one week were purchased from a local supermarket, transported on ice and processed on the same day or kept at 4°C for use within 24 h. The chicken breast meat was cut into 25 g samples under aseptic conditions and inoculated with approximately 10^8 cfu/mL of *S.* Typhimurium inoculum. The samples were left to stand for 20 min at room temperature in order to attach bacteria to the surface of the meat. Sodium metasilicate solutions 1% (pH 12.4 to 12.6) and 2% (12.5-12.7) were prepared in sterile de-ionized water and used as treatments for this study. The chicken breasts were marinated in solutions containing either 0% SMS (only sterile de-ionized water) and no inoculum (negative control), 0% SMS (only sterile de-ionized water) and inoculum (positive control), 1 and 2% SMS solutions plus inoculum. In each treatment, the chicken breast samples were packaged (one sample per bag) in the marinade solutions for the duration of storage period to ensure direct contact of the marinade with the meat.

Experiment 2. Higher concentrations of SMS were used than the currently approved levels to determine its efficacy as a carcass application. For this purpose, uncooked skin-on chicken breast meat samples were procured from the same source and treated under similar conditions except that the concentrations of SMS used were1 and 2% by weight of the meat not the marinade to simulate SMS carcass treatment applications. Negative and positive controls were included in the study as previously described for experiment one. In both experiments following the treatments, samples were aseptically packaged in sterile Fisher bags, mixed manually in the bags to ensure proper distribution of SMS and stored at 4 ± 1°C for 7 d. Duplicate samples for each

treatment were analyzed on 0, 1, 3, 5 and 7 d of storage for *Salmonella* recovery, psychrotrophic counts and pH measurements. Three separate trials were conducted for each experiment.

Microbiological and pH Analyses

For both experiments, 25 g samples of chicken breast were transferred aseptically from storage bags to 225 mL of sterile 0.1% peptone water in a sterile stomacher bag (01-002-56, Fisher Scientific, Pittsburgh, PA) and manually agitated for approximately 1 min to loosen and suspend bacteria in solution. Serial dilutions were prepared by transferring 1.0 mL of the sample homogenate to 9 mL of 0.1% sterile peptone water. A volume of 0.1 mL from each dilution was pipette onto duplicate prepoured XLT-4 agar plates (223420, Becton, Dickinson and Company, Sparks, MD) prepared with XLT-4 agar supplement (235310, Becton, Dickinson and Company, Sparks, MD) for *Salmonella* recovery and TSA plates for psychrotrophic microorganisms. The XLT-4 agar plates were incubated aerobically for 48 h at 35°C and the TSA plates were aerobically stored at 7 ±1°C for 10 d. Black colonies or colonies that were black-centered with a yellow periphery on XLT-4 agar were considered presumptively *S.* Typhimurium. After incubation, colony forming units (cfu) from each plate were counted, averaged and reported as log cfu/g of the sample. The pH for each sample homogenate was measured by placing the pH probe into the sample homogenate immediately after the microbiological analyses were completed. All pH measurements were recorded in duplicate using an Accumet pH meter (AB15 Accumet Basic, Fisher Scientific, Pittsburgh, PA).

Data Analyses

A total of 120 samples were analyzed per experiment. A complete randomized block design was used. Samples consisting of 4 treatments, 5 storage d, duplicate samples and 3 trials were analyzed by GLIMMIX procedures of SAS (SAS Institute, 2009) to analyze differences between trials, among treatments and storage d, and treatment × day interaction. The mean separation was performed using Tukey-Kramer and a level of significance of $\alpha = 0.05$ was used to determine any significant differences among means.

Results and Discussion

S. Typhimurium Recovery and Analysis

Experiment 1. No *Salmonella* was isolated in the negative control. In positive control, the counts of *S.* Typhimurium ranged from 5.01 to 6.33 log cfu/g d 0 through 7 d of storage (Table 3-1). No immediate antimicrobial effects of SMS were observed for 1 and 2% SMS treatments, as evidenced by similar ($P > 0.05$) *Salmonella* counts among the positive control and SMS treatments on d 0. Skinless and boneless chicken breasts marinated with 1 and 2 % SMS had lower ($P < 0.05$) *Salmonella* counts when compared to the positive control on d 3, 5 and 7 of storage. Reduction in *Salmonella* counts for the 1% SMS treatment included 0.91, 0.83 and 0.86 log cfu/g and 1.04, 1.16 and 1.13 for 2% SMS treatment on d 3, 5 and 7, respectively. The SMS treatments were similar ($P > 0.05$) on all sampling d.

Experiment 2. No *Salmonella* was isolated in the negative control. The initial *Salmonella* count for the breast meat in the positive control was 6.09 log cfu/g, which verified the desired 5 to 6 log cfu/g concentration of *S.* Typhimurium in the chicken breast (Table 3-2). The 1 and 2% SMS treatments reduced ($P < 0.05$) *Salmonella*

counts when compared to the positive control on d 0, 1, 3, 5 and 7. The use of 1 and 2% SMS (by weight of the meat) as a carcass application on skin-on chicken breasts resulted in an immediate (d 0) reduction of 1.43 ($P < 0.05$) and 2.90 ($P < 0.05$) logs of *Salmonella* respectively. Treatment of chicken breasts with 2% SMS resulted in 2.48 to 4.11 log cfu/g reductions ($P < 0.05$) of *S.* Typhimurium from d 0 to d 7 of storage. The reduction in *Salmonella* counts with 1% SMS treatment ranged from 1.11 to 1.43 log cfu/g from d 0 through d 7.

The findings from both experiments suggested that SMS is effective in reducing *Salmonella* in poultry meat, and its effect is enhanced when used at 2% concentration. The use of SMS could result in enhancing the microbiological safety of poultry meat. Although, this is the first work completed on the antimicrobial properties of SMS against *Salmonella* in poultry meat, similar findings were reported by Carlson et al. (2008) and Pohlman et al. (2009) for SMS in beef systems. Carlson et al. (2008) observed 2.6 log cfu/cm^2 reduction of *Salmonella* following the treatment of inoculated beef hides with a spray solution of 4% SMS (pH 12.94) at 23ºC whereas Pohlman et al. (2009) reported significant reduction of *Salmonella* (more than 1.5 log) in beef trimmings prior to grinding, inoculated with *S.* Typhimurium and treated with 4% SMS solution.

Psychrotrophic Microorganism Analysis

Experiment 1. Psychrotrophic counts increased with simultaneous increase in storage time for all treatments and controls (Table 3-3). No significant differences ($P > 0.05$) in the psychrotrophic counts were observed among controls and with 1 and 2 % SMS treatments on any day of storage. Poultry meat is considered to be spoiled when total psychrotrophic counts exceed 7 log cfu/g (Dainty and Mackey, 1992). This level of psychrotrophic counts were observed from d 3 through d 7 of storage in all treatments.

Experiment 2. Psychrotrophic counts increased with simultaneous increase in storage time for all treatments and controls (Table 3-4). Psychrotrophic counts were similar ($P > 0.05$) for chicken breasts treated with 1% SMS and the control samples on all storage d. Except for d 0, chicken breasts treated with 2% SMS had lower ($P < 0.05$) psychrotrophic counts when compared to the negative and positive controls on all storage d. Psychrotrophic counts were similar ($P > 0.05$) for chicken breasts treated with 1 and 2% SMS on all storage d. This observation revealed the effectiveness of SMS against psychrotrophic counts particularly when used at 2% concentration by weight of the meat. The data suggested that SMS could function in extending the shelf life of fresh poultry by retarding the growth of psychrotrophic microorganisms.

pH Analysis

Experiment 1. The pH values were similar ($P > 0.05$) for negative and positive controls from d 0 to d 7 (Table 3-5). Chicken breasts (skinless) treated with 1% SMS had higher ($P < 0.05$) pH values than the controls on d 0 and 1 only, whereas chicken breasts treated with 2% SMS had higher ($P < 0.05$) pH values than negative and positive controls on all sampling d. The reduction in *Salmonella* counts reported for chicken breast meat treated with 2% SMS may be largely attributed to the higher pH generated by 2% SMS. It was also observed that the pH of the 1% SMS treatment was insufficient to decrease psychrotrophic counts in the chicken breasts.

Experiment 2. The pH values were similar ($P > 0.05$) for negative and positive controls from d 0 to d 7 (Table 3-6). All chicken breasts (skin-on) treated with 1 and 2% SMS had higher ($P < 0.05$) pH values when compared to the negative and positive controls on all storage d. There was a difference of more than 2 pH units between the untreated and treated chicken breasts, which could be one possible explanation for

higher ($P < 0.05$) reductions (more than 3 log units) in *Salmonella* counts (Table 3-2), when compared to only 1log unit reduction of *Salmonella* populations in skinless chicken breasts treated with SMS (by weight of marinade in Experiment 1) at the currently approved levels (Table 3-1). Except for d 1, chicken breasts treated with 2% SMS had higher ($P < 0.05$) pH values when compared to the samples treated with 1% SMS.

In summary, SMS was effective in reducing *Salmonella* populations in skinless chicken breasts but had no effect on the growth of psychrotrophs when it was used as a marinade. The antimicrobial efficacy of SMS against *Salmonella* increased with an increase in concentration from 1 to 2% in marinades, with 2% SMS (by weight of the marinade) being the most effective level as a marinade application. Sodium metasilicate was effective in reducing *Salmonella* counts ($P < 0.05$) when applied at higher concentrations than the currently approved levels in skin-on chicken breasts, which suggests its future application as a carcass treatment in addition to the currently approved application as a marinade. The antimicrobial effectiveness of SMS increased as its concentration increased from 1 to 2% (by weight of meat) with the most effective antimicrobial level being 2% as a carcass application, which resulted in more than 3 log unit reductions in *Salmonella* counts in skin-on chicken breasts. The data from this study also revealed that SMS was effective in restricting the growth of psychrotrophs when used at 2% SMS by weight of the meat. The study also pointed out that the antimicrobial properties of SMS could be due to its high pH.

The data in this study revealed that SMS has significant antimicrobial potential against *S.* Typhimurium in chicken meat and should function to safeguard the

consumer's health by providing an additional intervention for poultry processing to enhance food safety. The data also suggested that SMS could function in extending the shelf life of poultry by retarding the growth of psychrotrophs, which are the primary spoilage microflora of fresh poultry.

Table 3-1. *Salmonella* counts (log cfu/g) in uncooked skinless and boneless chicken breasts inoculated with *S.* Typhimurium, treated with sodium metasilicate (by weight of marinade) and stored at 4 ± 1°C for 7 d: Experiment 1

	Day of Sampling				
Treatment	0	1	3	5	7
Negative Control	ND	ND	ND	ND	ND
Positive control	6.33[a, x]	6.05[a, x]	5.97[a, x]	5.59[a, y]	5.01[a, z]
1% SMS	5.97[a, w]	5.57[ab, x]	5.06[b, y]	4.76[b, y]	4.15[b, z]
2% SMS	5.76[a, w]	5.25[b, x]	4.93[b, x]	4.43[b, y]	3.88[b, z]

[a-b] Means within a column lacking a common superscript differ ($P < 0.05$)
[w-z] Means within a row lacking a common superscript differ ($P < 0.05$)
ND = Not detected
n= 6 values per mean
SMS = Sodium metasilicate

Table 3-2. *Salmonella* counts (log cfu/g) in uncooked chicken breasts (skin-on) inoculated with *S.* Typhimurium, treated with sodium metasilicate (by weight of meat) and stored at 4 ± 1°C for 7 d: Experiment 2

	Day of Sampling				
Treatment	0	1	3	5	7
Negative control	ND	ND	ND	ND	ND
Positive control	6.09[a, x]	5.48[a, y]	5.19[a, y]	4.88[a, y]	4.35[a, z]
1% SMS	4.66[b, x]	4.12[b, y]	3.93[b, y]	3.76[b, y]	3.24[b, z]
2% SMS	3.19[c, x]	3.0[c, x]	1.65[c, y]	0.77[c, z]	0.77[c, z]

[a-c] Means within a column lacking a common superscript differ ($P < 0.05$)
[x-z] Means within a row lacking a common superscript differ ($P < 0.05$)
ND = Not detected
n= 6 values per mean
SMS = Sodium metasilicate
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Table 3-3. Psychrotrophic counts (log cfu/g) in uncooked skinless and boneless chicken breasts inoculated with *S.* Typhimurium, treated with sodium metasilicate (by weight of marinade) and stored at 4 ± 1°C for 7 d: Experiment 1

	Day of Sampling				
Treatment	0	1	3	5	7
Negative control	4.82 [a, z]	5.64 [a, y]	7.59 [a, x]	8.58 [a, w]	9.28 [a, v]
Positive control	4.85 [a, z]	5.81 [a, y]	7.60 [a, x]	8.39 [a, w]	9.07 [a, v]
1% SMS	4.24 [a, z]	4.98 [a, y]	6.73 [a, x]	8.48 [a, w]	9.06 [a, v]
2% SMS	5.25 [a, z]	5.67 [a, z]	7.49 [a, y]	8.38 [a, x]	9.20 [a, w]

[a-b] Means within a column lacking a common superscript differ ($P < 0.05$)
[v-z] Means within a row lacking a common superscript differ ($P < 0.05$)
n= 6 values per mean
SMS = Sodium metasilicate

Table 3-4. Psychrotrophic counts (log cfu/g) in chicken breasts (skin-on) inoculated with *S.* Typhimurium, treated with sodium metasilicate (by weight of meat) and stored at 4 ± 1°C for 7 d: Experiment 2

	Day of Sampling				
Treatment	0	1	3	5	7
Negative control	5.01 [a, y]	6.13 [a, y]	8.07 [a, x]	8.43 [a, x]	9.01 [a, x]
Positive control	4.93 [a, z]	6.28 [a, y]	7.77 [a, x]	8.56 [a, x]	9.13 [a, x]
1% SMS	4.39 [a, z]	5.19 [a b, z y]	6.38 [a b, y x]	7.18 [a b, x]	7.79 [a b, x]
2% SMS	3.47 [a, z y]	3.30 [b, z]	4.64 [b, y x]	6.21 [b, x]	5.97 [b, x]

[a-c] Means within a column lacking a common superscript differ ($P < 0.05$)
[x-z] Means within a row lacking a common superscript differ ($P < 0.05$)
n= 6 values per mean
SMS = Sodium metasilicate

Table 3-5. pH measurements for uncooked skinless and boneless chicken breasts inoculated with *S.* Typhimurium, treated with sodium metasilicate (by weight of marinade) and stored at 4 ± 1°C for 7 d: Experiment 1

	Day of Sampling				
Treatment	0	1	3	5	7
Negative control	6.31 $^{c, y}$	6.32 $^{c, y}$	6.36 ^{b,y}	6.36 $^{b, y}$	6.56 $^{b, x}$
Positive control	6.31 $^{c, x}$	6.29 $^{c, x}$	6.39 $^{b, x}$	6.31 $^{b, x}$	6.45 $^{b, x}$
1% SMS	6.61 $^{b, x}$	6.56 $^{b, x}$	6.57 $^{b, x}$	6.53 $^{a\ b, x}$	6.63 $^{a\ b, x}$
2% SMS	6.92 $^{a, x}$	6.80 $^{a, x}$	6.86 $^{a, x}$	6.73 $^{a, x}$	6.83 $^{a, x}$

$^{a-c}$ Means within a column lacking a common superscript differ ($P < 0.05$)
$^{x-y}$ Means within a row lacking a common superscript differ ($P < 0.05$)
n= 6 values per mean
SMS = Sodium metasilicate

Table 3-6. pH measurements for chicken breasts (skin-on) inoculated with *S.* Typhimurium, treated with sodium metasilicate (by weight of meat) and stored at 4 ± 1°C for 7 d: Experiment 2

	Day of Sampling				
Treatment	0	1	3	5	7
Negative control	6.54 $^{c, x}$	6.50 $^{b, x}$	6.49 $^{c, x}$	6.52 $^{c, x}$	6.80 $^{c, x}$
Positive control	6.47 $^{c, x}$	6.38 $^{b, x}$	6.37 $^{c, x}$	6.44 $^{c, x}$	6.64 $^{c, x}$
1% SMS	9.40 $^{b, x}$	9.11 $^{a, x}$	8.84 $^{b, x}$	8.93 $^{b, x}$	8.79 $^{b, x}$
2% SMS	10.20 $^{a, x}$	9.84 $^{a, x}$	9.90 $^{a, x}$	9.82 $^{a, x}$	9.92 $^{a, x}$

$^{a-c}$ Means within a column lacking a common superscript differ ($P < 0.05$)
$^{x-y}$ Means within a row lacking a common superscript differ ($P < 0.05$)
n= 6 values per mean
SMS = Sodium metasilicate

CHAPTER 4
SODIUM METASILICATE AFFECTS *CAMPYLOBACTER JEJUNI IN VITRO* AND IN CHICKEN BREASTS STORED AT FOUR DEGREES CELSIUS FOR SEVEN DAYS

Campylobacteriosis caused by *Campylobacter* spp. is one of the leading causes of foodborne illnesses in the US. In 2009, the CDC reported *Campylobacter* as the second leading cause of foodborne infections, being responsible for 13.02 cases of illnesses per 100,000 persons (CDC, 2010b). Of the various *Campylobacter* species recognized, *Campylobacter jejuni* is the most pathogenic to man. Poultry is the principal reservoir of this microorganism in the food chain and it has been reported that in most countries, up to 80% of the chicken carcasses sold at retail levels are contaminated with *C. jejuni* (Hernandez, 1993; International Commission on Microbiological Specification for Foods, 2005). Based on the high prevalence of *Campylobacter* in poultry it is not surprising that poultry products are among the most common foods implicated in the cause of campylobacteriosis (Bryan and Doyle, 1995; Altekruse et al., 1999). As poultry and poultry products per capita consumption increase annually, the risk associated with foodborne illnesses from *C. jejuni* contaminated poultry products will also increase.

Due to public health significance of *C. jejuni,* USDA FSIS has introduced performance standards for *Campylobacter* for the first time in chicken carcasses since the inception of the Final Rule on Pathogen Reduction and Hazard Analysis and Critical Control Point (PR/HACCP rule) in 1996 (USDA FSIS, 2010). There is a great need to decrease the prevalence of *C. jejuni* in poultry products. Despite the use of various antimicrobial interventions such as acidified sodium chlorite (Kemp et al., 2001; Oyarzabal et al., 2004), organic acids (Zhao and Doyle, 2006; Bauermeister et al., 2008; Birk et al., 2010), trisodium phosphate (Whyte et al., 2001; Arritt et al., 2002; Riedel et al., 2009) and natural antimicrobials (de Mello, 2002; Friedman et al., 2002;

Fisher and Phillips, 2006; Piskernik et al., 2011) during poultry processing, poultry carcasses are frequently contaminated with *Campylobacter* spp. Therefore, there is tremendous need for additional interventions for use along with existing preventive measures to further restrict the occurrence of *C. jejuni* in poultry carcasses.

Sodium metasilicate (SMS) is a highly soluble chemical and the pH of a 1% solution ranges from 12.5 to 13.0. Sodium metasilicate is USDA FSIS approved as a processing aid for poultry products and can be used as an antimicrobial component of marinades for poultry products up to 2 % by weight of the marinades (USDA FSIS, 2011). Its application can provide an additional intervention in poultry processing to ensure the final removal or restriction of the growth of *C. jejuni* and thereby provide additional protection. Sodium metasilicate has not been evaluated for its efficacy against *C. jejuni* in poultry meat. The objectives of this study were to determine the antimicrobial properties of SMS against *C. jejuni in vitro* and to assess the antimicrobial effects of SMS against *C. jejuni* and psychrotrophic microorganisms in fresh chicken breasts inoculated with *C. jejuni*. In addition the effects of SMS treatments on pH during refrigerated storage of chicken breasts were also studied.

Materials and Methods

Two different studies were conducted to assess the antimicrobial effects of SMS against *C. jejuni*. In the first study, SMS was evaluated for its efficacy against *C. jejuni* in suspension and in second study the antimicrobial effects of SMS were determined against *C. jejuni* and psychrotrophs in chicken meat. The second study was designed and carried out in two different experiments during which SMS (Avgard®XP, Danisco, USA Inc.) was used and tested for its efficacy as a food antimicrobial when applied as a marinade (Experiment 1 - skinless and boneless chicken breasts) and carcass

application (Experiment 2 - skin-on) to treat chicken breasts inoculated with *C. jejuni*. These studies were carried out at the Meat Microbiology Laboratory, University of Florida, Gainesville, FL. Except for sample type, and application method, the same procedures were followed in both trials.

Bacterial Strain and Exposure of Cells to SMS and High pH

A reference strain of *C. jejuni* subsp. *jejuni* (ATCC 33560) was obtained from American Type Culture Collection (ATCC, Manassas, VA) and used to evaluate the antimicrobial properties of SMS. Microaerophilic conditions (5% oxygen, 10% carbon dioxide, and 85% nitrogen), required for the growth of *C. jejuni*, were produced by placing packs of GasPak™ EZ Campy container system (260680, Becton, Dickinson and Company, Sparks, MD) in BBL gas jars (11-814-39, Becton, Dickinson and Company, Sparks, MD). Stock cultures were prepared as instructed by ATCC. The freeze-dried reference strain was transferred to a test tube containing 6 mL of Brucella broth (211088, Becton, Dickinson and Company, Sparks, MD) and colonies were isolated on TSA with 5% defibrinated sheep blood (R01200 Remel, Lenexa, KS) with incubation at 37°C for 24 h under microaerophilic conditions. Identification of *C. jejuni* colonies was based on colony morphology, catalase, oxidase tests and hippurate hydrolysis. The cultures from the plates were frozen using the guidelines in the US FDA Bacteriological Analytical Manual (BAM, 2001) and incubated at -80°C till further use. The working cultures were prepared by thawing the frozen cultures at room temperature for 5 to 10 min, inoculating a loopful onto TSA blood agar and incubation at 42°C for 24 h under microaerophilic conditions. Two colonies were selected and transferred to 10 mL of *Campylobacter* Enrichment Broth (7526A, Neogen Corporation, Lansing, MI) containing lysed horse blood (15-14-0250-24, Hema Resource and Supply Inc., Aurora,

OR) and antibiotics (vancomycin, trimethoprim, cefoperazone, and cycloheximide) and incubated at 42°C for 24 h. After incubation, the cultures were centrifuged in sterile15-mL centrifuge tubes (05-539-5, Fisher Scientific, Pittsburgh, PA) at 5,000 g for 10 min at 4°C (Sorvall RC-5, Dupont Instruments, Newton, CT). Pellets were harvested, washed twice and re-suspended in 10 mL of treatment solution at room temperature containing either (i) 0.1% peptone water (control); (ii) 0.5% (pH 12.1), 1% (pH 12.4) and 2% (pH 12.5) SMS solutions, and (iii) 0.1 N NaOH (pH 12.5) solution for 1 and 30 min.

Cell Viability

Treated cells were tested for their viability by transferring 1 mL aliquots from each treated cell suspension and serially diluting in 9 mL of sterile 0.1% peptone water. A volume of 0.1mL from each dilution was pipetted onto duplicate pre-poured *Campylobacter* agar base plates (CM 0689, Oxoid Ltd., Basingstoke, Hampshire, England) supplemented with lysed horse blood and *Campylobacter* selective supplement (Blaser-Wang) (SR0098E, Oxoid Ltd., Basingstoke, Hampshire, England) for *C. jejuni* recovery. The *Campylobacter* agar plates were incubated for 48 h at 42°C under microaerophilic conditions as described above. After incubation, typical *C. jejuni* colonies from each plate were counted, averaged and reported as logarithmic colony-forming units per gram (log cfu/g) of the sample. Duplicate samples for each treatment were analyzed and the experiment was repeated three times.

Inoculum Preparation for Inoculation Studies

The working cultures were prepared by thawing the frozen cultures at room temperature for 5 to 10 min, inoculating a loopful onto TSA blood agar and incubation at 42°C for 24 h under microaerophilic conditions. Two colonies were selected and transferred to 10 mL of *Campylobacter* Enrichment Broth (7526A, Neogen Corporation,

Lansing, MI) containing lysed horse blood (15-14-0250-24, Hema Resource and Supply Inc., Aurora, OR) and antibiotics (vancomycin, trimethoprim, cefoperazone, and cycloheximide) and incubated at 42°C for 24 h. After incubation, the cultures were centrifuged in sterile15-mL centrifuge tubes (05-539-5, Fisher Scientific, Pittsburgh, PA) at 5,000 g for 10 min at 4°C (Sorvall RC-5, Dupont Instruments, Newton, CT). Pellets were harvested, washed twice and re-suspended in 10 mL of sterile phosphate buffered saline pH 7.4 (PBS) and used for inoculating the chicken breasts. Preliminary experiments were conducted to insure that a final concentration of inoculum between 4 to 5 log cfu/g was achieved in the chicken beast samples.

Sample Preparation, Inoculation and Treatment

Experiment 1. Uncooked skinless and boneless chicken breast meat samples, with expiration date of at least one week, were purchased from a local supermarket, transported on ice and processed on the same day or kept at 4°C for use within 24 h. Chicken breasts were cut into 25 g samples under aseptic conditions and inoculated with approximately 10^7 cfu/mL of *C. jejuni* in order to recover 4 to 5 log cfu/g. The samples were left to stand for 20 min at room temperature in order to allow for attachment of bacteria to the meat surface. One (pH 12.4 to 12.6) and 2% (pH 12.5-12.7) SMS solution were prepared in sterile de-ionized water and used as marinade treatments in this study. The 2% treatment represented the maximum concentration of SMS that can be used in a marinade for fresh poultry. The treatments included marinating the chicken breasts in solutions containing either 0% SMS (only sterile de-ionized water) and no inoculum (negative control), 0% SMS (only sterile de-ionized water) and inoculum (positive control), and 1 and 2% SMS solutions plus inoculum. In each treatment, the chicken breast samples were packaged (one sample per bag) in the

marinade solutions for the duration of the storage period to ensure direct contact of the marinade with the meat.

Experiment 2. Higher concentrations of SMS were used than the currently approved levels to determine its efficacy as a carcass application. For this purpose, uncooked skin-on chicken breast meat samples were procured from the same source and treated under similar conditions except that the concentrations of SMS used were 1 and 2% by weight of the meat not the marinade to simulate SMS carcass treatment applications. Negative and positive controls were included in the study as previously described for experiment 1. In both experiments following the treatments, samples were aseptically packaged in sterile Fisher bags, mixed manually in the bags to ensure proper distribution of SMS and stored at 4 ± 1°C for 7 d. Duplicate samples for each treatment were analyzed on 0, 1, 3, 5 and 7 d of storage for *C. jejuni* recovery, psychrotrophic counts and pH measurements. Three separate trials were conducted for each experiment.

Microbiological and pH Analyses

For both experiments, 25 g chicken breast samples were transferred aseptically from storage bags to 225 mL of sterile 0.1% peptone water in a sterile stomacher bag (01-002-56, Fisher Scientific, Pittsburgh, PA) and manually agitated for approximately 1 min in order to loosen and suspend bacteria in solution. Serial dilutions were prepared by transferring 1.0 mL of the sample homogenate to 9 mL of 0.1% sterile peptone water. A volume of 0.1mL from each dilution was pipetted onto duplicate pre-poured *Campylobacter* agar base plates (CM 0689, Oxoid Ltd., Basingstoke, Hampshire, England) supplemented with lysed horse blood and *Campylobacter* selective supplement (Blaser-Wang) (SR0098E, Oxoid Ltd., Basingstoke, Hampshire, England)

for *C. jejuni* recovery, and TSA (MP Biomedicals, LLC, Solon, OH) plates for isolation of psychrotrophic microorganisms. The *Campylobacter* agar plates were incubated for 48 h at 42°C under microaerophilic conditions as described above and TSA plates were aerobically stored at 7 ±1°C for 10 d. After incubation, typical *C. jejuni* colonies and psychrotrophs from each plate were counted, averaged and reported as logarithmic colony-forming units per gram (log cfu/g) of the sample. The pH for each sample homogenate was measured by placing the pH probe into the sample homogenate immediately after the microbiological analyses were completed. All pH measurements were recorded in duplicate using an Accumet pH meter (AB15 Accumet Basic, Fisher Scientific).

Data Analyses

Data for the *in vitro* cell viability experiment were analyzed by GLIMMIX procedures of SAS (SAS Institute, 2009) to analyze differences between trials, among treatments and exposure time, and treatment × time interaction. The mean separation was performed using Tukey's and a level of significance of $\alpha = 0.05$ was used to determine any significant differences among means.

For chicken breast experiments, a total of 120 samples were analyzed per experiment. A complete randomized block design was used consisting of 4 treatments, 5 storage d, duplicate samples and 3 trials. Data were analyzed by GLIMMIX procedures of SAS (SAS Institute, 2009) to analyze differences between trials, among treatments and storage d, and treatment × day interaction. The mean separation was performed using Tukey-Kramer and a level of significance of $\alpha = 0.05$ was used to determine any significant differences among means.

Results and Discussion

Effect of SMS and High pH on Cell Viability

This study revealed that SMS resulted in more than 4 log reduction of *C. jejuni* with concentration of SMS as low as 0.5% (Table 4-1). Treating *C. jejuni* with 0.5, 1 and 2% SMS and 0.1N NaOH for 1 and 30 min resulted in similar reduction *(P > 0.05)* of *C. jejuni* populations. The results indicate that SMS possess antimicrobial effects against *C. jejuni.* The reduction was similar with high pH treatments that highlight the potential role of high pH of SMS as its antimicrobial properties. In an earlier study it was proven that SMS was unable to exert any antimicrobial effects against *Salmonella* following pH neutralization (with 6.0 N HCl) in chicken breasts inoculated with *S.* Typhimurium, which indicates that it's the high pH of SMS that is responsible for bactericidal effects against *S.* Typhimurium.

The effect of high pH on viability has been studied. Exposing *C. jejuni* cultures to 0.1% potassium hydroxide solution (pH 12.2) *in vitro* resulted in similar reduction (more than 4 logs) of *C. jejuni* (Hinton et al., 2006). High pH has been reported to cause rapid death of Gram-negative pathogens such as *Salmonella* (Mendonca et al., 1994; Sampathkumar et al., 2003). But *C. jejuni* seems to resist high pH better than *Salmonella* as seen earlier where the latter was immediately inactivated following exposure to SMS but *C. jejuni* was still surviving after 30 min treatment with SMS as high as 2% SMS.

Campylobacter jejuni Recovery and Analysis

Experiment 1. *Campylobacter jejuni* counts were similar ($P > 0.05$) for all treatments when compared to the positive control (Table 4-2). *C. jejuni* counts ranged from 4.01 to 4.83 for all treatments. No *C. jejuni* was isolated in the negative control.

Experiment 2. No *C. jejuni* was detected in the negative control. The *C. jejuni* counts ranged from 4.42 to 4.71 log cfu/g d 0 through 7 d of storage for the positive control (Table 4-3). The 1 and 2% SMS treatments reduced ($P < 0.05$) *C. jejuni* counts on all storage days when compared to the positive control. The use of 1 and 2% SMS (by weight of the meat) as a carcass application on skin-on chicken breasts resulted in immediate (on d 0) log reductions of 1.12 ($P < 0.05$) and 3.27 ($P < 0.05$) for *C. jejuni,* respectively. Treatment of chicken breasts with 2% SMS resulted in 3.27 to 3.79 log cfu/g reductions ($P < 0.05$) of *C. jejuni* from d 0 to d 7of storage. The reduction in *C. jejuni* counts with 1% SMS treatment ranged from 1.12 to 1.26 log cfu/g from d 0 through d 7.

The findings from both experiments suggest that SMS is effective in reducing *C. jejuni* in poultry meat, and its effect is enhanced when used at 2% concentration (by weight of meat). The use of SMS could result in enhancing the microbiological safety of poultry meat. Although, this is the first work completed on the antimicrobial properties of SMS against *C. jejuni* in poultry meat, SMS has been reported by Carlson et al. (2008) and Pohlman et al. (2009) for its effectiveness against *Salmonella* in beef systems. Carlson et al. (2008) observed 2.6 log cfu/cm^2 reduction of *Salmonella* following the treatment of inoculated beef hides with a spray solution of 4% SMS (pH 12.94) at 23ºC whereas Pohlman et al. (2009) reported significant reduction of *Salmonella* (more than 1.5 log) in beef trimmings prior to grinding, inoculated with *S.* Typhimurium and treated with 4% SMS solution.

Psychrotrophic Microorganism Analysis

Experiment 1. Psychrotrophic counts increased with simultaneous increase in storage time for all treatments and controls (Table 4-4). Psychrotrophic counts were

similar ($P > 0.05$) among controls and breast meat treated with 1 and 2% SMS on all storage days. Poultry meat is considered to be spoiled when total psychrotrophic counts exceed 7 log cfu/g (Dainty and Mackey, 1992) which was observed from d 3 through d 7 for all treatments.

Experiment 2. Psychrotrophic counts increased with storage time for all treatments and controls (Table 4-5). Psychrotrophic counts were similar ($P > 0.05$) for chicken breasts treated with 1% SMS and the control samples on all storage d. Except for d 0, chicken breasts treated with 2% SMS had lower ($P < 0.05$) psychrotrophic counts when compared to the negative and positive controls on all storage d. Psychrotrophic counts were similar ($P > 0.05$) for chicken breasts treated with 1 and 2% SMS on all storage d. This observation revealed the effectiveness of SMS against psychrotrophic counts, particularly when used at 2% concentration by weight of the meat. The data suggested that SMS could function in extending the shelf life of fresh poultry by retarding the growth of psychrotrophic microorganisms.

pH Analysis

Experiment 1. The pH values were similar ($P > 0.05$) for negative and positive controls from d 0 to d 7 (Table 4-6). Chicken breasts (skinless) treated with 1% SMS had higher ($P < 0.05$) pH values than the controls on d 0 and 3 only, whereas pH values for chicken breasts treated with 2% SMS were higher ($P < 0.05$) than the negative and positive controls on all storage d. It was observed that the pH of the 1 and 2% SMS treatments were insufficient to decrease *C. jejuni* and psychrotrophic counts in the chicken breasts.

Experiment 2. The pH values were similar ($P > 0.05$) for negative and positive controls from d 0 to d 7 (Table 4-7). All chicken breasts (skin-on) treated with 1 and 2%

SMS had higher ($P < 0.05$) pH values when compared to the negative and positive controls on all storage d. There was a difference of more than 3 pH units between the untreated and chicken breasts treated with 2% SMS (by weight of meat), which could be one possible explanation for higher ($P < 0.05$) reductions (more than 3 logs) in *C. jejuni* counts (Table 4-3) as compared to no effect on *C. jejuni* populations in skinless chicken breasts treated with SMS (by weight of marinade in Experiment 1) at the currently approved levels as previously discussed. Except for d 1, chicken breasts treated with 2% SMS had higher ($P < 0.05$) pH values when compared to the samples treated with 1% SMS.

In conclusion, SMS was effective in reducing *C. jejuni* populations and psychrotrophic organisms in skin-on chicken breasts but had no effect on the growth of *C. jejuni* and psychrotrophs when it was used as a marinade in skinless chicken breasts. The study also pointed out that the antimicrobial properties of SMS could be due to its high pH. The antimicrobial effectiveness of SMS increased as its concentration increased from 1 to 2 % (by weight of meat) with the most effective antimicrobial level being 2% as a carcass application, which resulted in more than 3 log reductions in *C. jejuni* counts in skin-on chicken breasts. These findings suggest the future application of SMS as a carcass treatment in addition to the currently approved application as a marinade. The data also revealed that SMS could function to safeguard the consumer's health by providing an additional intervention for poultry processing to enhance food safety. The SMS treatment was also effective in retarding the growth of psychrotrophs when used at 2% SMS by weight of the meat. The SMS treatment could

function in extending the shelf life of poultry by retarding the growth of psychrotrophs, which are the primary spoilage microflora of fresh poultry.

Table 4-1. Recovery of *Campylobacter jejuni* (log cfu/mL) after exposure to sodium metasilicate and sodium hydroxide solutions

Treatment	1 min	30 min
Positive control	7.24[a, x]	7.85[a, y]
0.5% SMS	2.81[b, x]	3.05[b, x]
1% SMS	2.75[b, x]	2.89[b, x]
2% SMS	2.53[b, x]	2.86[b, x]
0.1 N NaOH	2.76[b, x]	2.88[b, x]

[a-b] Means within a column lacking a common superscript differ ($P < 0.05$)
[x-y] Means within a row lacking a common superscript differ ($P < 0.05$)
n = 6 values per mean
SMS = Sodium metasilicate

Table 4-2. *Campylobacter jejuni* counts (log cfu/g) in uncooked skinless and boneless chicken breasts inoculated with *C. jejuni*, treated with sodium metasilicate (by weight of marinade) and stored at 4 ± 1°C for 7 d: Experiment 1

	Day of Sampling				
Treatment	0	1	3	5	7
Negative Control	ND	ND	ND	ND	ND
Positive control	4.83[a, w]	4.73 [a, w]	4.66[a, w]	4.42[a ,w]	4.39[a ,w]
1% SMS	4.79 [a, w]	4.54[a, w]	4.49 [a ,w]	4.25 [a ,w]	4.19 [a ,w]
2% SMS	4.49 [a, w]	4.45 [a, w]	4.24 [a ,w]	4.13 [a ,w]	4.01 [a ,w]

[a-b] Means within a column lacking a common superscript differ ($P < 0.05$)
[w-z] Means within a row lacking a common superscript differ ($P < 0.05$)
ND = Not detected
n = 6 values per mean
SMS = Sodium metasilicate

Table 4-3. *Campylobacter jejuni* counts (log cfu/g) in uncooked skin-on chicken breasts inoculated with *C. jejuni,* treated with sodium metasilicate and stored at 4 ± 1°C for 7 d: Experiment 2

	Day of Sampling				
Treatment	0	1	3	5	7
Negative control	ND	ND	ND	ND	ND
Positive control	4.71 [a, x]	4.66 [a, x]	4.57 [a, x]	4.47 [a, x]	4.42 [a, x]
1% SMS	3.59 [b, x]	3.49 [b, x]	3.4 [b, x]	3.21 [b, x]	3.24 [b, x]
2% SMS	1.44 [c, x]	1.32 [c, x]	1.21 [c, x]	0.68 [c, y]	0.68 [c, y]

[a-c] Means within a column lacking a common superscript differ ($P < 0.05$)
[x-y] Means within a row lacking a common superscript differ ($P < 0.05$)
ND =Not detected
n= 6 values per mean
SMS = Sodium metasilicate

Table 4-4. Psychrotrophic counts (log cfu/g) in uncooked skinless and boneless chicken breasts inoculated with *Campylobacter jejuni*, treated with sodium metasilicate and stored at 4 ± 1°C for 7 d: Experiment 1

	Day of Sampling				
Treatment	0	1	3	5	7
Negative control	4.53 [a, z]	5.49 [a, z]	7.11 [a, y]	8.39 [a, x]	9.40 [a, x]
Positive control	4.49 [a, z]	5.50 [a, z]	7.27 [a, y]	8.67 [a, x]	9.57 [a, x]
1% SMS	3.95 [a, z]	5.25 [a, y z]	6.72 [a, y]	8.61 [a, x]	9.15 [a, x]
2% SMS	4.31 [a, z]	5.44 [a, y z]	6.92 [a, y]	8.63 [a, x]	9.59 [a, x]

[a-b] Means within a column lacking a common superscript differ ($P < 0.05$)
[x-z] Means within a row lacking a common superscript differ ($P < 0.05$)
n= 6 values per mean
SMS = Sodium metasilicate

Table 4-5. Psychrotrophic counts (log cfu/g) in chicken breasts (skin-on) inoculated with *Campylobacter jejuni*, treated with sodium metasilicate and stored at 4 ± 1°C for 7 d: Experiment 2

	Day of Sampling				
Treatments	0	1	3	5	7
Negative control	4.51 [a, z]	5.33 [a, z]	7.13 [a, y]	8.68 [a, x]	9.13 [a, x]
Positive control	4.41 [a, y]	5.53 [a, y]	7.46 [a, x]	8.74 [a, x]	9.11 [a, x]
1% SMS	3.47 [a, y]	4.23 [a b, y]	5.38 [a b, y]	7.23 [a b, x]	8.22 [a b, x]
2% SMS	2.53 [a, z]	2.79 [b, z]	4.38 [b, y z]	5.74 [b, y x]	6.65 [b, x]

[a-b] Means within a column lacking a common superscript differ ($P < 0.05$)
[x-z] Means within a row lacking a common superscript differ ($P < 0.05$)
n= 6 values per mean
SMS = Sodium metasilicate

Table 4-6. pH measurements for uncooked skinless and boneless chicken breasts inoculated with *Campylobacter jejuni*, treated with sodium metasilicate and stored at 4 ± 1°C for 7 d: Experiment 1

	Day of Sampling				
Treatment	0	1	3	5	7
Negative control	6.34 [c, x]	6.39 [c, x]	6.28 [c, x]	6.32 [c, x]	6.33 [b, x]
Positive control	6.29 [c, x]	6.20 [b c, x]	6.23 [c, x]	6.37 [b c, x]	6.42 [b, x]
1% SMS	6.70 [b, x]	6.57 [b, x]	6.53 [b, x]	6.55 [b, x]	6.53 [b , x]
2% SMS	7.11 [a, x]	6.81 [a, y]	6.78 [a, y]	6.76 [a, y]	6.78 [a, y]

[a-c] Means within a column lacking a common superscript differ ($P < 0.05$)
[x-y] Means within a row lacking a common superscript differ ($P < 0.05$)
n= 6 values per mean
SMS = Sodium metasilicate

Table 4-7. pH measurements for chicken breasts (skin-on) inoculated with *Campylobacter jejuni*, treated with sodium metasilicate (by weight of meat) and stored at 4 ± 1°C for 7 d: Experiment 2

	Day of Sampling				
Treatment	0	1	3	5	7
Negative control	6.06 [c, x]	6.12 [b, x]	6.2 [c, x]	6.3 [c, x]	6.38 [c, x]
Positive control	6.00 [c, x]	6.13 [b, x]	6.12 [c, x]	6.24 [c, x]	6.31 [c, x]
1% SMS	9.82 [b, x]	9.52 [a, x y]	9.16 [b, x y]	8.79 [b, y]	8.74 [b, y]
2% SMS	10.79 [a, x]	10.09 [a, x]	10.07 [a, x]	9.95 [a, x]	10.00 [a, x]

[a-c] Means within a column lacking a common superscript differ ($P < 0.05$)
[x-y] Means within a row lacking a common superscript differ ($P < 0.05$)
n= 6 values per mean
SMS = Sodium metasilicate

CHAPTER 5
FATE OF *LISTERIA MONOCYTOGENES* INOCULATED ONTO TURKEY HAM AND TREATED WITH SODIUM METASILICATE AT FOUR DEGREES CELSIUS FOR TWENTY EIGHT DAYS

Listeria monocytogenes is a foodborne pathogen of high public health significance because of its high case fatality rate (CDC, 2010a). Outbreaks have been reported with consumption of ready-to-eat (RTE) poultry products in the United States (CDC, 2002), which highlights the public health importance of this pathogen in RTE poultry products. Due to its public health significance, USDA FSIS maintains a zero-tolerance policy for *L. monocytogenes* in RTE meat and poultry products. Pregnant women, newborns, and immunocompromised persons are at the highest risk for contacting listeriosis due to *L. monocytogenes.* It is estimated that every year approximately 2,500 cases of illness and 500 deaths occur due to foodborne listeriosis in the US (Mead et al., 1999).

Approximately 13 different serotypes of *L. monocytogenes* have been described, and serotypes 1/2a, 1/2b, and 4b are the most commonly encountered in illnesses caused by *L. monocytogenes* (Kathariou, 2002; Gorski et al., 2006). The presence of *L. monocytogenes* in RTE products usually involves post-processing contamination and is of high significance in terms of food safety. Lot of research has been conducted to control the *L. monocytogenes* contamination of food products. Various antimicrobial compounds have been tested for their efficacy to reduce or eliminate *L. monocytogenes* in RTE foods. Organic acids, bacteriocins, and natural antimicrobials such as nisin and rosemary have been tested in RTE meat and poultry products for their anti-*Listeria* properties and were reported to be effective in restricting growth of *L. monocytogenes* (Burt, 2004; Lungu and Johnson, 2005; Over et al., 2009; Ruiz et al., 2009).

Sodium metasilicate (SMS) is a white anhydrous fast dissolving, fine granular chemical with pH of 1% solution ranging from 12.5 to 13.0. Sodium metasilicate is USDA FSIS approved as a processing aid and can be used as an antimicrobial component of marinades for meat and poultry products up to 2% by weight of the marinades (USDA FSIS, 2011). It has also been approved for use in RTE meat and poultry products where it can be used up to a 6% solution applied on the surface of the product at a rate not exceeding 300 ppm of the finished product (USDA FSIS, 2011). Limited studies have been conducted to evaluate the antimicrobial activity of SMS in meat systems and *in vitro,* but not in poultry products (Weber et al., 2004; Carlson et al., 2008; Pohlman et al., 2009). The objectives of this study were to investigate the efficacy of SMS as anti-*Listeria* compound to reduce *L. monocytogenes* populations on turkey ham after 28 d of storage at 4°C and to ascertain the effects of various treatments on the pH.

Materials and Methods

This study was carried out to determine anti-*Listeria* properties of SMS (Avgard®XP, Danisco, USA Inc.) as a food antimicrobial when applied to treat turkey ham inoculated with *L. monocytogenes*. The study was carried out at the Meat Microbiology Laboratory, University of Florida, Gainesville, FL.

Bacterial Strain and Inoculum Preparation

Reference strains of *L. monocytogenes* ½ a, ½ b, 4b, Scott A and 19115 obtained from ABC Research Corporation in Gainesville, FL, were used as the inoculum in this study to evaluate the antimicrobial properties of SMS. Stock cultures were prepared by transferring each reference strain to test tubes containing 10 mL of TSB followed by incubation at 35°C for 24 h. After incubation, the cultures were centrifuged in sterile15

mL centrifuge tubes (05-539-5, Fisher Scientific, Pittsburgh, PA) at 5,000g for 10 min at 4°C (Sorvall RC-5, Dupont Instruments, Newton, CT). The supernatant was discarded and the pellets were resuspended in 10 mL of sterile 0.1% peptone water (CM009, Oxoid Ltd., Basingstoke, Hampshire, England), centrifuged again and pellets were suspended in 1 mL of TSB supplemented with 30% glycerol in a 2 mL cryogenic vial (430488, Corning Inc., Corning, NY), stored at –80°C, and used as the stock culture for the inoculation studies. The working cultures were prepared by thawing the deep-frozen culture at room temperature for 5 to 10 min, and a loopful was streaked onto TSA (MP Biomedicals, LLC, Solon, OH) followed by incubation at 35°C for 24 h. One colony was selected and transferred to 10 mL of TSB and incubated at 35°Cfor 20 h. Cells were pelleted by centrifugation at 5,000 g for 10 min at 4°C harvested, washed and re-suspended in sterile 0.1% peptone water. A cocktail of all these strains was prepared by mixing equal amount of inoculum for each strain and used as the final inoculum for the study. Preliminary experiments were carried out to determine the final concentration of inoculum between 5 to 6 log cfu/g in turkey ham samples.

Sample Preparation, Inoculation and Treatment

Ready-to-eat (RTE) turkey ham samples with expiration date of at least 2 months were purchased from a local supermarket, transported on ice and processed on the same day or kept at 4°C for use within 24 h. The turkey ham meat was cut into 25 g samples under aseptic conditions and inoculated with approximately 10^8 cfu/mL of *L. monocytogenes* cocktail inoculum to recover 5 to 6 log cfu/g. The samples were left to stand for 20 min at room temperature in order to attach bacteria to the meat surface. Sodium metasilicate solution 6% (pH 13.0 to 13.1) was prepared in sterile de-ionized water and used as treatments for this study. The turkey ham samples were treated with

either 0% SMS (only sterile de-ionized water) and no inoculum (negative control), 0% SMS (only sterile de-ionized water) and inoculum (positive control), and 6% SMS solution with final concentration of 300 ppm and 600 ppm of SMS in turkey ham samples plus inoculum. In each treatment, the turkey ham samples were vacuum packaged (one sample per bag) and stored at 4°C for the rest of the study.

Microbiological and pH Analyses

Samples were analyzed on d 0, 7, 14, 21 and 28 of storage for presence of *L. monocytogenes* and pH. Turkey ham samples (25 g each) were transferred aseptically from storage bags to 225 mL of sterile 0.1% peptone water in a sterile stomacher bag (01-002-56, Fisher Scientific, Pittsburgh, PA) and manually agitated for approximately1 min to loosen and suspend bacteria in solution. Serial dilutions were prepared by transferring1.0 mL of the sample homogenate to 9 mL of 0.1% sterile peptone water. A volume of 0.1 mL from each dilution was pipette onto duplicate pre-poured modified oxford medium agar plates prepared with MOX supplement for *L. monocytogenes* recovery. The plates were incubated aerobically for 48 h at 35°C. After incubation, cfu from each plate were counted, averaged and reported as log cfu/g of the sample. The pH for each sample homogenate was measured by placing the pH probe into the sample homogenate immediately after the microbiological analyses were completed. All pH measurements were recorded in duplicate using an Accumet pH meter (AB15 Accumet Basic, Fisher Scientific, Pittsburgh, PA).

Data Analyses

A total of 120 samples were analyzed and a complete randomized block design was used. Samples consisting of 4 treatments, 5 storage d, duplicate samples and 3 trials were analyzed by GLIMMIX procedures of SAS (SAS Institute, 2009) to analyze

differences between trials, among treatments and storage d, and treatment × day interaction. The mean separation was performed using Tukey-Kramer and a level of significance of α = 0.05 was used to determine any significant differences among means.

Results and Discussion

L. monocytogenes Recovery and Analysis

No *L. monocytogenes* were isolated in the negative control. In positive control the counts of *L. monocytogenes* ranged from 6.34 to 7.29 log cfu/g d 0 through 7 d of storage (Table 5-1). No antimicrobial effects of SMS were observed for 300 and 600 ppm concentrations of 6% SMS treatments, as evidenced by similar ($P > 0.05$) *L. monocytogenes* counts among the positive control and SMS treatments on all sampling d. The SMS treatments were similar ($P > 0.05$) on all sampling d. However, *L. monocytogenes* was able to significantly grow and populate even with SMS treatments as evidenced from almost 1 log unit increase in *L. monocytogenes* population on d 28 as compared to initial inoculum. This increase in growth of *L. monocytogenes* was correspondent to significant decrease in pH during that period (Table 5-2). The findings from this study suggested that SMS is ineffective in reducing *L. monocytogenes* in turkey ham, at the currently approved levels.

pH Analysis

The pH values were similar ($P > 0.05$) for all treatments from d 0 to d 28 in this study (Table 5-2). The reduction in *L. monocytogenes* populations reported in pure culture experiment (data not shown) was as a result of high pH generated by SMS solutions, which was not observed in this study. It can be assumed that the pH of the 6% SMS treatment with final levels of 300 and 600 ppm were insufficient to cause

enough elevation in pH so as to have any effect on growth of *L. monocytogenes* in turkey ham.

In summary, SMS was ineffective in reducing *L. monocytogenes* populations in turkey ham. The antimicrobial efficacy of SMS against *L. monocytogenes* had no effect with an increase in concentration from 300 to 600 ppm of 6% SMS in turkey ham, which suggests that higher concentrations of SMS may be needed to observe some anti-*Listeria* effect of SMS in RTE poultry products.

Table 5-1. *Listeria monocytogenes* counts (log cfu/g) in turkey ham inoculated with *L. monocytogenes*, treated with sodium metasilicate and stored at 4 ± 1°C for 28 d

	Day of Sampling				
Treatment	0	7	14	21	28
Negative control	ND	ND	ND	ND	ND
Positive control	6.47 [a, x]	6.34 [a, x]	6.40 [a, x]	6.64 [a, x]	7.29 [a, x]
6% SMS 300 ppm	6.22 [a, y]	6.12 [a, y]	6.40 [a, y, x]	6.76 [a, y, x]	7.27 [x]
6% SMS 600 ppm	6.11 [a, y]	6.01 [a, y]	6.51 [a, y, x]	6.89 [a, y, x]	7.29 [x]

[a-b] Means within a column lacking a common superscript differ ($P < 0.05$)
[x-z] Means within a row lacking a common superscript differ ($P < 0.05$)
ND =Not detected
N = 6 values per mean
SMS = Sodium metasilicate

Table 5-2. pH measurements for turkey ham inoculated with *Listeria monocytogenes*, treated with sodium metasilicate and stored at 4 ± 1°C for 28 d

	Day of Sampling				
Treatment	0	7	14	21	28
Negative control	6.86 [a, x]	6.88 [a, x]	6.65 [a, x]	6.59 [a, x]	6.59 [a, x]
Positive control	6.80 [a, x]	6.87 [a, x]	6.62 [a, x, y]	6.59 [a, x, y]	6.45 [a, y]
6% SMS 300 ppm	6.92 [a, x]	6.94 [a, x]	6.72 [a, x, y]	6.71 [a, x, y]	6.54 [a, y]
6% SMS 600 ppm	6.99 [a, x]	7.01 [a, x]	6.79 [a, x, y]	6.77 [a, x, y]	6.62 [a, y]

[a-b] Means within a column lacking a common superscript differ ($P < 0.05$)
[x-z] Means within a row lacking a common superscript differ ($P < 0.05$)
ND =Not detected
N = 6 values per mean
SMS = Sodium metasilicate

CHAPTER 6
RECOVERY OF *SALMONELLA* TYPHIMURIUM FROM CHICKEN BREASTS TREATED WITH SODIUM METASILICATE AFTER PH NEUTRALIZATION

Nontyphoidal *Salmonella* is the leading bacterial cause responsible for majority of foodborne infections in the US (Scallan et al., 2011). Poultry has been commonly linked as the vehicle for illnesses attributed to foodborne salmonellosis (CDC, 2010a). Therefore it is important to control the contamination of poultry products with *Salmonella*. Sodium metasilicate (SMS) has been approved by USDA FSIS as a processing aid in poultry processing, which can be used as an antimicrobial component of marinades for poultry products up to 2% by weight of the marinades (USDA FSIS, 2011). The use of SMS as an antimicrobial has shown reduction of *Salmonella* in chicken breasts and beef trimmings (Weber et al., 2004; Carlson et al., 2008; Pohlman et al., 2009). However, the mechanism of antimicrobial action of sodium metasilicate is not fully understood. Sodium metasilicate is a highly alkaline compound and pH of 1% solution is around 12.5. It is possible that such high pH of SMS is responsible for its antimicrobial properties. The objectives of this study were to compare the antimicrobial properties of pH neutralized SMS treatment solutions to sodium hydroxide solutions against *S.* Typhimurium inoculated on fresh chicken breasts, and to ascertain the effects of the treatments on pH.

Materials and Methods

This study was designed and carried out to determine efficacy of SMS (Avgard®XP, Danisco, USA Inc.) as a food antimicrobial after neutralizing its pH and compare the antimicrobial efficacy of sodium hydroxide at pH corresponding to 1 and 2% SMS solutions. The study was carried out at the Meat Microbiology Laboratory, University of Florida, Gainesville, FL.

Bacterial Strain and Inoculum Preparation

The selection of bacterial strain and inoculum preparation were carried out as described earlier. Preliminary recovery studies were carried out with different volumes of inoculum used to determine the final concentration of inoculum between 5 to 6 log cfu/g in chicken beast samples. It was found that using 100 µL of 10^8 cfu/mL of inoculum onto 25 g of chicken breast meat resulted in desired final concentration of inoculum.

Sample Preparation, Inoculation and Treatment

Uncooked skinless and boneless chicken breast meat samples with expiration date of at least one week were purchased from a local supermarket, transported on ice and processed on the same day or kept at 4°C for use within 24 h. The chicken breast meat was cut into 25 g samples under aseptic conditions and inoculated with approximately 10^8 cfu/mL of *S.* Typhimurium inoculum. The samples were left to stand for 20 min at room temperature in order to attach bacteria to the meat surface. Sodium metasilicate 1% (pH 12.4 to 12.6) and 2% (12.5-12.7) solutions were prepared in sterile de-ionized water and their pH was adjusted to 7.0 ± 0.2 with 6N HCl. Solutions of 0.01 N (pH 12.0) and 0.1 N (pH 12.5) NaOH were prepared in sterile de-ionized water and also included as treatments. A pH 7.0 buffer was prepared by add 29.1 mL of 0.1 molar NaOH to 50 mL 0.1 molar potassium dihydrogen phosphate (KH_2PO_4) and included as treatment in the study. The chicken breasts were marinated in solutions containing either 0% SMS (only sterile de-ionized water) and no inoculum (negative control), 0% SMS (only sterile de-ionized water) and inoculum (positive control), 1 and 2% SMS solutions (with pH adjusted to 7.0 ± 0.2) plus inoculum, 0.01 N and 0.1 N NaOH solutions (pH 12 and 12.5, respectively) plus inoculum and NaOH - potassium dihydrogen phosphate buffer (pH 7.0) plus inoculum. For each treatment, the chicken

breast samples were aseptically packaged in sterile Fisher bags (one sample per bag) in the treatment solutions for the duration of storage period to ensure direct contact of the marinade with the meat and stored at 4 ± 1°C for 7 d. Duplicate samples for each treatment were analyzed on 0, 1, 3, 5 and 7 d of storage for *Salmonella* recovery, and pH measurements. Three separate trials were conducted for this study.

Microbiological and pH Analyses

Chicken breast samples (25 g) were transferred aseptically from storage bags to 225 mL of sterile 0.1% peptone water in a sterile stomacher bag (01-002-56, Fisher Scientific, Pittsburgh, PA) and manually agitated for approximately 1 min to loosen and suspend bacteria in solution. Serial dilutions were prepared by transferring 1.0 mL of the sample homogenate to 9.0 mL of 0.1% sterile peptone water. A volume of 0.1 mL from each dilution was pipette onto duplicate prepoured XLT-4 agar plates (223420, Becton, Dickinson and Company, Sparks, MD) prepared with XLT-4 agar supplement (235310, Becton, Dickinson and Company, Sparks, MD) for *Salmonella* recovery. The XLT-4 agar plates were incubated aerobically for 48 h at 35°C and black colonies or colonies that were black-centered with a yellow periphery on XLT-4 agar were considered presumptively *S.* Typhimurium. After incubation, cfu from each plate were counted, averaged and reported as log cfu/g of the sample. The pH for each sample homogenate was measured by placing the pH probe into the sample homogenate immediately after the microbiological analyses were completed. All pH measurements were recorded in duplicate using an Accumet pH meter (AB15 Accumet Basic, Fisher Scientific).

Data Analysis

A total of 210 samples were analyzed and a complete randomized block design was used. Samples consisting of 7 treatments, 5 storage d, duplicate samples and 3

trials were analyzed by GLIMMIX procedures of SAS (SAS Institute, 2009) to analyze differences between trials, among treatments and storage d, and treatment × day interaction. The mean separation was performed using Tukey-Kramer and a level of significance of α = 0.05 was used to determine any significant differences among means.

Results and Discussion

S. Typhimurium Recovery and Analysis

No *Salmonella* was isolated in the negative control. In positive control, the counts of *S.* Typhimurium ranged from 4.43 to 5.71 log cfu/g d 0 through 7 d of storage (Table 6-1). No immediate antimicrobial effects were observed for any treatments, as evidenced by similar ($P > 0.05$) *Salmonella* counts among the positive control and various treatments on d 0 through d 3. Skinless and boneless chicken breasts marinated with 0.01 N and 0.1 N NaOH solutions had lower ($P < 0.05$) *Salmonella* counts when compared to the positive control on d 5 of storage. However on d 7 only chicken breasts treated with 0.1 N NaOH has significant reduction ($P < 0.05$) in *Salmonella* population as compared to positive control. Reduction in *Salmonella* counts for the 0.1 N NaOH treatment included 0.82 and 0.80 log cfu/g on d 5 and 7, respectively whereas chicken breast treated with 0.01 N NaOH had reduction in *Salmonella* counts only on d 5 (0.79 log). The SMS and buffer treatments had similar ($P > 0.05$) *Salmonella* counts on all sampling d.

The findings from both experiments suggested that SMS is ineffective in reducing *Salmonella* in poultry meat when its pH is adjusted to neutral, which was confirmed by using a pH 7.0 buffer that also gave similar results as with 1 and 2% SMS solutions (with their pH adjusted to 7.0 ± 0.2). Also we treated the *Salmonella* inoculated chicken

breasts with 0.01 N and 0.1 N NaOH solutions (having pH strengths comparable to 1 and 2% SMS solutions) to compare the effect of high pH on reduction in *Salmonella* in chicken meat. The results obtained after NaOH treatments suggested that the high pH generated by SMS was the major contributing factor in the antimicrobial effects against *Salmonella* in chicken breasts as reported earlier.

pH Analysis

Except for chicken breasts (skinless) treated with 0.1 N NaOH, pH values were similar ($P > 0.05$) for all treatments from d 0 to d 7. Chicken breasts (skinless) treated with 0.1 N NaOH had higher pH ($P < 0.05$) when compared to the positive control on d 1 and d 7 (Table 6-2). The reduction in *Salmonella* counts reported for chicken breast meat treated with 0.1 N NaOH may be largely attributed to the higher pH generated by 0.1 N NaOH solutions.

In summary, SMS was ineffective in reducing *Salmonella* populations in skinless chicken breasts when the pH of the 1 and 2% SMS marinade solutions was adjusted to near neutral. However significant reduction in *Salmonella* populations in skinless chicken breasts was observed with 0.01 N and 0.1 N NaOH solutions when applied as marinade, which suggests that high pH is effective in restricting the *Salmonella* growth in chicken breasts.

Table 6-1. *Salmonella* counts (log cfu/g) in uncooked skinless and boneless chicken breasts inoculated with *S.* Typhimurium, treated with sodium metasilicate (pH neutralized), pH 7.0 buffer, sodium hydroxide and stored at 4 ± 1°C for 7 d

	Day of Sampling				
Treatment	0	1	3	5	7
Negative control	ND	ND	ND	ND	ND
Positive control	5.71 ^{a,x}	5.62 ^{a,x}	5.06 ^{a,x,y}	4.95 ^{a,y}	4.43 ^{a,y}
1% SMS (pH 7.0 ± 0.2)	5.67 ^{a,x}	5.62 ^{a,x}	5.29 ^{a,x}	4.98 ^{a,x,y}	4.50 ^{a,y}
2% SMS (pH 7.0 ± 0.2)	5.77 ^{a,x}	5.66 ^{a,x}	4.98 ^{a,y}	4.89 ^{a,y}	4.64 ^{a,y}
NaOH - KH_2PO_4 Buffer (pH 7.0)	5.61 ^{a,x}	5.32 ^{a,x,y}	4.98 ^{a,x,y}	4.89 ^{a,y}	4.51 ^{a,y}
0.01 N NaOH (pH 12.0)	5.54 ^{a,x}	5.51 ^{a,x}	4.85 ^{a,y}	4.16 ^{b,z}	3.84 ^{a,b,z}
0.1 N NaOH (pH 12.5)	5.40 ^{a,x}	5.10 ^{a,x}	4.74 ^{a,x,y}	4.13 ^{b,y,z}	3.63 ^{b,z}

$^{a-b}$ Means within a column lacking a common superscript differ ($P < 0.05$)
$^{x-z}$ Means within a row lacking a common superscript differ ($P < 0.05$)
ND =Not detected
n = 6 values per mean
SMS = Sodium metasilicate

Table 6-2. pH measurements for uncooked skinless and boneless chicken breasts inoculated with *S.* Typhimurium, treated with sodium metasilicate (pH neutralized), pH 7.0 buffer, sodium hydroxide and stored at 4 ± 1°C for 7 d

	Day of Sampling				
Treatment	0	1	3	5	7
Negative control	6.18 ^{a,x}	6.13 ^{a,b,x}	6.14 ^{a,x}	6.17 ^{a,x}	6.29 ^{a,b,x}
Positive control	6.03 ^{a,x}	5.95 ^{a,x}	6.04 ^{a,x}	6.07 ^{a,x}	6.07 ^{a,x}
1% SMS (pH 7.0 ± 0.2)	6.22 ^{a,x}	6.05 ^{a,b,x}	6.07 ^{a,x}	6.25 ^{a,x}	6.19 ^{a,b,x}
2% SMS (pH 7.0 ± 0.2)	6.24 ^{a,x}	6.11 ^{a,b,x}	6.09 ^{a,x}	6.17 ^{a,x}	6.34 ^{a,b,x}
NaOH - KH_2PO_4 Buffer (pH 7.0)	6.25 ^{a,x}	6.20 ^{a,b,x}	6.15 ^{a,x}	6.18 ^{a,x}	6.28 ^{a,b,x}
0.01 N NaOH (pH 12.0)	6.28 ^{a,x}	6.23 ^{a,b,x}	6.35 ^{a,x}	6.39 ^{a,x}	6.26 ^{a,b,x}
0.1 N NaOH (pH 12.5)	6.33 ^{a,x}	6.31 ^{b,x}	6.31 ^{a,x}	6.35 ^{a,x}	6.44 ^{b,x}

$^{a-b}$ Means within a column lacking a common superscript differ ($P < 0.05$)
$^{x-z}$ Means within a row lacking a common superscript differ ($P < 0.05$)
n = 6 values per mean
SMS = Sodium metasilicate

CHAPTER 7
INACTIVATION AND KILLING OF *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM BY SODIUM METASILICATE INVOLVES CYTOPLASMIC MEMBRANE DISRUPTION

The use of SMS as an antimicrobial has shown efficacy against Gram-negative foodborne pathogens such as *Salmonella* and *E. coli* O157:H7 *in vitro* and meat systems (Weber et al., 2004; Carlson et al., 2008; Pohlman et al., 2009). In a previous study conducted on chicken breasts, it was noticed that SMS has little or no action against *S.* Typhimurium when high pH of SMS is neutralized which indicate that it could be the high alkaline conditions generated by SMS which are responsible for its antimicrobial activity. *Salmonella* is susceptible to high pH and increasing the pH and exposure time to alkaline conditions result in great reduction in viability of *Salmonella* (Mendonca et al., 1994; Sampathkumar et al., 2003). However, the exact mechanism by which sodium metasilicate exerts its antimicrobial action is not fully understood and very limited scientific literature has been documented on its bactericidal or bacteriostatic mechanism of action. The objectives of the present study were to determine the antimicrobial efficacy of SMS against *S.* Typhimurium in suspension and elucidate the mechanism of antimicrobial action of SMS. Sodium metasilicate is a highly alkaline compound and pH of 1% solution is around 12.5. It is possible that such high pH of SMS is responsible for its antimicrobial properties. Previous studies have explained the effect of high pH effect on gram negative foodborne pathogens such as *Salmonella* by postulating that the action of alkaline conditions result in loss of membrane integrity as a result of disruption of cytoplasmic membrane, changes in cell morphology and leakage of cytoplasmic contents leading to cell death (Mendonca et al., 1994; Sampathkumar et al., 2003). The combination of flow cytometry with fluorescent stains has also been used

to assess variety of cell functions such as cell viability based on membrane integrity and other physiological functions (Davey and Kell, 1996; Gruden et al., 2004; Paparella et al., 2008; Kim et al., 2009). In the present study a combination of propidium iodide and SYTO9 dyes was used with flow cytometry and transmission electron microscopy to assess the changes in membrane integrity and internal cellular structures of *S.* Typhimurium following SMS treatment.

Material and Methods

Bacterial Strain and Exposure of Cells to SMS and High pH

Reference strain of *S.* Typhimurium (ATCC 14028) obtained from ABC Research Corporation in Gainesville, FL, was used in this study to elucidate the mechanism of antimicrobial action of SMS. Stock and working cultures were prepared as described earlier. One colony was selected from the working culture and transferred to 10 mL of TSB and incubated at 35°Cfor 20 h. Cells were pelleted by centrifugation at 5,000 g for 10 min at 4°C harvested, washed and re-suspended in 10 mL of treatment solution at room temperature containing either (i) 0.1% peptone water (control); (ii) 0.5% (pH 12.1), 1% (pH 12.4) and 2% (pH 12.5) SMS solutions, and (iii) 0.1 N NaOH (pH 12.5) solution for 1, 10 and 30 min.

Cell Viability

Treated cells were tested for their viability by transferring 1 mL aliquots from each treated cell suspension and serially diluting in 9 mL of sterile 0.1% peptone water. A volume of 0.1 mL from each dilution was pipette onto duplicate prepoured XLT-4 agar plates (223420, Becton, Dickinson and Company, Sparks, MD) prepared with XLT-4 agar supplement (235310, Becton, Dickinson and Company, Sparks, MD) for *Salmonella* recovery. The XLT-4 agar plates were incubated aerobically for 48 h at 35°C

and black colonies or colonies that were black-centered with a yellow periphery on XLT-4 agar were considered presumptively *S.* Typhimurium. After incubation, colony forming units (cfu) from each plate were counted, averaged and reported as log cfu/mL of the sample. Resuscitation experiment was done to isolate injured or sublethally treated cells by transferring 1 mL of the treated suspension into 10 mL of universal pre-enrichment broth (Becton, Dickinson and Company, Sparks, MD) and incubating at 35°C for 24 h. After incubation 1.0 mL was transferred to 10 mL of tetrathionate broth (Becton, Dickinson and Company, Sparks, MD) to isolate injured or sublethally treated cells. Duplicate samples for each treatment were analyzed and the experiment was repeated three times.

Flow Cytometry Analysis of the Membrane Integrity

Fluorescent dyes: Propidium Iodide (PI) (Invitrogen, Eugene, OR) and SYTO9 (Invitrogen) were used in this study to differentiate live and dead bacterial cells based on the membrane integrity. Both of these dyes bind and stain the nucleic acids, PI is a red fluorescent stain that can only enter and stain the cells presumed to be dead with damaged or permeabilized cytoplasmic membranes and is normally excluded by live cells while SYTO 9, which emits green fluorescence is able to stain both live and dead cells (Kim et al., 2009). After the treatment of cells to 0.5, 1, 2% SMS and 0.1 N NaOH for 1, 10 and 30 min, each sample (10^8cfu/mL) was serially diluted to 10^6cfu/mL in phosphate buffered saline (PBS; NaCl 8 g/l, KCl 0.2g/l, Na_2HPO_4 1.44 g/l, KH_2PO_4 0.24 g/l; pH 7.4) and 1 mL of the diluted sample was then stained with 2.5 µl of PI (1.5 mM) and SYTO9 (0.5 mM) either single or in combination and incubated at room temperature for 30 min. Samples were run in FACSort machine (BD Biosciences) with emission wavelengths for SYTO9 and PI at 530 +/-15 nm and 585 +/- 21 nm, respectively. Data

were analyzed by Cell Quest Software, Version 3.3 (BD Biosciences). The flow cytometry analysis was repeated twice for each treatment and the results were found to be reproducible. Samples were run and data were analyzed in the flow cytometry section of Interdisciplinary Center for Biotechnology Research, University of Florida, Gainesville, FL.

Transmission Electron Microscopy

Transmission electron microscopy (TEM) was performed to observe the changes at cellular level following exposure of *S.* Typhimurium cell suspensions to SMS and high pH treatment. Treated cells were centrifuged to pellet by centrifugation at 12000g for 10 min and fixed in 4% paraformaldehyde + 2% glutaraldehyde in 0.1M sodium cacodylate, pH 7.24. Fixed cells were processed with the aid of a Pelco BioWave laboratory microwave. The samples were washed in 0.1M sodium cacodylate buffer, and encapsulated in molten 3% low-temperature gelling agarose Type IV (A3643 Sigma-Aldrich, Saint-Louis, MO) post fixed with 1% osmium tetroxide (OsO_4), water washed and dehydrated in a graded ethanol series followed by 100% acetone. Dehydrated samples were infiltrated in graded acetone/Spurrs epoxy resin and cured at 60°C. Cured resin blocks were trimmed, thin sectioned and collected on formvar copper 200 mesh grids, post-stained with 2% aq. Uranyl acetate and Reynold's lead citrate. Sections were examined with a Hitachi H-7000 TEM (Hitachi High Technologies America, Inc. Schaumburg, IL) and digital images acquired with Veleta camera and iTEM software (Veleta- Olympus Soft-Imaging Solutions Corp, Lakewood, CO). All the samples were analyzed in the Electron Microscopy and Bioimaging section of Interdisciplinary Center for Biotechnology Research, University of Florida, Gainesville, FL.

Results and Discussion

Effect of SMS and High pH on Cell Viability

This study revealed that SMS resulted in more than 8 log reduction and immediate inactivation of *S.* Typhimurium with concentration of SMS as low as 0.5% with no detectable surviving *S.* Typhimurium (Table 7-1), which was confirmed by resuscitation studies. Treating *S.* Typhimurium with 1 and 2% SMS and 0.1N NaOH for 1, 10 and 30 min resulted in similar reduction and rapid killing of *S.* Typhimurium. In a similar study, exposure of *E. coli* O157:H7 to 0.6% SMS (5 to 10 s) pH 12.1, resulted in 100% inhibition with no recoverable E. coli O157:H7 (Weber et al., 2004). High pH has been reported to cause rapid death of Gram-negative pathogens such as *Salmonella* (Mendonca et al., 1994; Sampathkumar et al., 2003). In an earlier study it was proven that SMS was unable to exert any antimicrobial effects against *Salmonella* following pH neutralization (with 6.0 N HCl) in chicken breasts inoculated with *S.* Typhimurium, which indicates that it's the high pH of SMS that is responsible for bactericidal effects against *S.* Typhimurium.

Flow Cytometry Analysis of SMS and High pH Treated Cells

The effect of SMS (different concentrations and time of exposure) on the membrane integrity of *S.* Typhimurium was determined by use of PI and Syto9 with subsequent flow cytometry. The fluorescent signals obtained from the PI and SYTO9 after different treatments are presented in two-dimensional dot plots (Figure 7-1, 7-2 and 7-3). The cell population on the dot plot was clustered and identified in three different zones viz. PI positive, SYTO9 positive and unstained. The cell populations present in PI positive zone were stained with PI and their cytoplasmic membrane integrity was compromised and were presumed to be dead. It should be noted that as

the concentration of SMS increased from 0.5% to 2% and exposure time of *S.* Typhimurium to different concentrations of SMS increased from 1 to 30 min, there was a decrease in PI positive cell population for every treatment (Fig 7-4). This could possibly be due to the harsh conditions generated by high alkaline (pH 12.5) environment for longer exposure of cells resulting in complete loss of membrane integrity and severe cell lysis causing leakage of cytoplasmic contents including nucleic acids thereby low intensity signal from red fluorescent PI dye. Also PI binds with double stranded nucleic acids, and binds with a stoichiometry of one dye molecule per 4-5 base pairs of DNA, so extensive lysis and destruction could have destroyed the DNA to such an extent that PI couldn't stain enough DNA following SMS and sodium hydroxide treatments.

It was also observed that the intensity of fluorescent signals of PI positive cells increased when PI and SYTO9 were added together as a mixture as compared to PI added alone for every treatment. The most possible explanation could be the quenching effect or fluorescence energy transfer when SYTO9 and PI dyes were added together This phenomenon has been reported in literature whereby the emission properties of the stain mixture (such as PI and SYTO9 in this case) changes due to displacement of SYTO9 by PI and quenching of SYTO9 emissions by fluorescence resonance energy transfer resulting in different fluorescent intensities as compared to the effects seen when added alone (Stocks, 2004; Berney et al., 2007). SYTO9, when added alone, did not provide any significant information on cells with compromised membranes but it was able to stain more cells with increase in time of exposure and concentration of SMS.

The results from flow cytometry did not compare with those of the plate count experiment. There were no surviving populations of *S.* Typhimurium on plate counts

with any of the SMS and sodium hydroxide treatments that suggest that percentage of membrane permeabilized cells as PI positive should be 100%, which was never the case in any of the treatment in flow cytometry experiment. The results from this study indicate that flow cytometry didn't provide reliable information on differentiation of live and dead *S.* Typhimurium cells by use PI/SYTO9 particularly following high alkaline treatments with chemicals like SMS and sodium hydroxide. Combination of SYTO9 and PI does not always provide distinct differentiation of live and dead bacterial cells as some intermediate states are also observed (Berney et al., 2007; Kim et al., 2009)

The permeability of the cytoplasmic membrane of *S.* Enteritidis as a result of alkaline environment (Sampathkumar et al., 2003), was also confirmed by the uptake of PI dye. Kim et al. (2009) also used combination of PI/SYTO9 and subsequent flow cytometry to determine the effect of supercritical carbon dioxide on the membrane integrity and live and dead population of *S.* Typhimurium. However, this study represents first work on the combination of PI/SYTO9 and flow cytometry to determine the efficacy of SMS and high pH on membrane integrity and differentiation of live and dead *S.* Typhimurium populations.

Transmission Electron Microscopy

S. Typhimurium (ATCC 14028) exposed to SMS and sodium hydroxide (pH 12.5) appeared wrinkled, vacuolated, and lysed as compared to the untreated cells (Figure 7-5). The cytoplasmic membranes of *S.* Typhimurium treated with 1 and 2% SMS were ruptured, and the cells appeared to be lysed with cytoplasm leaking into extracellular matrix (Figure 7-5 D). After the treatment, intracellular material became more electron dense and there was a clear gap between electron dense cytoplasm and cytoplasmic membrane. In contrast the cytoplasm of untreated cells was homogenous with intact cell

membrane and intracellular material was observed (Figure 7-5 A). Cells exposed to high pH (12.5) corresponding to pH of 1 and 2% SMS solutions showed some dissimilarities as compared to cells treated with SMS. Cells had more electron dense cytoplasm with somewhat less rupturing and lysis. However, wrinkled, vacuolated cells with breaks between cell membranes and leakage of intracellular material was also seen with following treatment with 0.1 N NaOH. Electron dense material observed on electron micrographs could be the result of alkaline pH. It has been reported that high pH treatment causes denaturation and precipitation of cytoplasmic proteins and other cytoplasmic constituents (Mendonca et al., 1994; Sampathkumar et al., 2003). However, the phase separation phenomena of dark and light electron dense zones reported in these studies due to effect of high pH were not observed extensively in this study especially with SMS treatments.

Previous studies have attempted to elucidate the high pH effect on foodborne pathogens by postulating that the action of alkaline conditions result in loss of membrane integrity as a result of disruption of cytoplasmic membrane, changes in cell morphology and leakage of cytoplasmic contents leading to cell death (Mendonca et al., 1994; Sampathkumar et al., 2003).

Mendonca et al. (1994) studied the mechanism of action of high pH against common foodborne pathogens. They exposed *S.* Enteritidis, *E. coli* O157:H7 and *L. monocytogenes* to high pH conditions generated with sodium hydroxide and sodium bicarbonate buffers for different time periods. They reported the reductions in *Salmonella* and *E. coli* as a result of cell death due to rupturing of cell membrane. Transmission electron microscopy of cells treated with high pH (12.0) revealed the

separation of outer membranes of *S.* Enteritidis from the cell wall at various points. In a similar study, Sampathkumar et al. (2003) determined that treating *S.* Enteritidis with TSP (pH 11.0) resulted in rapid cell death with no detectable survivors after 1 h. Electron microscopy revealed that there was disruption of cytoplasmic and outer membranes of *S.* Enteritidis as a result of the alkaline pH which led to permeabilization of cell membrane and leakage of intracellular contents and eventual cell death.

The results from this study indicate that SMS acts on the cytoplasmic membrane and causes lysis of the cells and leakage of intracellular contents. The breakage in membrane integrity was also seen by uptake of PI by cells treated with SMS with subsequent flow cytometry. The extensive lysis of *S.* Typhimurium cells observed with 2% SMS might also corroborate the findings from the flow cytometry experiment where less number of cells were stained with PI as compared to the 0.5 and 1% SMS treatments. This observation indicated that with increase in SMS concentration more DNA might have been destroyed leaving few nucleotides for PI dye molecules to attach. In addition, the different electron micrographic findings between SMS and sodium hydroxide treated cells suggest that these two highly alkaline chemicals might function differently in killing *S.* Typhimurium. Finally, a more in depth study is needed to elucidate the differences between the mode of action of SMS and sodium hydroxide treatments, if any.

Table 7-1. Recovery of *Salmonella* (log cfu/mL) after exposure to sodium metasilicate and sodium hydroxide solutions for one, ten and thirty minutes

	Time of Exposure		
Treatment	1 min	10 min	30 min
Positive control	8.78	8.75	8.76
0.5% SMS	ND	ND	ND
1% SMS	ND	ND	ND
2% SMS	ND	ND	ND
0.1 N NaOH	ND	ND	ND

n = 6 values per mean
SMS = Sodium metasilicate
ND = Not detected

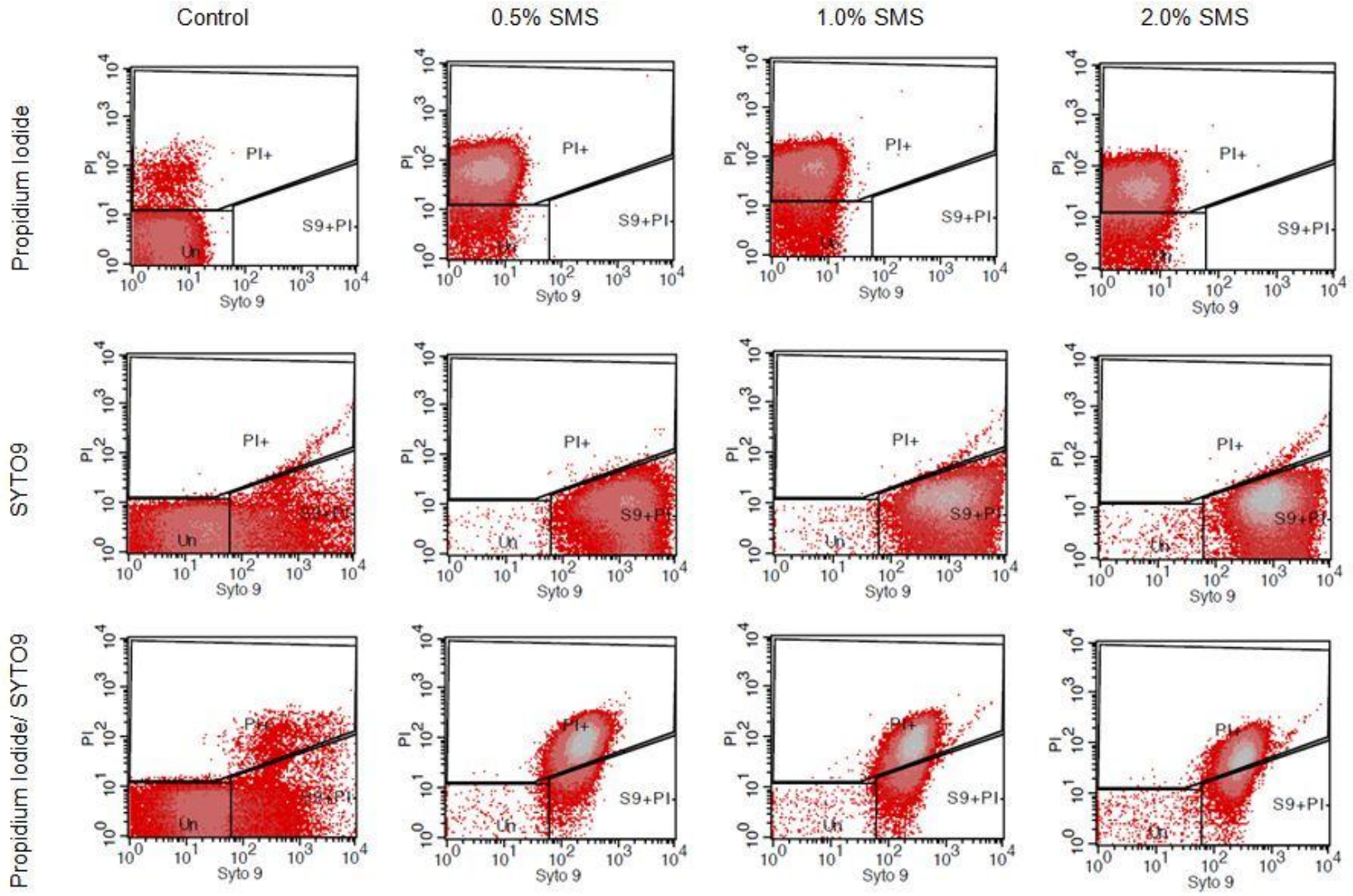


Figure 7-1. Flow cytometric analysis of *S.* Typhimurium (ATCC 14028) treated with SMS and stained with PI, SYTO9 and PI/SYTO9. The bacterial cells were untreated (control) or treated with SMS at the indicated concentrations and a time exposure for 1 min. The blocked regions PI+, S9+PI and Un represents the PI-positive cells (loss of membrane integrity), SYTO9-positive cells and unstained cells, respectively.

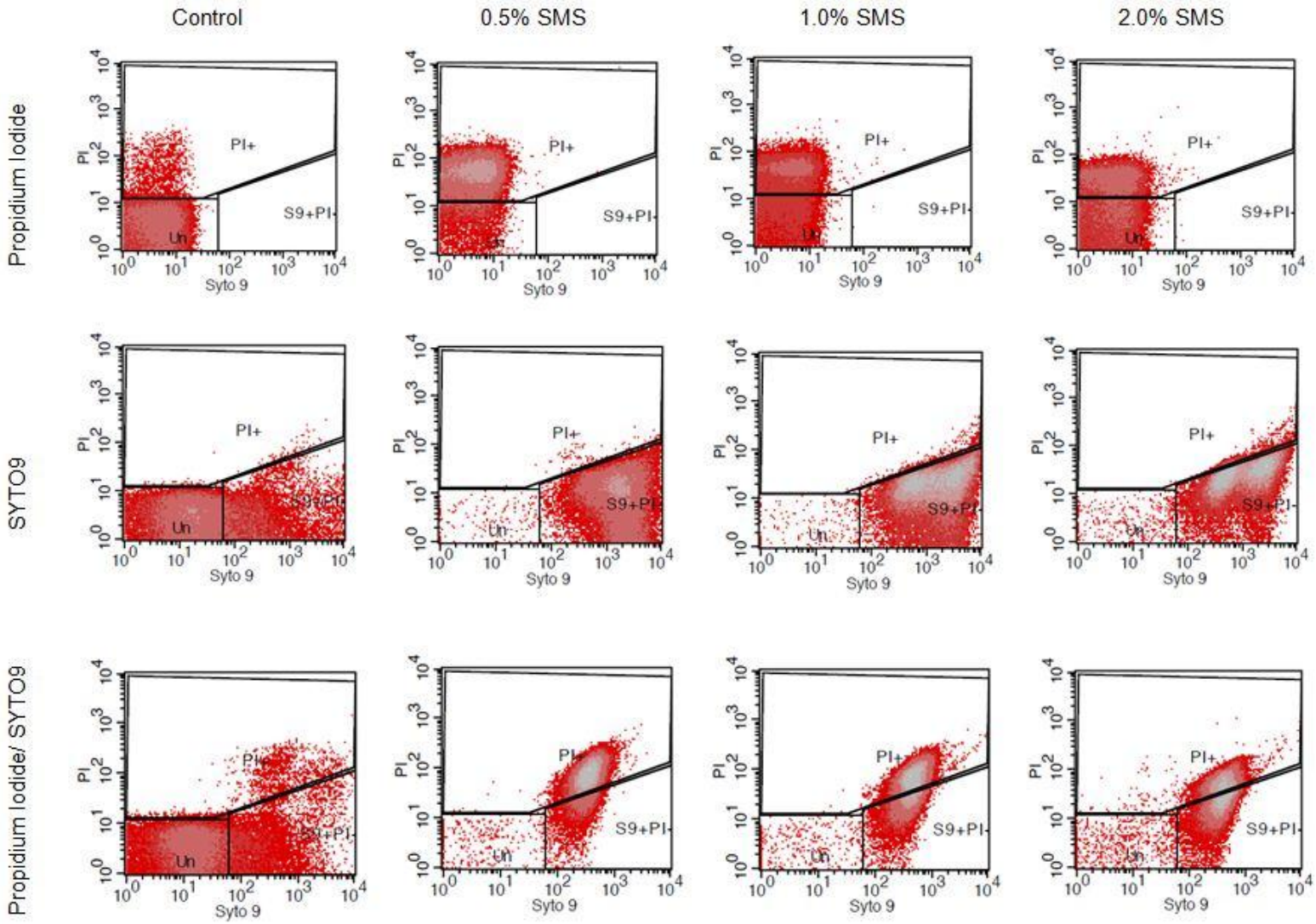


Figure 7-2. Flow cytometric analysis of S. Typhimurium (ATCC 14028) treated with SMS and stained with PI, SYTO9 and PI/SYTO9. The bacterial cells were untreated (control) or treated with SMS at the indicated concentrations and a time exposure for 10 min. The blocked regions PI+, S9+PI and Un represents the PI-positive cells (loss of membrane integrity), SYTO9-positive cells and unstained cells, respectively.

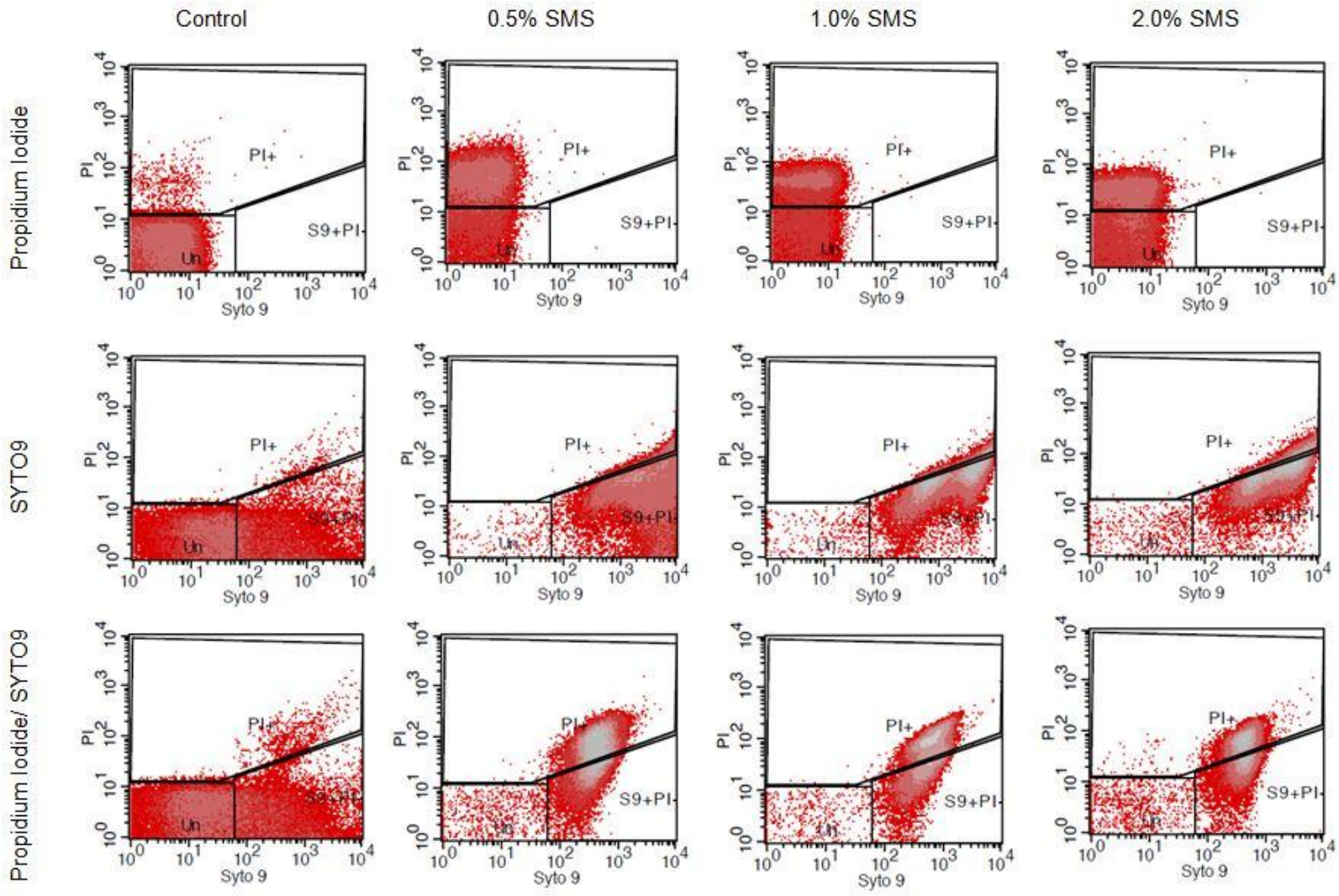
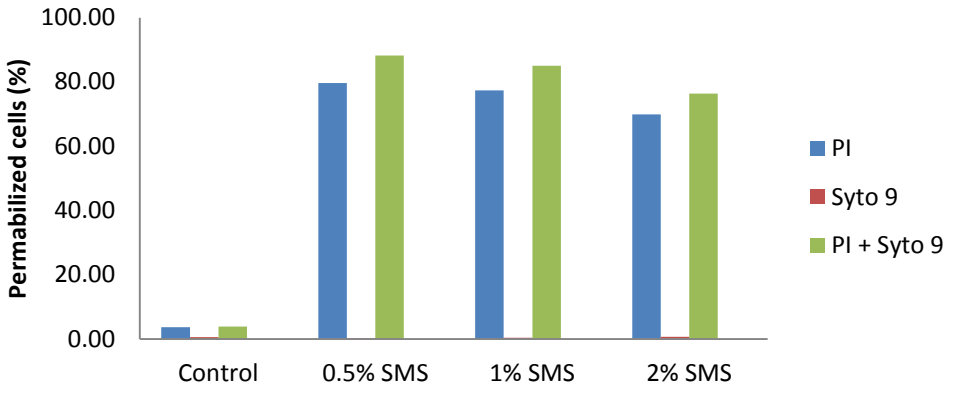
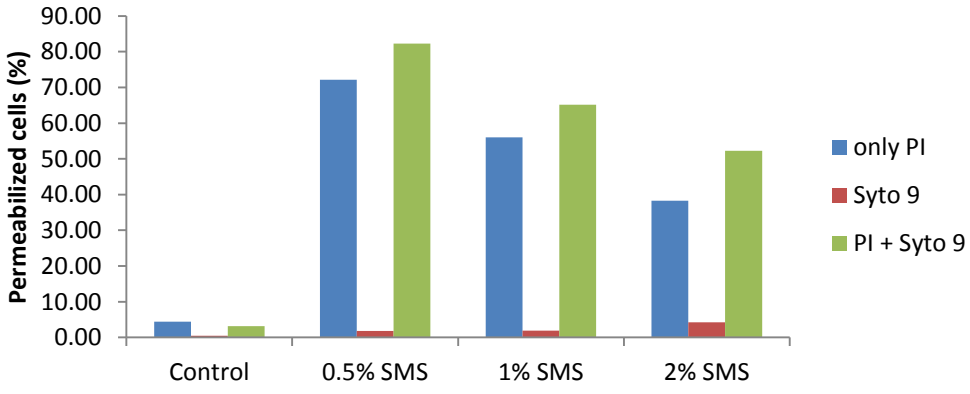


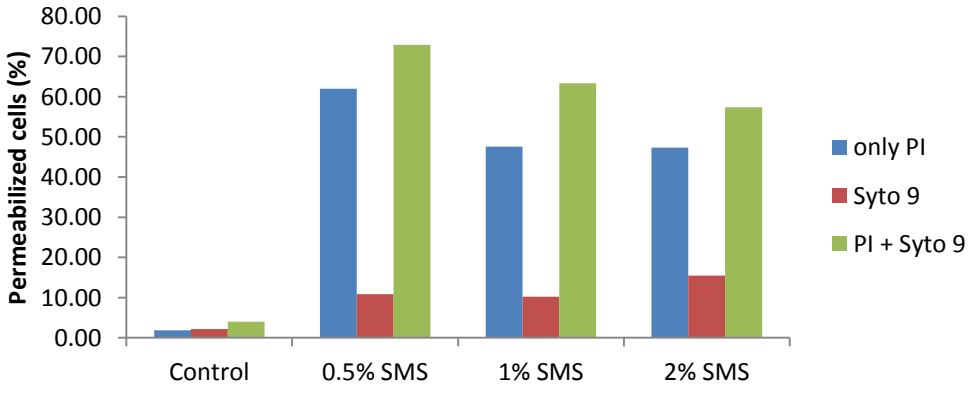
Figure 7-3. Flow cytometric analysis of S. Typhimurium (ATCC 14028) treated with SMS and stained with PI, SYTO9 and PI/SYTO9. The bacterial cells were untreated (control) or treated with SMS at the indicated concentrations and a time exposure for 30 min. The blocked regions PI+, S9+PI and Un represents the PI-positive cells (loss of membrane integrity), SYTO9-positive cells and unstained cells, respectively.



A

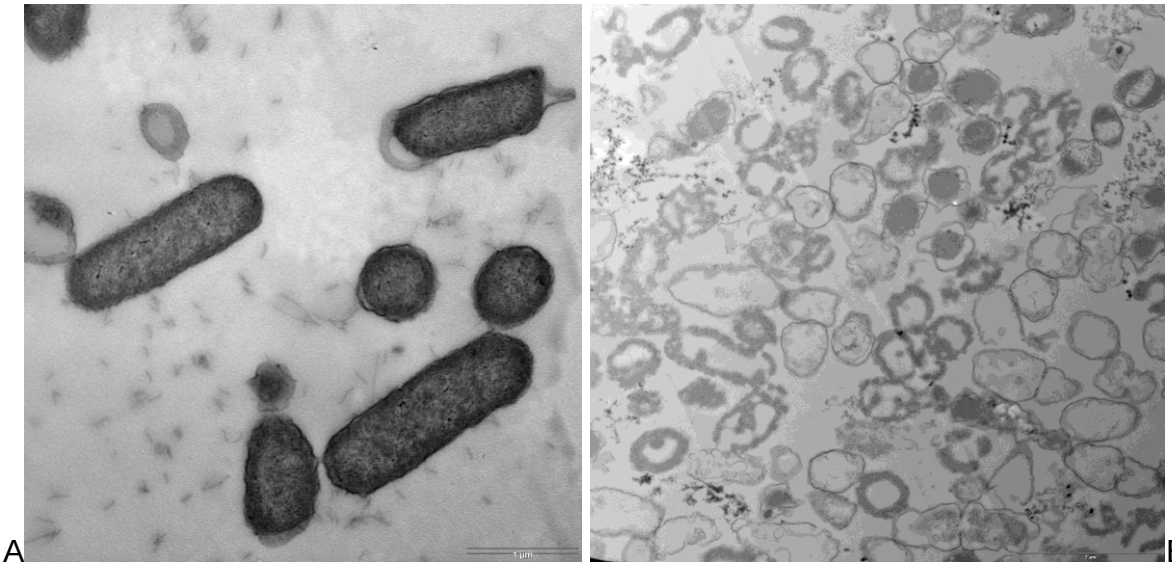


B



C

Figure 7-4. The percentage of membrane permeabilized *S*. Typhimurium cells (PI positive) following treatment with different concentration of SMS at room temperature for A) 1 min, B) 10 min and C) 30 min.



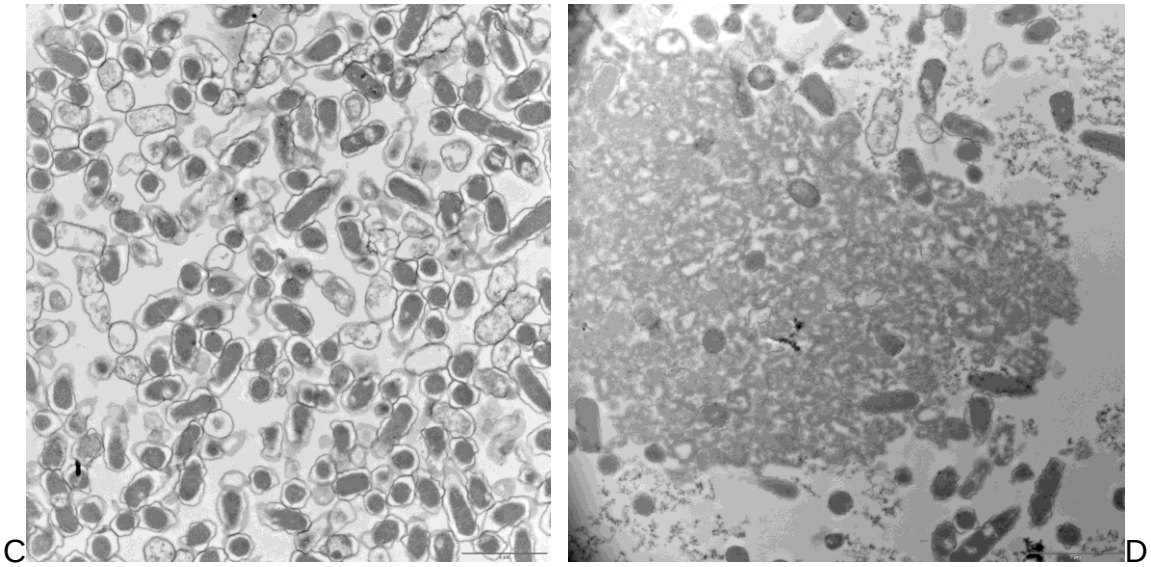


Figure 7-5. Transmission electron micrographs of *S*. Typhimurium (ATCC 14028) exposed to A) control, B) 2% SMS for 1 min, C) 0.1 N NaOH and D) 2% SMS for 30 min at room temperature

CHAPTER 8
EFFECT OF SODIUM METASILICATE ON VIABILITY OF *LISTERIA MONOCYTOGENES*

Listeria monocytogenes is a major concern in ready-to-eat (RTE) meat and poultry products and many outbreaks have been attributed to consumption of RTE poultry products contaminated with *L. monocytogenes.* Lot of interest is still there in terms of food safety to control the occurrence of *L. monocytogenes* in RTE foods. *L. monocytogenes* has been known to be susceptible to high pH (Laird et al., 1991; Mendonca et al., 1992; Taormina and Beuchat, 2002). Sodium metasilicate (SMS) is a highly alkaline chemical and has been approved for its use as antimicrobial in RTE meat and poultry products (USDA FSIS 2011). Sodium metasilicate is known to possess antimicrobial properties against Gram-negative foodborne pathogens such as *Salmonella* and *E. coli* O157:H7 (Weber et al., 2004; Carlson et al., 2008; Pohlman et al., 2009). However, the antimicrobial efficacy of SMS against *L. monocytogenes* needs to be studied and also the mechanism by which sodium metasilicate exerts its antimicrobial action is not fully understood. The objectives of the present study were to determine the antimicrobial efficacy of SMS and high pH against *L. monocytogenes in vitro* and to determine the mechanism of antimicrobial action of SMS against *L. monocytogenes*, if any. In a previous study we found that it is the high pH of SMS which was responsible for its antimicrobial properties against *Salmonella* and SMS has no effect against *S.* Typhimurium when its high pH was neutralized, which indicate that it could be the high alkaline conditions generated by SMS that are responsible for its antimicrobial activity. The combination of flow cytometry with fluorescent stains has been used to assess variety of cell functions such as cell viability based on membrane integrity and other physiological functions (Davey and Kell, 1996; Gruden et al., 2004;

Paparella et al., 2008; Kim et al., 2009). In the present study we used combination of propidium iodide and SYTO9 dyes with flow cytometry and transmission electron microscopy to assess the effect of SMS and high pH on changes in membrane integrity and intracellular structures of *L. monocytogenes*.

Material and Methods

Bacterial Culture and Exposure of Cells to SMS and High pH

Reference strain of *L. monocytogenes* (Scott A) obtained from ABC Research Corporation in Gainesville, FL, was used as in this study to evaluate the antimicrobial properties of SMS. Stock and working cultures were prepared as described earlier. One colony from the working culture was selected and transferred to 10 mL of TSB and incubated at 35° C for 20 h. Cells were pelleted by centrifugation at 5,000 g for 10 min at 4°C harvested, washed and re-suspended in 10 mL of treatment solution at room temperature containing either (i) 0.1% peptone water (control); (ii) 1% (pH 12.5), 2% (pH 12.6) 3% (pH 12.8), 4% (pH 12.9), 5% (pH 13.0) and 6% (pH 13.1) SMS solutions, and (iii) 0.1N (pH 12.6), 0.2N (pH 12.8), and 0.3N (pH12.9) NaOH solution for 1, 10 and 30 min.

Cell Viability

Treated cells were tested for their viability by taking out 1 mL aliquot from each treated cell suspensions and serially diluting in 9 mL of sterile 0.1% peptone water. A volume of 0.1 mL from each dilution was pipette onto duplicate prepoured modified Oxford medium (MOX) plates by mixing Oxford medium (222530, Becton, Dickinson and Company, Sparks, MD) supplemented with modified oxford antimicrobic supplement (211763**,** Becton, Dickinson and Company, Sparks, MD) for *L. monocytogenes* recovery. The MOX agar plates were incubated aerobically for 48 h at

35°C and after incubation, colony forming unit (cfu) from each plate were counted, averaged and reported as log cfu/mL of the sample. Duplicate samples for each treatment were analyzed and the experiment was repeated three times.

Flow Cytometry Analysis of the Membrane Integrity

Fluorescent dyes: Propium Iodide (PI) (Invitrogen, Eugene, OR) and SYTO9 (Invitrogen) were used in this study to differentiate live and dead bacterial cells based on the membrane integrity. Both of these dyes bind and stain the nucleic acids, PI is a red fluorescent stain that can only enter and stain the cells presumed to be dead with damaged or permeabilized cytoplasmic membranes and is normally excluded by live cells while SYTO 9, which emits green fluorescence is able to stain both live and dead cells (Kim et al., 2009). After the treatment of cells to 1%, 2%, 3%, and 4% SMS for 1 and 30 min, each sample (10^8 cfu/mL) was serially diluted to 10^6cfu/mL in phosphate buffered saline (PBS; NaCl 8 g/l, KCl 0.2 g/l, Na_2HPO_4 1.44 g/l, KH_2PO_4 0.24 g/l; pH 7.4) and 1 mL of the diluted sample was then stained with 2.5 µl of PI (1.5 mM) and SYTO9 (0.5 mM) either single or in combination and incubated at room temperature for 30 min. Samples were run in FACSort machine (BD Biosciences) with emission wavelengths for SYTO9 and PI at 530 +/-15 nm and 585 +/- 21 nm, respectively. Data were analyzed by Cell Quest Software, Version 3.3 (BD Biosciences). The flow cytometry analysis was repeated twice for each treatment and the results were found to be reproducible. All the samples were analyzed in flow cytometry section of Interdisciplinary Center for Biotechnological Research, University of Florida, Gainesville, FL.

Transmission Electron Microscopy

Transmission electron microscopy (TEM) was performed to observe the changes at cellular level following exposure of *L. monocytogenes* cell suspensions to SMS and high pH treatment. Cells were treated and processed as described earlier under material and methods section for TEM of *Salmonella.*

Data Analysis

Data for the cell viability experiment were analyzed by GLIMMIX procedures of SAS (SAS Institute, 2009) to analyze differences between trials, among treatments and exposure time, and treatment × time interaction. The mean separation was performed using Tukey's and a level of significance of $\alpha = 0.05$ was used to determine any significant differences among means.

Results and Discussion

Effect of SMS and High pH on Cell Viability

This study revealed that SMS had concentration and time effect on inactivation of *L. monocytogenes* in suspension (Table 8-1). No significant reduction in *L. monocytogenes* (*P > 0.05*) was observed with 1 min exposure to 1% SMS. Concentrations of 1, 2 and 3% SMS reduced *L. monocytogenes* by more than 5 log after 30 min. Exposure for 1 min to 4 and 5% SMS reduced *L. monocytogenes* by more than 5 logs. *L. monocytogenes* was not detected after 1 min exposure to 6% SMS. Treating *L. monocytogenes* with 0.1, 0.2 and 0.3N NaOH for 1, 10 and 30 min. followed similar trend in reduction of *L. monocytogenes* population (Table 8-2) or in other words high pH exerted a concentration and time effect on reduction of *L. monocytogenes in vitro*. In a similar study, exposure of *L. monocytogenes* to pH as high as 12 for 15 min resulted in only 1 log reduction (Mendonca et al., 1994). In an earlier study it was found

that SMS was unable to exert any antimicrobial effects against *Salmonella* following pH neutralization (with 6.0 N HCl) in chicken breasts inoculated with *S.* Typhimurium which dictates that it could be the high pH of SMS that is responsible for bactericidal effects against *S.* Typhimurium, which could be true for its antimicrobial effect against *L. monocytogenes.* As seen from findings in this study when high pH solutions of NaOH were used corresponding to pH values of 2%, 3% and 4% SMS solutions similar response was observed in terms of reduction of *L. monocytogenes* numbers in suspension.

Flow Cytometry Analysis of SMS and High pH Treated Cells

The effect of SMS (different concentrations and time of exposure) on the membrane integrity of *L. monocytogenes* was determined by use of PI and SYTO9 with subsequent flow cytometry. The fluorescent signals obtained from the PI and SYTO9 after different treatments are presented in two-dimensional dot plots (Figure 8-1 and 8-2). The cell population on the dot plot was clustered and identified in three different zones viz. PI positive, SYTO9 positive and unstained. The cell populations present in PI positive zone were stained with PI and their cytoplasmic membrane integrity was compromised and were presumed to be dead. It should be noted that as the concentration of SMS increased from 2% to 4% at exposure time of 1 min, there was an increase in PI positive cell population for every increase in SMS concentration (Fig 8-3 A). These findings corroborate the observations from plate counts where we found that increasing the SMS concentration result in more inactivation of *L. monocytogenes* cell populations. It was also observed that the intensity of fluorescent signals of PI positive cells increased when PI and SYTO9 were added together as a mixture as compared to PI added alone for every treatment. It probably could be due to the quenching effect or

fluorescence energy transfer between SYTO9 and PI dyes when they were added together (Stocks, 2004; Berney et al., 2007). SYTO9 when added alone didn't provide any significant information on cells with compromised membranes.

However when *L. monocytogenes* cells were exposed to different concentrations of SMS for 30 min, there was a decrease in PI positive cell population for every increase in SMS concentration (Fig 8-3 B). This could possibly due to the longer exposure of *L. monocytogenes* to harsh alkaline conditions generated by SMS resulting in more damage to membrane integrity and causing leakage of cytoplasmic contents including nucleic acids thereby low intensity signal from red fluorescent PI dye. In order to produce red fluorescent signals, PI binds with double stranded nucleic acids with a stoichiometry of one dye molecule per 4-5 base pairs of DNA, so more loss of DNA following exposure to SMS for longer period might not have allowed PI to stain enough DNA.

The results from flow cytometry experiment particularly the 1 min exposure of *L. monocytogenes* to SMS matched with those of the plate count experiment, thereby proving there is concentration effect of SMS on viability of *L. monocytogenes*. However, flow cytometry findings from 30 min exposure of *L. monocytogenes* to different concentration of SMS didn't match the observations from plate count experiments. This finding indicates that flow cytometry didn't provide reliable information on differentiation of live and dead *L. monocytogenes* cells by use PI/SYTO9 particularly following long exposure to high alkaline environments with chemicals like SMS and sodium hydroxide. It has been reported in literature that combining SYTO9 and PI does not always provide distinct differentiation of live and dead bacterial cells with much accuracy (Berney et al.,

2007; Kim et al., 2009). We reported the use of combination of PI/SYTO9 and flow cytometry to determine the efficacy of SMS and high pH on membrane integrity and differentiation of live and dead *L. monocytogenes* cell populations for the first time.

Transmission Electron Microscopy

L. monocytogenes (Scott A) cells exposed to 2% SMS and 0.1N NaOH although had different intracellular morphology than untreated cells but they seem to retain their overall shape (Figure 8-4). Cells treated with SMS have lost the normal electron dense cytoplasm which became lighter after the treatment, also cell wall and cytoplasmic membrane of some cells appeared to be disrupted particularly following 30 min exposure to SMS and NaOH as compared to the untreated cells (Figure 8-4). There were distinct clear and dark zones seen in the cytoplasm particularly following treatment with 2% SMS for 1 min and 30 min exposures to NaOH. However with 2% SMS treatment for 30 min there was hardly any clear and dark zones. In contrast the cytoplasm of untreated cells was homogenous with intact thick cell walls and indistinct cell membranes. Cells exposed to high pH (12.5) corresponding to pH of 1 and 2% SMS solutions with 0.1 N NaOH showed some dissimilarities as compared to cells treated with SMS. In a similar study following the treatment of *L. monocytogenes* with pH 12, the cytoplasm had distinct clear and dark zones, with bulging of cytoplasmic membrane against the cell wall and no discharge of intracellular material (Mendonca et al., 1994). In previous study we found that SMS and high pH caused extensive lysis of *S.* Typhimurium and there was significant leakage of cytoplasmic contents on electron micrographs but in this study the effect of SMS and high pH on *L. monocytogenes*, which is a Gram-positive is not as extensive as seen with *Salmonella* in the previous study. This difference in findings indicates that Gram-negative and Gram-positive

bacteria most likely differ in their sensitivities to high pH owing to their differences in their cell wall structures (Mendonca et al., 1994). As reported earlier by Mendonca et al. (1994) the thick peptidoglycan layer in the cell walls of Gram-positive bacteria, in this case *L. monocytogenes* help the intact cells to stabilize their weakened cytoplasmic membrane as a result of highly alkaline environment.

The results from flow cytometry experiment and transmission electron microscopy study indicate that following SMS treatment, the membrane integrity of *L. monocytogenes* is compromised leading to leakage of intracellular contents and eventually cell death. The breakage in membrane integrity was also seen by uptake of PI by cells treated with SMS with subsequent flow cytometry. In addition, it could be possible that SMS and NaOH act differently in their killing/inactivation mechanism *of L. monocytogenes* as seen in the differences of electron micrographic findings between SMS and sodium hydroxide treated cells. Further studies may be necessary to reveal the difference in their mechanism of action.

Table 8-1. Effect of sodium metasilicate on *Listeria monocytogenes* in suspension (log cfu/mL)

Treatment	1 min	10 min	30 min
Positive control	8.72 [a, x]	8.70 [a, x]	8.77 [a, x]
1% SMS (pH 12.5)	8.53 [a, x]	5.47 [b, y]	3.48 [b, z]
2% SMS (pH 12.6)	7.97 [b, x]	5.19 [b, y]	1.74 [c, z]
3% SMS (pH 12.8)	5.68 [c, x]	4.44 [c, y]	0.98 [d, z]
4% SMS (pH 12.9)	3.56 [d, x]	1.91 [d, y]	ND
5% SMS (pH 13.0)	1.05 [e]	ND	ND
6% SMS (pH 13.1)	ND	ND	ND

[a-e] Means within a column lacking a common superscript differ ($P < 0.05$)
n = 6 values per mean; ND = Not detected
SMS = Sodium metasilicate

Table 8-2. Effect of high pH on *Listeria monocytogenes* in suspension (log cfu/mL)

Treatment	1 min	10 min	30 min
Positive control	8.84 [a, x]	8.85 [a, x]	8.83 [a, x]
0.1 N NaOH (pH 12.6)	8.65 [a, x]	6.44 [b, y]	2.64 [b, z]
0.2 N NaOH (pH 12.8)	7.61 [b, x]	2.63 [c, y]	1.53 [c, z]
0.3 N NaOH (pH 12.9)	6.88 [c, x]	1.11 [d, y]	ND

[a-e] Means within a column lacking a common superscript differ ($P < 0.05$)
n = 6 values per mean; ND = Not detected

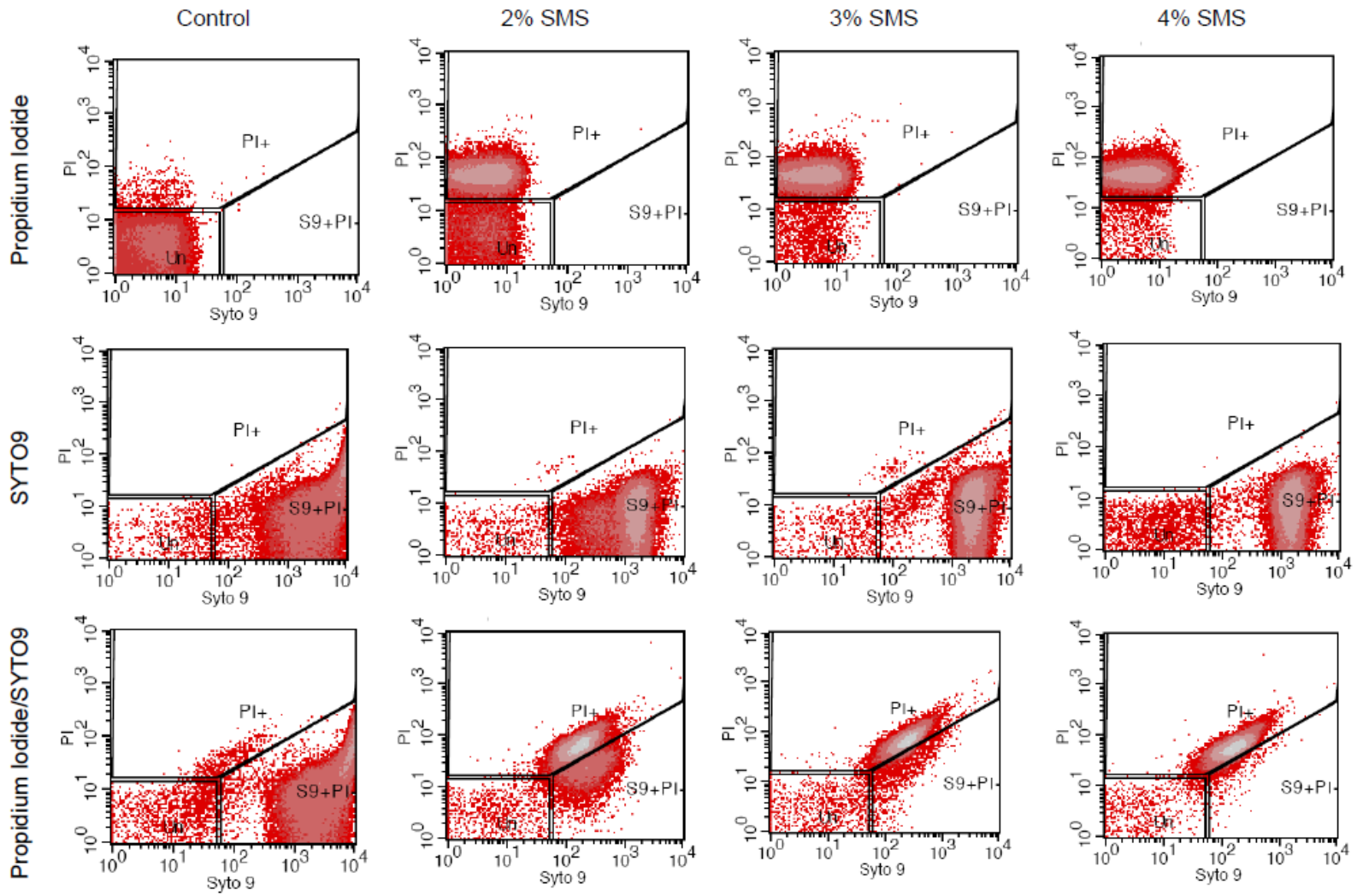


Figure 8-1. Flow cytometric analysis of *L. monocytogenes* (Scott A) treated with SMS and stained with PI, SYTO9 and PI/SYTO9. The bacterial cells were untreated (control) or treated with SMS at the indicated concentrations and a time exposure for 1 min. The blocked regions PI+, S9+PI- and Un represents the PI-positive cells (loss of membrane integrity), SYTO9-positive cells and unstained cells, respectively.

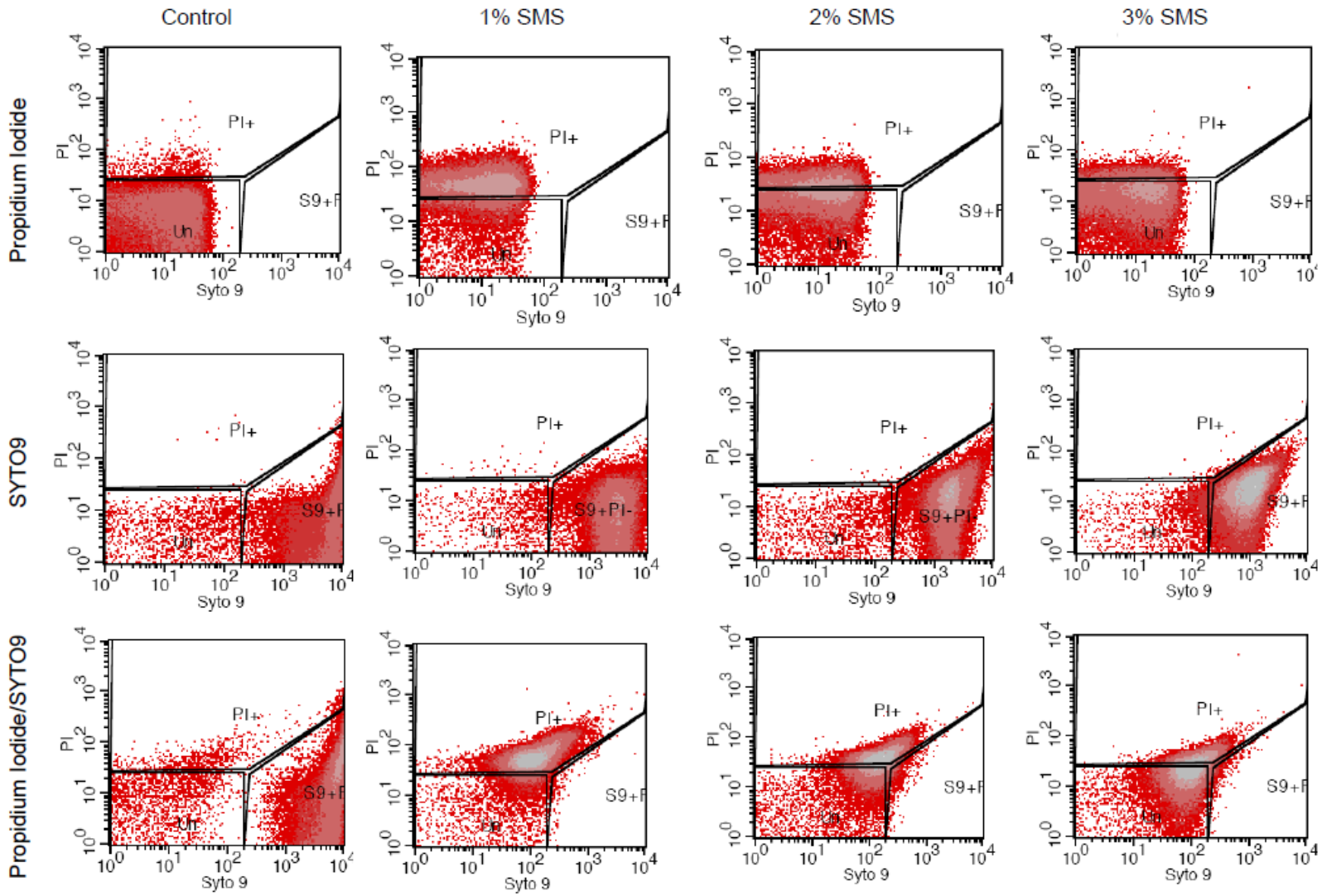
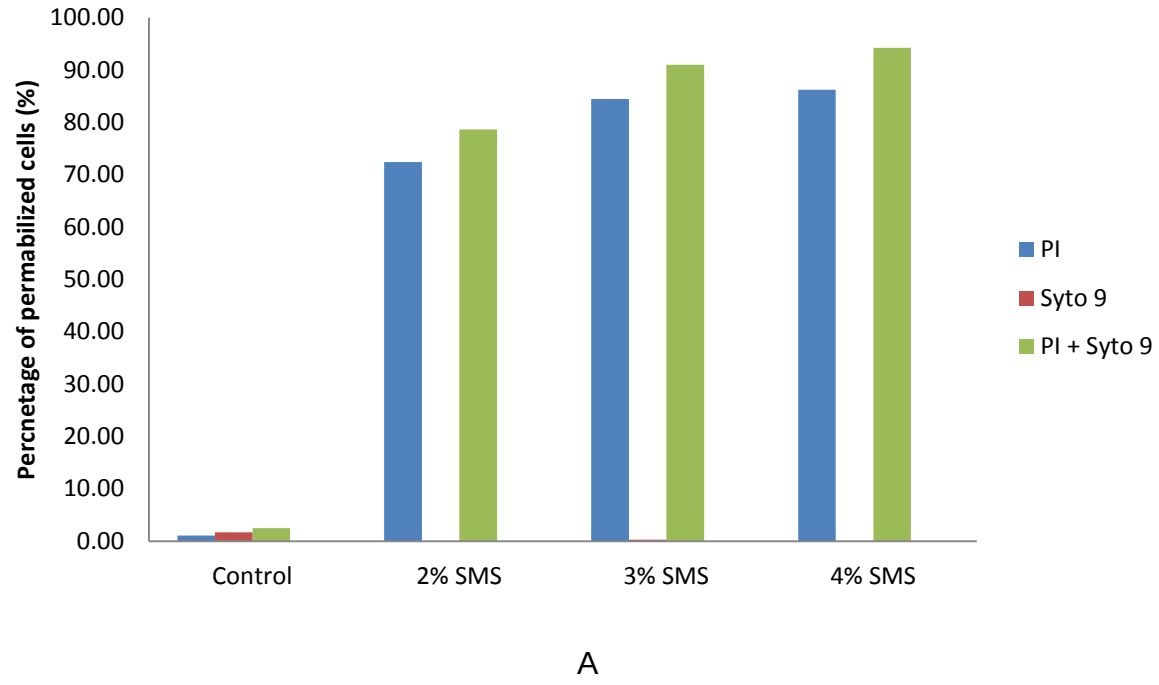
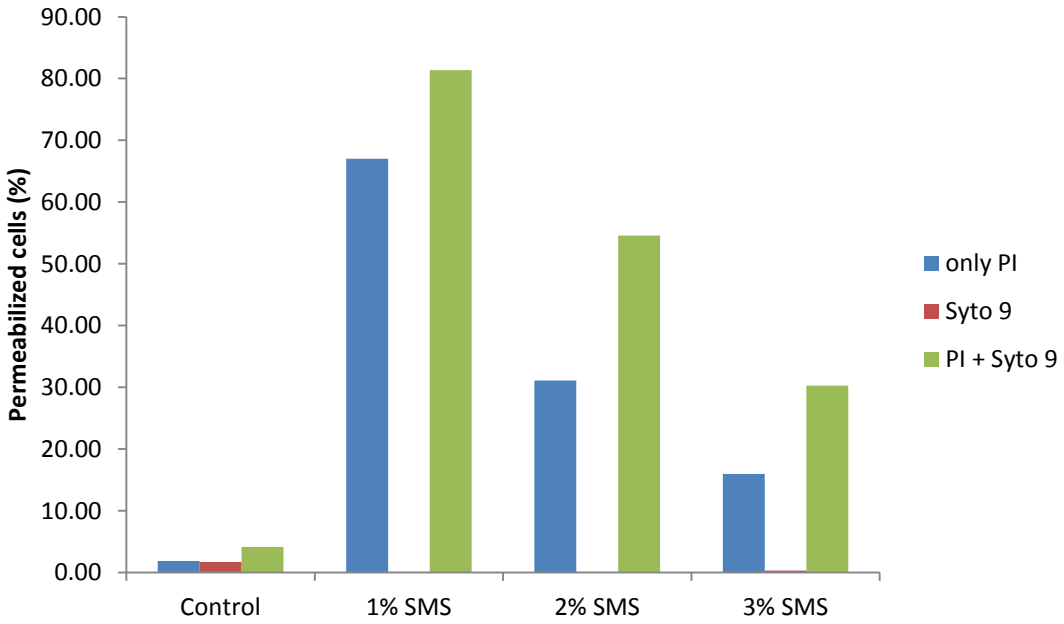


Figure 8-2. Flow cytometric analysis of *L. monocytogenes* (Scott A) treated with SMS and stained with PI, SYTO9 and PI/SYTO9. The bacterial cells were untreated (control) or treated with SMS at the indicated concentrations and a time exposure for 30 min. The blocked regions PI+, S9+PI- and Un represents the PI-positive cells (loss of membrane integrity), SYTO9-positive cells and unstained cells, respectively.

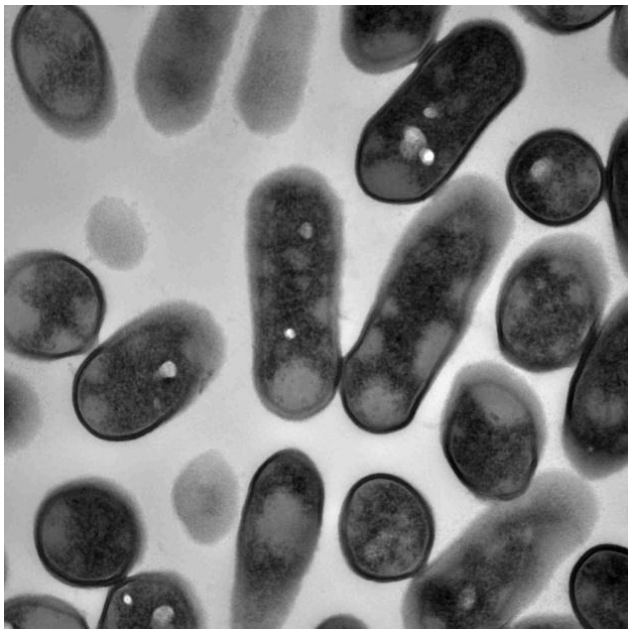


A

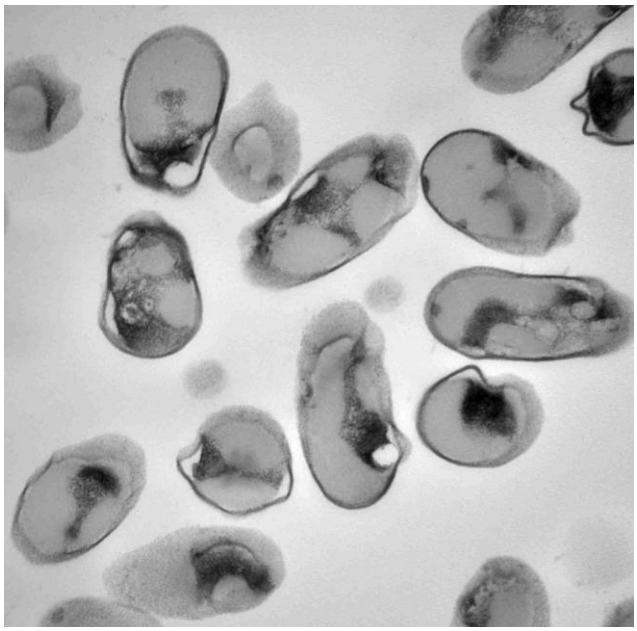


B

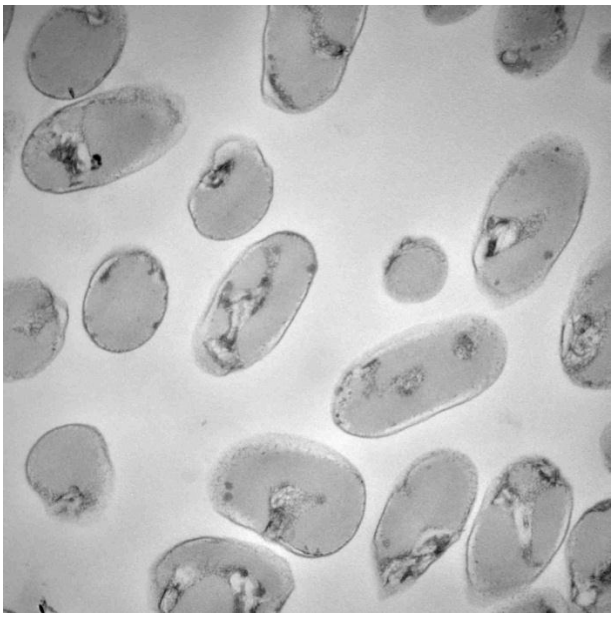
Figure 8-3. The percentage of membrane permeabilized *L. monocytogenes* cells (PI positive) following treatment with different concentration of SMS and 0.1 N NaOH at room temperature for A) 1 min and B) 30 min.



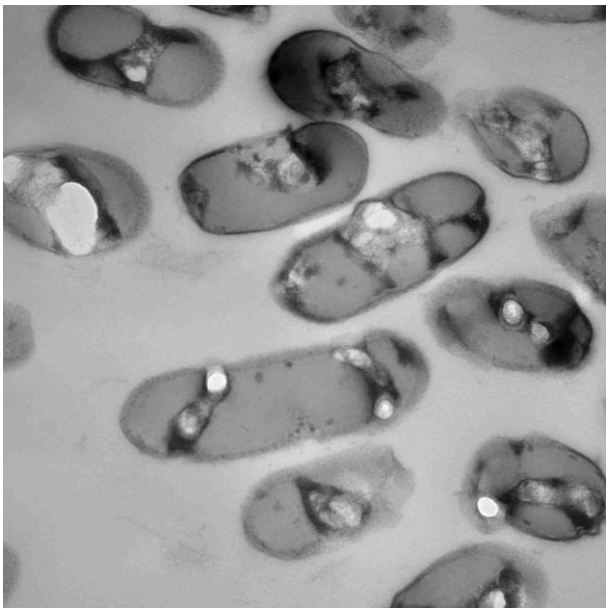
A



B



C



D

Figure 8-4. Transmission electron micrographs of *L. monocytogenes* (Scott A) exposed to A) control, B) 2% SMS for 1 min C) 2% SMS for 30 min and D) 0.1 N NaOH for 30 min at room temperature

CHAPTER 9
CONCLUSIONS

Findings from the present study indicate that sodium metasilicate was able to inactivate *Salmonella* completely *in vitro* with concentration as low as 0.5% and as early as 1 min of exposure. Sodium metasilicate when applied as a marinade at a concentration of 2% of the weight of marinade was able to reduce *Salmonella* by about 1 log cfu/g in chicken breasts (skinless and boneless) but had no effect on psychrotrophs. However, SMS when used at a concentration of 2% by weight of the meat which is higher than the currently approved levels, higher reduction in numbers of *S.* Typhimurium (more than 3 log reduction) was achieved and it also significantly reduced growth of psychrotrophs in chicken breasts (skin-on). These findings highlight the potential use of SMS as a carcass application (for which it is not currently approved).

There appears to be difference in susceptibility between *S.* Typhimurium and *C. jejuni* to SMS *in vitro* as the latter seemed to survive better under similar conditions. More studies are required to determine this difference between these two Gram-negative pathogens. However, SMS reduced *C. jejuni* more than 4 log cfu/mL *in vitro* with no significant difference among SMS concentrations used up to 2% concentration for 30 min. Sodium metasilicate when used as marinade (up to 2% of the weight of marinade) had no effect on growth of *C. jejuni* and psychrotrophs in chicken breasts (skinless and boneless). However, SMS when used at a concentration of 2% by weight of the meat, which is higher than the currently approved levels, significant reduction in *C. jejuni* (more than 3 log reduction) cell population was achieved and it also significantly reduced growth of psychrotrophs in chicken breasts (skin-on). The results

from these studies indicate that SMS does not have any effect on growth of *C. jejuni* in chicken breasts at the currently approved levels of 2% of the weight of marinade and higher concentration of SMS may be needed to control *C. jejuni* in fresh poultry carcasses. This study revealed that SMS could function to control the foodborne pathogens *S.* Typhimurium and *C. jejuni* and can also extend the shelf life of fresh poultry by retarding the growth of psychrotrophic bacteria, which are the primary spoilage organisms in fresh poultry.

Listeria monocytogenes was susceptible to SMS in suspension and there was a concentration and time effect on reduction of *L. monocytogenes* with SMS *in vitro.* The susceptibility of *L. monocytogenes* to SMS was reduced significantly as there was no effect of SMS on *L. monocytogenes* when *L. monocytogenes* was inoculated onto RTE turkey ham, which suggested that higher concentrations of SMS may be needed to control growth of *L. monocytogenes* on RTE poultry products.

The antimicrobial effect of SMS against *S.* Typhimurium, *C. jejuni* and *L. monocytogenes* observed in this study may be due to high pH of SMS. This observation can be corroborated with the similar results seen with sodium hydroxide against *S.* Typhimurium and *C. jejuni* and also inability of SMS to exert any effect on growth of *S.* Typhimurium in chicken breasts after its pH neutralization.

Loss of cell viability and membrane integrity was evidenced by uptake and staining of *S.* Typhimurium and *L. monocytogenes* cell populations with propidium iodide following treatment with SMS. Transmission electron microscopic examination of SMS treated *S.* Typhimurium cells showed disruption in cytoplasmic membrane and leakage of cytoplasmic contents outside the cells whereas cells treated with high pH revealed

wrinkled cell morphology with electron dense cytoplasm. The present study indicates that *Salmonella* exposed to SMS treatment causes permeabilization and disruption of the cytoplasmic and outer membranes resulting in leakage of cytoplasmic contents and eventually cell death. Similar but less destructive changes were also seen in electron micrographs of *L. monocytogenes* cells treated with SMS. *L. monocytogenes* cells seemed to retain the overall cellular morphology because of the thick peptidoglycan layer in their cell wall and also phase separation of light and dark zones was observed. It was also evident that SMS treatments caused disruption in membrane and cell death.

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BIOGRAPHICAL SKETCH

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