SURVIVAL, ATTACHMENT AND INTERNALIZATION OF *Salmonella agona* AND *Salmonella gaminara* ON ORANGE SURFACES

A Thesis

by

REEMA SINGH

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2004

Major Subject: Food Science and Technology

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Approved as to style and content by:

Suresh D. Pillai (Chair of Committee)	Michael E. Hume (Member)
Suryakant Waghela (Member)	Alan R. Sams (Head of Department)

Rhonda K. Miller
(Chair of Food Science and Technology Faculty)

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ABSTRACT

Survival, Attachment and Internalization of *Salmonella agona* and *Salmonella gaminara* on Orange Surfaces. (August 2004)

Reema Singh, B.S., Jamia Millia Islamia;

M.S., Jamia Millia Islamia, India

Chair of Advisory Committee: Dr. Suresh. D. Pillai

Salmonella outbreaks associated with orange juices have been reported in the past. Though there have been studies on the internalization of *Salmonella* into oranges there is inadequate information on the ability of this pathogen to survive on orange surfaces, become internalized, and survive the low pH internal conditions. The objective of this work was to study the survival of *Salmonella gaminara* and *Salmonella agona* on oranges obtained from the field and retail outlets and investigate their attachment and internalization potential. These studies showed that oranges obtained from both the field and retail outlets harbored relatively high concentrations of aerobic heterotrophic bacterial populations. There were significant differences in the survival of *Salmonella agona* and *Salmonella gaminara at* 4°C, room temperature (25°C) and 37°C. Survival was highest at 37°C and lowest at 4°C for both *Salmonella gaminara and Salmonella agona. Salmonella agona* and *Salmonella gaminara* showed significant differences in recovery when the cells were treated with pH 4.0, 7.0 and 9.5 buffers. The internalization studies suggest that a negative temperature differential favors the internalization of *Salmonella* cells into the fruit. Significant differences in the

internalization of *Salmonella* into field and market oranges were observed with more internalization in the field oranges as compared to the market oranges. These results suggest that to prevent *Salmonella* contamination of orange juices adequate pre-harvest protection against pathogen contamination and post-harvest cleaning and disinfection strategies need to be employed.

DEDICATION

To my parents whose support and guidance gave me the ability to complete my project.

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CHAPTER I

INTRODUCTION

1. Rationale

Salmonella is a facultative anaerobic, Gram-negative bacterium that does not require strict conditions for its growth. It is capable of proliferating and surviving in diverse ecosystems including food production and processing facilities. Environmental stresses such as osmotic shock, oxidative stress, heat shock, low pH and nutrient starvation are contributing factors in the capability of *Salmonella* to proliferate in diverse ecosystems (Kwon and Rickie, 1998). Infection in humans is characterized by gastroenteritis, which manifests itself as diarrhea, vomiting, fever, abdominal cramps headache and fever. Persons at risk include infants, the elderly, as well as immuno-compromised individuals, in whose case complications can result in meningitis, septicemia, Reiter syndrome and death (Pavia and Tauxe, 1991).

At least seven *Salmonella* outbreaks associated with consumption of orange juice have been reported since 1944. *Salmonella* outbreaks associated with orange juice may have at its source the cross contamination of oranges in the field by infected animal manure (Liang *et al*., 2002). The intact orange surface serves as a barrier to the invasion of *Salmonella* in the fruit. The barrier is removed during the production of juice and *Salmonella* has a chance to invade the interior and contaminate the juice, resulting in the establishment of high

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This thesis follows the style and format of Molecular Microbiology.

population of *Salmonella* within the orange juice and facilitating outbreaks (Gawande and Bhagwat 2002a). Therefore, it is important to investigate the attachment and survival of *Salmonella* on the orange surfaces. One way of approaching the problem of attachment is to develop rapid and efficacious methods to remove this pathogen from the surface. The probability of the pathogen to infiltrate interior tissues, mechanisms shielding this pathogen from the effect of sanitizers such as chlorine and physical removal such as washing may pose as the biggest hurdles in developing efficacious methods to remove the pathogens from the surface. Results from studies conducted with tomatoes have shown that a negative temperature difference between the outside and the inner areas of the fruit increases the internalization of *Salmonella* via the stem scar tissue (Burnett *et al*., 2000). In the produce houses where the oranges are washed, the difference in the temperature between the oranges and the water used for washing them could be one of the reasons for the internalization of *Salmonella* into the oranges (Zhuang *et al.*, 1995).

The ability of a pathogen such as *Salmonella* to survive, become internalized and proliferate within oranges becomes a significant hurdle to intervention strategies to remove this contaminant. To address issues such as survival and persistence of *Salmonella* on orange surfaces, it is critical to understand the survival of this pathogen on orange surfaces and identify the factors controlling the attachment and detachment of the pathogen from the surfaces. The overall objective of this study was to investigate the survival, attachment and internalization of *Salmonella gaminara* and *Salmonella agona* on oranges. The ultimate goal of this research is to identify these factors and conditions so that appropriate *Salmonella* intervention strategies could be implemented to reduce the potential for *Salmonella* food-borne outbreaks through the consumption of contaminated oranges.

2. Overall Objective

The overall objective of this study was to understand the survival and attachment of *Salmonella agona* and *Salmonella gaminara* on orange surfaces and determine whether they could be internalized into the oranges.

3. Specific Objectives

1. Determine the survival and attachment of *Salmonella* agona and *Salmonella gaminara* on orange surfaces in the presence and absence of indigenous microbial populations.
2. Determine whether *Salmonella agona* and *Salmonella gaminara* can get internalized into the oranges.

CHAPTER II

LITERATURE REVIEW

1. Food - Borne Outbreaks

Today food- borne diseases are a key concern of the food industry and the U.S government. Bacteria, viruses, prions and parasites are some of the causes of the food-borne illness (Moreno-Lopez, 2002). Symptoms of the food-borne illness include gastroenteritis, renal and hepatic disorder and neurological syndromes. Each year, approximately 76 million cases of food–borne illness, 325,000 hospitalizations, and about 5,000 deaths are reported in United States (Mead *et al.,* 1999). Pathogens that are the main concern today are *Salmonella, Campylobacter jejuni, E.coli* O157: H7*, Listeria monocytogenes* and *Cyclospora cayetanensis.* Data published in 1999 states that each year about 38.6 million illnesses were caused by the known pathogens out of which about 13.8 million (36%) cases were food-borne. Out of these 13.018 million cases, 13% were caused by bacteria, 7% by parasites and 80% by viruses. Most of the acute gastroenteritis cases reported are caused by food-borne transmission and bacterial pathogens contribute about 60%, parasites contribute about 5% and viruses are responsible for about 34% of the hospitalizations (Mead *et al.,* 1999)

Food-related deaths are of big concern and reports issued by the Center of Disease Control and Prevention (CDC) show that of all the pathogens that cause food-borne transmissions, five pathogens responsible for causing 90% of the deaths i.e., *Salmonella, Listeria, Toxoplasma,* Norwalk–like viruses, *Toxoplasma, Campylobacter* and *E.coli* O157: H7. Of all deaths, *Salmonella* causes 31%, *Listeria* causes about 28%, *Toxoplasma* cause 21%, Norwalk-like viruses are responsible for 7%, *and Campylobacter* contributes 5% of the

death and *E.coli* O157: H7 are responsible for 3% of the deaths (Mead *et al.*, 1999). Frequency of the food–borne illness is shown in Figure 1.

2. Food–Borne Outbreaks Associated with Fruits and Vegetables

In recent years, the number of food-borne disease outbreak cases associated with the consumption of raw fruits and un-pasteurized juices have increased. Numerous microorganisms capable of causing human illnesses have been isolated from fresh fruits (Beuchat and Ryu, 1997) such as tomatoes, strawberries, apples and oranges (Burnett *et al.,* 2001). Some of the pathogens associated with animals have now also been linked to fruits and vegetables and some of these pathogens are listed in Table 1.

Bacteria, viruses and protozoans have been linked to the food borne outbreaks associated with fresh produce. Most of the bacterial contamination has been associated with *Salmonella* and *E.coli* O157 .The viruses involved in outbreaks include Noro-virus and the protozoa that have been mostly involved in the outbreaks include *Cyclospora*, *Giardia* and *Cryptosporidium.*

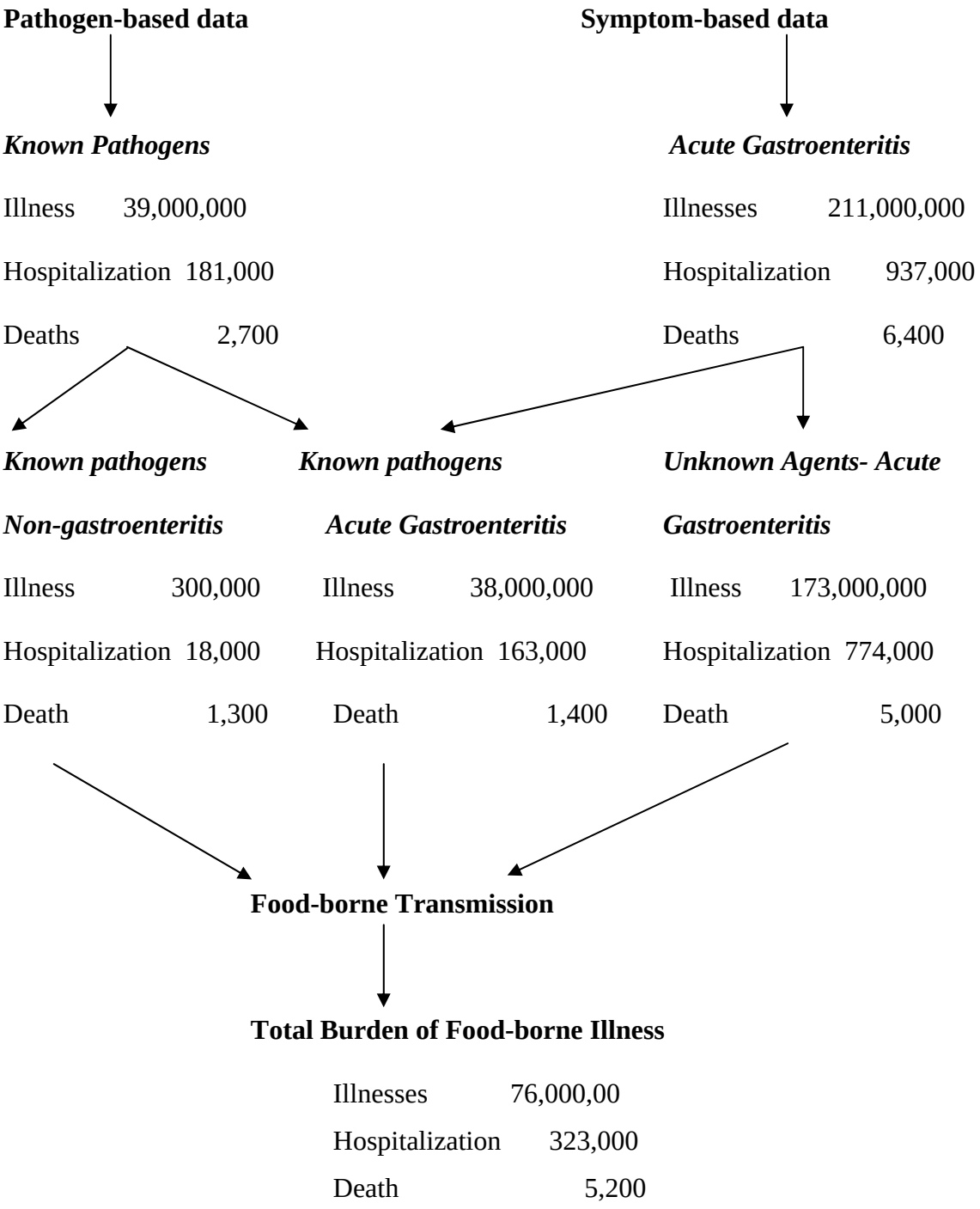

Figure1: Frequency of Food-borne Illness in the United States (Mead *et al.*, 1999)

Table 1. Outbreaks associated with raw vegetables and fruits and unpasturized products between 1980 - 2000 (Beuchat, 2002)

Microorganism	**Location**	**Type of produce**
Bacteria		
Clostridium botulinum	USA	Cabbage
E. coli O157: H7	USA, Japan	Apple cider, lettuce, Apple juice, alfalfa-sprouts,
Listeria monocytogenes	USA, Canada	Celery, lettuce, Tomato, cabbage
Salmonella		
miami	USA	Watermelon
typhimurium	USA	Apple cider
saint-paul	UK	Watermelon
chester	USA	Cantaloupes
poona	USA, Canada	Cantaloupes
montevideo	USA	Tomatoes
hartford/gaminara	USA	Orange juice
rubislaw	USA	Orange juice
stanley	USA	Alfalfa sprouts
typhi	USA	Mamey
Shigella	UK	Fruit salad
Vibrio cholerae	USA, Israel	Vegetables
Viruses		
Norwalk and Norwalk-like	UK, USA	Melon, lettuce, celery
Hepatitis A	UK, USA	Lettuce, tomatoes, Strawberries (frozen), Raspberries (frozen)
Parasites		
Cyclospora	USA, Canada, Peru	Raspberries, lettuce, basil, raw vegetables
Cryptosporidium	USA, Peru	Apple cider, celery

The North York Public health department, Canada reported a food-borne outbreak of cyclosporiasis and the product implicated was Guatemalan raspberries. Another case of cyclosporiasis was reported in May 1996. This outbreak of cyclosporiasis was reported among 49 people who had attended a private by catered luncheon. When investigated about the possible cause of the cyclosporiasis, it was found that eating the strawberry flan, decorated with strawberries, blueberries and raspberries was one of the possible reasons of the outbreak (Manuel *et al.*, 1999).

Listeria monocytogenes is one of the common food-borne pathogens that have been associated with a number of outbreaks. This food-borne pathogen is responsible for causing illness such as meningitis in humans and although the total cases of listeriosis is less than most food-borne pathogens, the case fatality rate is very high (Norrung *et al.*, 2000).

Enteric viruses are responsible for a number of illnesses in humans and include viral contaminations of herbs such as cilantro and parsley (Endley *et al.*, 2003). Noroviruses belongs to the family of *Caliciviridae* and is one of the widely known causes of the food-borne outbreaks. The Center for Disease Control and Prevention (CDC) recently reported that about 96% of the total cases of gastroenteritis that has been caused by the nonbacterial agents have been caused by Noroviruses. The food-borne outbreaks due to Noroviruses have been associated with shellfish, lettuce, fruits and chicken. (Schwab *et al.,* 2000). Noroviruses have been responsible for more than 65% of the cases of non-bacterial gastroenteritis in the United States and a major health concern today (Mead *et al.,* 1999). These viruses are transmitted by agricultural practices and also through contamination from person to person contact (Richards, 2001). Consumption of uncooked shellfish is one of the main risks associated with the contamination of the Norovirus. The increase in the risk of contamination of fruits and vegetables occurs due to the change in the eating habits and contamination of

the fruits and vegetables during cultivation, processing or through food handlers (Mead *et al.,* 1999).

In the recent years, *Salmonella* has been found associated with tomatoes, orange juices, apple juices and sprouts (Burnett *et al.,* 2000). A case of typhoid fever reported in Florida during 1998-99 was associated with the consumption of a milk shake made with frozen mamey and the isolates obtained from the patient were identified as *Salmonella typhi* (Katz *et al*., 2002). *Salmonella* has been found associated with melons and there are reports of the outbreak of *Salmonella chester* in cantaloupe from Mexico, with *Salmonella poona* in cantaloupe from Texas and an outbreak of salmonellosis in California due to *Salmonella saphara*. Non-typhoidal salmonellosis has been reported to increase in the past 20 years in the United States. Most of these outbreaks are due to consumption of contaminated fruits and vegetables. Approximately 1.41 million cases and 500 human deaths are reported in the United States due to *Salmonella* infection. Out of the total 30% deaths caused by the food-borne contamination in the United States, 95% deaths occur due to *Salmonella* infection (Santos *et al.,* 2003).

3. Survival of Pathogens on the Surface of Fruits and Vegetables

Number of factors such as the environmental conditions, agricultural practices, handling and packaging of the produce govern the presence of microorganism on the surface of fruits and vegetables. Association of the microorganisms with fruits is effected by many factors such as the environment in which plants are grown and the pH of the tissue, presence of antimicrobial factors on the surface or inside the fruits (Burnett *et al* .,2000).

A variety for pathogenic bacteria has been isolated from fruits and vegetables (Beuchat, 2002), some of these bacteria are listed in table 2.

Temperature plays an important role in the survival of the pathogens on the surface of fruits and vegetables (Gawande and Bhagwat, 2002a*). E. coli* O157:H7 was reported to survive in banana, orange, pineapple concentrate stored at -23°C and it was also reported that about 10^3 CFU/100ml of *E. coli* O157:H7 were detected in orange juice stored at -10°F after 147 days of inoculation (Oyarzabal *et al.,* 2003).

Studies done with *Salmonella typhimurium* show that it is sensitive to the acidic environment during the growth phase of the growth indicating that the growth phase also effect the survival of *Salmonella* (Gawande and Bhagwat, 2002b). Surface of contact is also known to effect the survival of the pathogens and studies show that as compared to free cell suspension, *Salmonella* exhibit more resistant to antimicrobial agents and temperature abuse when the bacteria are attached to surface (Dhir and Dodd, 1995). It's been reported that the bacterial cells are more resistant to stress when they move from the exponential phase to the stationary phase. The studies done on the effect of the growth phase on the survival of pathogens indicate that growth phase initiates some phenotypic and genotypic responses in bacteria that support the survival of pathogens under stress conditions (Taylor-Robinson *et al.,* 2003).

Table2: Pathogenic bacteria isolated from vegetables (Beuchat, 2002)

Vegetables	**Pathogens**
Alfalfa sprouts and beans	*S. havana, S. newport, Bacillus cereus, Aeromonas*
Cabbage	*L.* monocytogenes, *E.coli* O157:H7*, Salmonella, Vibrio cholerae*
Lettuce	*L.* monocytogenes, *Campylobacter, Salmonella, Staphylococcus,*
Parsley	*Shigella, Campylobacter, Salmonella, Staphylococcus*
Spinach	*Campylobacter, Salmonella, Aeromonas*
Tomato	*Salmonella, L.* monocytogenes

Zhuang *et al.,* reported that there was no significant increase in the number of *Salmonella montevideo* when it was inoculated on the tomatoes at 10°C for 18 days, but a significant increase was observed with in 7 days of inoculation at 20°C with 2 log reduction after another 7 days (Zhuang *et al* 1995). Guo *et al.,* observed that *Salmonella* numbers were decreased by 4-log after 14 days of spot-inoculation on tomatoes at 20°C. Apparently the difference in the survival was observed to be effected by the inoculation procedure as well survival of *Salmonella* was seen to be enhanced in tomatoes when they were dipped in the cell suspension as compared to the spot inoculation (Guo *et al.,* 2000).

4. Attachment of Pathogens to Fruits and Vegetables

Survival and growth of the bacteria on fruits depends on many factors, one of which is whether the bacteria are able to attach to the surface. The attachment of the bacteria on the surface of the fruit is governed by number of factors such as temperature, pH of the fruit, water activity and the medium in which the bacteria are grown (Iturriaga *et al*., 2003). Information available today regarding the attachment of the bacteria on the fruit surface is limited and most of the studies done previously relied on synthetic surfaces such as polypropylene, meat and glass (Gawande and Bhagwat, 2002b). Thus, complete information about the attachment mechanism of the bacteria is still not properly clear, however, research indicates that bacterial attach to the surface of fruits may be similar to that of the attachment of the plant pathogenic bacteria (Iturriaga *et al*., 2003). Plant pathogenic bacteria attach to the surface through both reversible and irreversible attachment, which involves the weak Van der Waal force of attraction between the cells and the surface (Iturriaga *et al*., 2003). It was reported that adsorption of the microorganism to the surface is related to hydrophobicity of

the bacterial strain and it was said that the least hydrophobic bacteria show the least adsorption (Burnett *et al.,* 2000).

Salmonella have been found to grow on the freshly peeled orange surface when the oranges were kept at room temperature. Isolation of *Salmonella* from orange juices indicates that *Salmonella* are able to survive the refrigerated temperature for a couple of days and also it has been reported that the bacteria survive in the acidic environment of the orange juice in numbers sufficient to cause a health danger (Sharma *et al*., 2001). It was reported that *Salmonella* can survive a temperature range of 4°C to 54°C and are able to survive at pH ranging from 4.0 to 9.5. *Salmonella* survived and grew in the wounded, chopped and cut tomatoes whose pH was recorded to be pH 4.31 to 4.52 (Iturriga *et al*., 2003). These results show that *Salmonella* is able to survive on tomatoes at low pH when these tomatoes were stored at temperature 20°C and 30°C. Experiments done in apple juice have shown that *Salmonella* can survive as long as 30 days and can grow in low pH. After studying the survival of *Salmonella* in low pH researchers found that, *Salmonella* can survive at pH below 3.8 for about 2 days and can survive for more than 30 days at 22°C. Studies on survival have shown that when *Salmonella* was inoculated in the apple juices, the number reached 10^9 and the number stayed that high for approximately 10 days, after which the number started decreasing (Goverd *et al.,* 1979).

5. Internalization of Pathogens into Fruits and Vegetables

Internalization of microorganisms into fruits and vegetables takes place through the stomata, lenticles and punctures on the surface of the fruits (Reina *et al.,* 2002). Studies shown that if there is a temperature difference between the fruit and a suspension, which contains pathogens such as *Salmonella* (and the suspension is at a lower temperature than the

fruit) pathogens can enter fruits and vegetables (Zhuang *et al.,* 1995). The internalization of *Salmonella* has been studied on mangoes, tomatoes, apple and lettuce (Penteado *et al.,* 2004; Buchanan *et al.,*1999; Zhuang *et al.,* 1995; Reina *et al.,* 2002). Confocal laser scanning microscopy shows that infiltration of the bacterial cells takes place through the floral tubes that are attached to the internal seeds and tissues (Warriner *et al.,* 2003).

An internalization study done by Zhuang *et al.,* on tomatoes showed that infiltration of *Salmonella montevideo* takes place from the surface of the tomatoes to the core tissues when there is a negative temperature difference between the tomatoes and the water used for washing. Higher internalization was observed when there was a -15°C differential (Zhuang *et al.,* 1995).

Internalization of *E. coli* O157:H7 was reported into iceberg lettuce by Takeuchi *et al.,* and they observed that on lettuce leaves, *E.coli* O157:H7 attachment takes place at the cracks in the cuticles, stomata and the trichomes. Internalization of *E. coli* O157:H7 was observed through the cuts located at the junction of cells and no internalization was observed through intact cells. The plant cell wall protects the internalization of the pathogens into the tissues by forming a boundary between the pathogens and the core tissues (Takeuchi *et al.,* 2000).

E. coli O157:H7 contamination was observed in the intact apples by Buchanan *et al.,* after the apples were immersed in cold water. The internalization of *E. coli* was not uniform with the most internalization being observed through the blossom end area. The internalization of *E. coli* was increased when there was negative temperature difference between the apples and the water (Buchanan *et al.,* 1999)

Mangos are sterilized by a heat disinfection process and after sterilization the mangos are cooled for storage. This whole heating and cooling procedure could lead to the

internalization of *Salmonella* into mangos. It was observed by Penteado *et al,* that when the heating and cooling procedure results in the negative temperature differential between the mangos and the water used for disinfection and this leads to internalization of *Salmonella* results mostly through the stem - end area of the mangos (Penteado *et al.*, 2004).

Various internalization studies have been done in the past to show the possible pathway of the bacteria into the plant cell and the bioluminescent studies have shown that the bacteria could colonize easily in the roots of the germinating beans. It has been reported that the bacteria can enter the plant cell through any crack in the epidermis and also through the fissures that are formed due to the lateral roots (Warriner *et al.,* 2003).

Internalization studies show that to avoid the internalization of the pathogens/ microorganisms, it is advisable to wash the fruits in running water. Experiments have been done to find an effective way to remove the pathogens from fruit surfaces. When fruits were rubbed with agitation for approximately 30 sec to 1 min followed by washing with water there was a 5-log reduction in the surface microbial population (Parnell *et al.,* 2003).

6. *Salmonella* Contamination of Orange Juice

Unpasteurized orange juice has been reported as a vehicle for a *Salmonella* outbreak. In 1995, the New Jersey Health Department reported case of salmonellosis in which the product implicated was unpasturized orange juice consumed in a park in Florida. It was estimated that about 1200 to 6400 individuals were infected (Bell, 2000)
and *Salmonella hartford* and *Salmonella gaminara* were found as prime reasons for the outbreak (Cook *et al.,* 1999). The orange juice consumed in the park was not pasteurized and the fruit that was used to obtain the juice was cut in half. Cutting the fruit in half increased

the area of contact between the surface and the pulp was accounted as the possible reasons for the contamination of the juice with *Salmonella* (Bell., 2000)

In 1999, the state health departments in Washington and Oregon reported salmonellosis cases that were linked to orange juice. In Washington, about 85 persons were reported to have been affected after consuming the unpasteurized orange juice (Boase *et al.*, 1999). In Oregon, fifty seven persons reportedly fell sick after consuming orange juice which was prepared by the same company that manufactured the drink reported in the outbreak in Washington (Bell., 2000). *Salmonella munchen* was isolated from persons with diarrhea, bloody diarrhea, abdominal cramps and fever. In 1999, drinking unpasturized orange juice infected more than 400 people in southern Australia, and the organism that was identified was *Salmonella typhimurium* PT135a (Bell., 2000)

The possible route of the pathogens into plant cell could be the bruised and cut surface of the fruits and vegetables. These cut surfaces may release fluids that can have nutrients or antimicrobials could effect on the growth of naturally occurring microorganism and pathogens. The cut on the surface of the fruits and vegetables can come in contact with the soil or other type of contamination and the pathogens present there can find a way to penetrate the fruit. It has been found that such conditions lead to the growth of molds and mold growth can increase the pH of the fruits and vegetable. This increased pH might help the pathogens to grow and multiply in the fruits. Pathogens colonize and make bio-film that protect them from the deteriorating effect of the internal environment of the fruit and could promote the growth of the spoilage pathogens (Beuchat, 2002)

There could be many reasons for an outbreak of *Salmonella* to occur following the consumption of fruit juices such as orange juices. One of the possible reasons could be the fruit picked for juice production. These fruits are handpicked from the trees and also the

workers pick fallen fruits from the ground. These fruits are collected together in a common place and from there they are shipped within 24 hours under room temperature to the processing plant. The fallen fruit can become contaminated with enteric- pathogens such as *Salmonella* (USDA, 1997). The infected oranges can cross-contaminate other oranges during the transport, washing, juice processing and during canning. The fruits that are washed and waxed for selling are usually high standard fruits. The fruits that are of low grade, such as those picked from the ground or those that have bruises or wounds are sometimes used for production of the juice and this could be one of the possible reasons for the presence of the pathogens in the orange juices (Bell,1946)

Other possible reasons for the outbreak could be contamination by the animals or improper cleaning of the processing plant or from the soils irrigated with contaminated streams (Okafo *et al.,* 2003). The processing plants sometimes have cracks and holes on the walls, ceilings and these acts as the passage for rodents, bird and other animals. Researchers have isolated *Salmonella newport* from the feces of frogs and *Salmonella hartford* has been isolated from toads and it has been reported that these toads and frogs were found outside the processing house. Researchers have isolated *Salmonella saintpaul* from unwashed oranges that were stored in the cold storage. The conveyer belt that is used for cleaning the oranges has also been reported as the dwelling place of the pathogens. When microbiological testing of the conveyer belt was done prior to washing of the oranges in a processing house, researchers found high number of *E.coli* and also a large number of microorganisms were found on the floor of the processing house (Bell, 1946).

Presence of *Salmonella* raises many questions as how the human pathogen can survive and attach on the oranges and what could be the factors that could favor the internalization of *Salmonella* into the oranges. Thus the main objective was to look at the

survival and attachment of *Salmonella* at different temperature and at low pH and what could be the factors that effect internalization.

CHAPTER III

SURVIVAL AND ATTACHMENT OF *Salmonella gaminara* AND *Salmonella agona* ON ORANGE SURFACE

1. Overview

Studies were done to determine the survival of *Salmonella agona* and *Salmonella gaminara* on orange surfaces as a function of temperature and to identify whether electrostatic forces were responsible for Salmonella attachment to orange surfaces. *Salmonella agona* and *Salmonella gaminara* populations showed significant decrease ($p<0.05$), when stored at 4ºC. The pathogen population, however, increased within 48 hours of incubation when stored at 25ºC and 37ºC. There were no significant differences ($p< 0.05$) in the recovery of inoculated *Salmonella* cells from orange surfaces after 96 hours when the cells were removed using buffers of different pH (4.0. 7.0., and 9.5) suggesting that acidic environment doesn't affect the attachment of *Salmonella* on oranges.

2. Introduction

Our understanding of the survival and growth of human pathogens on fresh produce have increased in the recent years (Jianghong *et al.,* 2002; National Advisory Committee on Microbiological Criteria for foods., 1999; Liao *et al.,* 2001; Bhagwat *et al.,* 2003). Fruits and vegetables are natural carriers of epiphytic microorganisms that are not pathogenic in nature. Fruits and vegetables become contaminated with the human pathogens during harvesting, transportation, and packing or processing.

Survival of *Salmonella* on the surface of oranges is influenced by a number of factors such as the presence of the indigenous microbial population, the surface structure, and

temperature (Iturriaga *et al.,* 2003; Schenzel *et al.,* 2002). Survival of the pathogenic bacteria depends on background microbial population and the production of certain signals that can support the cell-cell signaling in the microbial population (DeLisa). Growth and survival of the food- borne pathogens is also influenced by the environmental factors such as pH, temperature, water activity and the food microstructure (Hills *et al.,* 2001).

In order to understand the survival of a pathogen in an environment that is not its natural habitat, it is important to understand the levels of potential background microbial populations. The indigenous microbial population on the surface of fruits and vegetables, fresh cut product or minimally processed foods can pose a competitive challenge to human enteric pathogens and this could result in the suppression of the pathogens (Schuenzel *et al.,*2002).

The objectives of these studies were a) to determine the numbers of indigenous bacterial population on the surfaces of oranges obtained directly from an orchard and compare it to oranges obtained from a retail source, and b) to study the survival of *Salmonella agona* and *Salmonella gaminara* on orange surfaces.

3. Materials and Methods

3.1 Heterotrophic bacterial populations on orange surfaces

Sample collection: Oranges for the experiment were obtained from a local grocery store (College Station, Texas) and directly from the orchard of commercial field site in South Texas. The oranges were directly obtained from the orchard. Oranges from the commercial site were picked from the tree, stored on ice and were shipped by overnight courier to the laboratory in College Station, Texas. Three oranges were analyzed from the field and three from the grocery store to understand the levels of indigenous bacterial populations.

Sample processing: The oranges from field and the grocery were processed immediately after they were received in the laboratory. Each orange was placed in separate ziploc® bag (Nasco -Whirl-Pak, Ontario, CA) and was washed with 100 ml of sterile 0.1% peptone buffer. The entire orange surface was rubbed by hand for 2 minutes to ensure transfer of the microbial population from the surface into the buffer.

Microbiological analysis: The oranges washed were removed from the bags and the washes were used for serial dilution and plating. The washes were serially diluted in 0.1 % peptone and 0.1 ml of appropriate dilutions were spread plated in Tryptic Soy Agar (Beuchat and Ryu, 1997) and R2A plates (Atlas, 1996). The plates were then incubated at 37ºC for five days before performing the colony counts. The plates were incubated for 5 days to account for all slow growing heterotrophic organisms.

Statistical analysis: The data was analyzed using a T-test using Sigma Plot (Systat Software, Inc. Point Richmond, CA) to compare the microbial count obtained on TSA & R2A also to compare the microbial population on retail and field oranges.

3.2 Survival of *Salmonella gaminara* and *Salmonella agona* on orange surfaces

Sample collection: Oranges for these experiments were also obtained from the local grocery store (College Station., Texas) and from a field from South Texas and shipped to the laboratory as mentioned above.

Bacterial cultures: The *Salmonella* strains used for the experiments were *Salmonella agona* and *Salmonella gaminara*. These strains were obtained from the University of Florida and were adapted for growth in the presence of 80 µg /ml of rifampicin. These strains were involved in food-borne outbreaks in the past. *Salmonella agona* was isolated from an

outbreak associated with alfalfa sprouts (Parnell *et al.,* 2003) and *Salmonella gaminara* was isolated from an outbreak associated with orange juice(Cool *et al.*,1999).

***Inoculum preparation*:** Rifampicin resistant strains of *Salmonella, Salmonella agona* (strain # LJH618) and *Salmonella gaminara* (strain # LJH6161) were grown overnight at 37º C in 10 ml of Tryptic Soy Broth (TSB) supplemented with 80µg/ml of rifampicin. The overnight cultures were transferred twice at 24-hour intervals to TSB supplemented with 80µg/ml rifampicin. These cultures were then centrifuged at 8000 x rpm for 15 minutes. The supernatants were discarded and pellets were suspended in 10 ml of 0.1% peptone. The cells were again centrifuged at 8000 x rpm for 5minutes. The pellets were then resuspended in 25 ml of 5% bovine serum albumin (Parnell *et al.,* 2003).

***Inoculation on the orange surface and recovery of the cells*:** The inoculum was serially diluted in 9 ml of 0.1% peptone. Fifty microliters of the inoculum having approximately 10^5 CFU were inoculated on a 3 x 3-cm area on the orange surface; three replicates were used per sample. The inoculated oranges were incubated at 4ºC, 25ºC and 37ºC. The oranges were analyzed for the numbers of remaining *Salmonella* sp. at for 0hr, 24, 48, 72 & 96 hours. The surfaces of the oranges were swabbed with cotton swabs. These swabs were then dipped in 1 ml of 0.1% peptone and then serially diluted in 0.1% peptone. The 10^4 to 10^7 dilutions were plated on TSA plates supplemented with rifampicin (80µg/ml). These plates were incubated at 37º C for five days and the numbers of surviving *Salmonella agona* and *Salmonella gaminara* on the surface of orange were enumerated under the different incubation temperatures.

***Statistical analysis*:** The experiment was done in triplicate and T-test was performed for the statistical analysis of the survival of *Salmonella gaminara* and *Salmonella agona* at different incubation temperatures.

3.3 Role of surface charge on the bacterial attachment to the orange surfaces.

Orange samples: Oranges for the experiment were obtained from local grocery store and from the field in South Texas.

***Bacterial cultures and inoculum preparation*:** The *Salmonella* strains used for the experiment were *Salmonella agona* and *Salmonella gaminara* that were used in the previous studies. The inoculum was prepared as previously described.

Inoculation on the orange surface and recovery of the cells: The inoculum was serially diluted in 9ml of 0.1% peptone. Fifty microliters of the inoculum (~ 10^5 CFU) were inoculated on a 3 x 3 cm area on the orange surface. The oranges were stored at 25ºC for up to 96 hours (three replicates per treatment). At periodic intervals of 0, 24, 48 72, and 96 hours, the surface were swabbed with the cotton swabs dipped in a pH 4.0, 7.0 and 9.5 buffer. Buffers containing the cells were then serially diluted and dilutions (10^4 to 10^7) were plated on TSA plates supplemented with rifampicin (80 µg/ml). These plates were incubated at 37º C for five days and the recovery of *Salmonella agona* and *Salmonella gaminara* from the orange surfaces as a function of swabbing with different pH buffers was estimated.

***Statistical analysis*:** Three oranges were used for each pH treatment. The data was analyzed using the T-test to statistically evaluate if there is any significant difference between the data obtained after treatment with different pH buffers.

4. Results and Discussion

4.1 Heterotrophic bacterial populations on orange surfaces

The oranges harbor a large indigenous bacterial population on their surfaces (Figures 2,3 and 4). The data show that on an average 10^9 $\log_{10}$ CFU/orange were obtained from the retail oranges and about 10^{11} $\log_{10}$ CFU/orange were obtained from the farm oranges. The data show that there was significant difference in the microbial population obtained from field when plated on TSA and R2A.Significant difference was also observed in the microbial population obtained from market when plated on R2A and TSA. The result obtained show that there was a significant difference in the microbial population on oranges obtained from the field and the market.

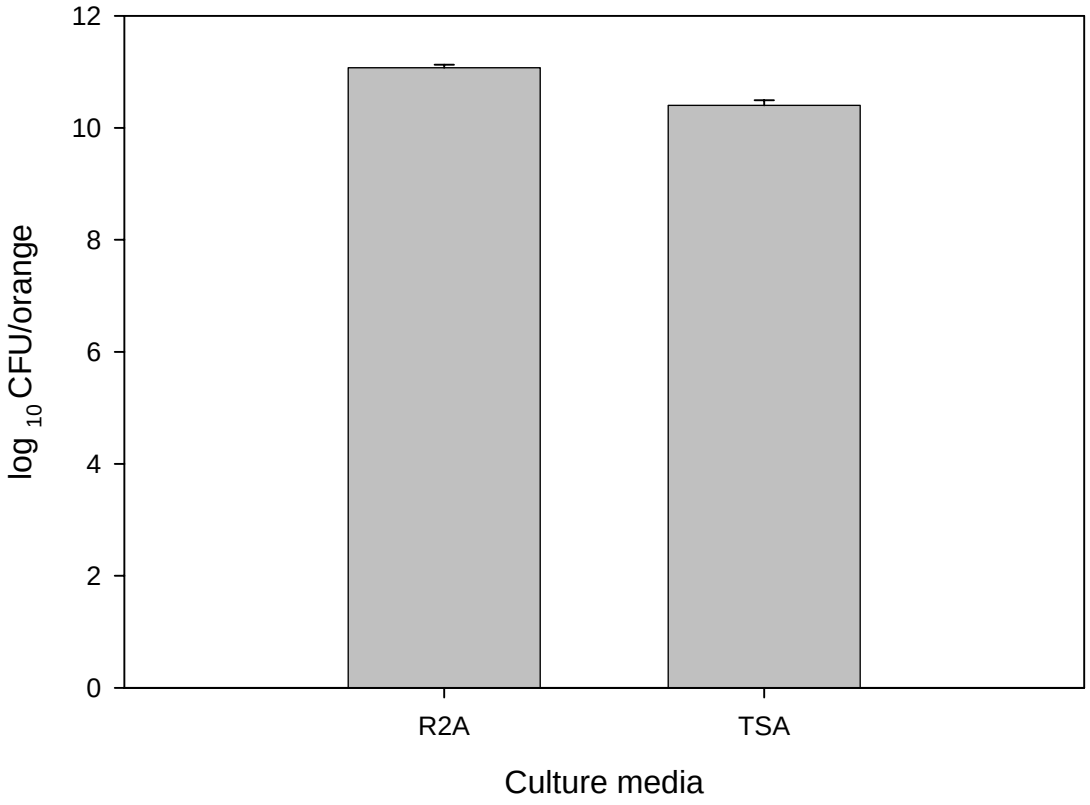

Figure 2: Recovery of indigenous microbial population on R2A and TSA of oranges from field

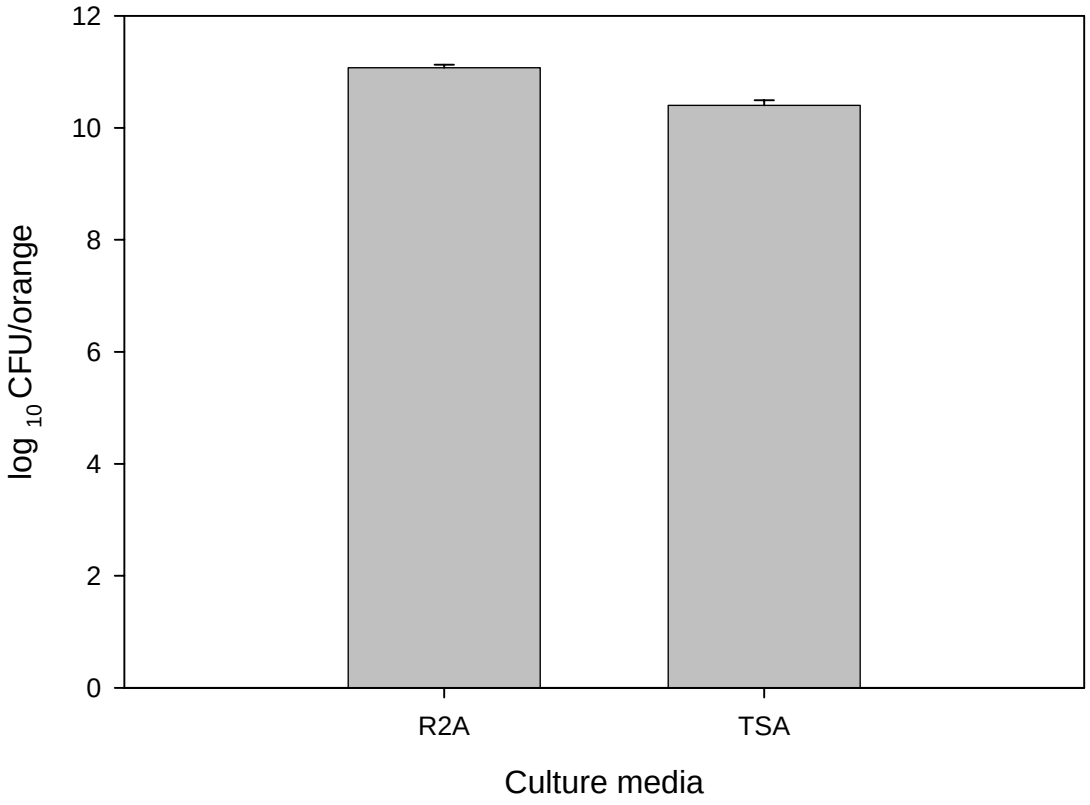

Figure 3: Recovery of indigenous microbial population on R2A and TSA of oranges from market

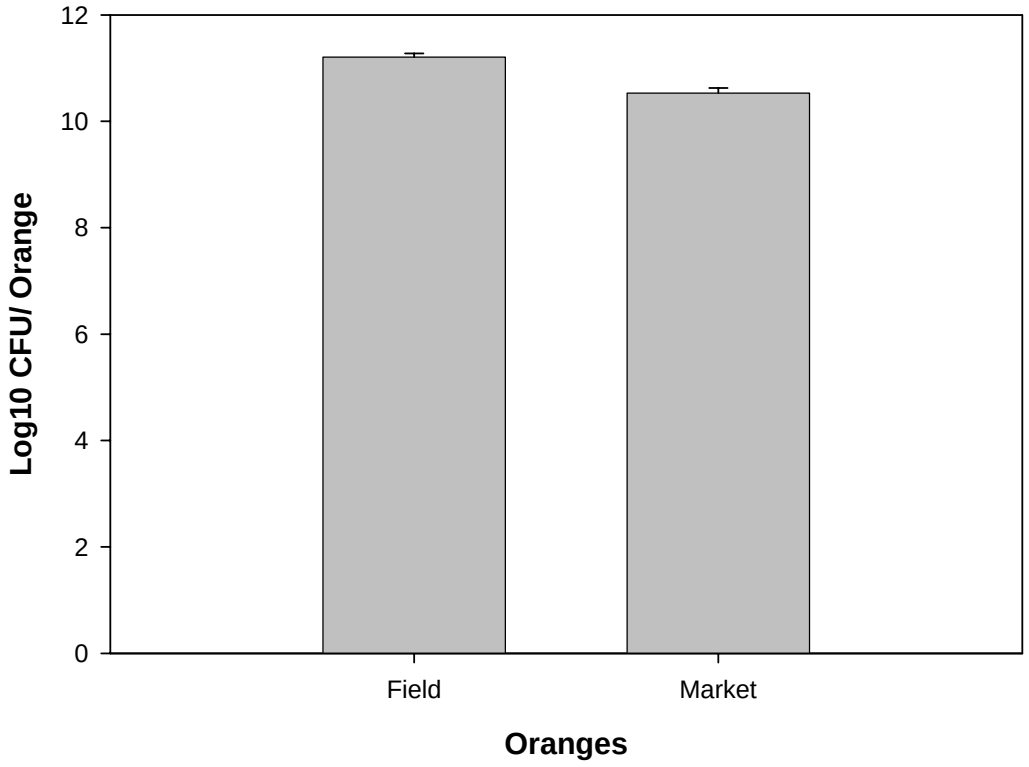

Figure 4: Recovery of indigenous microbial population of oranges from field and market

The oranges obtained from the retail sources showed less microbial population on their surfaces as compared to the farm oranges. The retail oranges are normally processed (washed with a disinfectant and waxed) before they are shipped to the retail outlets. Waxing the surface increases the attractiveness of the surface and thus makes it more appealing to customers and also presumably protects the surface (Kolattukudy *et al.*,1984). The levels of organisms on the farm oranges on the other hand represent the possible maximum bioburden that these fruits can be expected to harbor since they were not processed prior to analysis. The source of the microorganisms on the surface of oranges could be air, water, the containers used to store the fruits, biological vectors and/or human contact (Strayer *et al.*, 1994)

The significant difference in the microbial population on the R2A and TSA plates is probably due to the composition of the R2A and TSA media. The R2A contains MgSO4 (Magnesium sulfate), K_2HPO_4, and sodium pyruvate along with the yeast extract and glucose. The medium is recommended for the recovery of Gram-negative stressed microorganisms (Atlas, 1996). Tryptic Soy Agar (TSA) on the other hand is used primarily for the recovery of heterotrophic bacteria (Atlas , 1996).

The surface washing of oranges as performed in this study has been considered as the most efficient way to remove the microbial population from the surface (Beuchat, 2002). It has been reported the microorganism can be removed from the surface by the washing and rubbing the surface (Kenny *et al.,* 2001). It has been reported that for apples or oranges the approximately 100 ml of 0.1% peptone is required to retrieve the maximum microbial population from the surface (Beuchat, 2002).

4.2 Survival of *Salmonella agona* and *Salmonella gaminara* on orange surfaces

Survival of *Salmonella agona* on unwashed orange surface: Figure 5 shows the result obtained after inoculation of *Salmonella agona* on the unwashed surface at 4°C, room temperature and 37°C for 96 hours. The number of pathogens showed a sharp decline from initial 7.4 log $_{10}$ to 7.2 log $_{10,}$ 24 hours of incubation at 4°C, but after that there was gradual decline in the population. There was not a significant change in the population of pathogens incubated at room temperature. The pathogens showed a gradual decline throughout the incubation. The pathogens on the orange surface when incubated at 37° C showed a significant decrease in the population after 48 hours of incubation and after this initial decrease the numbers remained relatively constant. When the survival of the pathogen at different temperature of incubation was compared, it was observed that there was a significant difference in the numbers at room temperature and at 37ºC after 48 hours incubation. Beside that, there was not any significant difference ($P<0.05$) in the population of the pathogens at 4°C, room temperature and at 37°C incubation.

Survival of *Salmonella agona* on washed orange surface: Survival of *Salmonella agona* on the washed orange surface is shown in figure 6. The survival of *Salmonella agona* on the orange surface at 4°C show that there was sharp declines in the numbers until 72 of incubation. At room temperature the pathogens show a sharp decline until 48 hours of incubation and after that the numbers were constant until 96 hours of incubation. At 37° C, the pathogen numbers showed a gradual decline until 48 hours of incubation and after that a sharp decline until 72 hours of incubation when numbers become constant. When survival at 4°C, room temperature and 37° C was compared, there was no significant difference. However, there was a significant difference in pathogen count on inoculated oranges after 72

hours and 96 hours of incubation when the numbers at 37ºC incubation were compared to those that were incubated at room temperature.

Survival of *Salmonella gaminara* on unwashed orange surface: Figure 7 indicates the survival of *Salmonella gaminara* on the unwashed oranges. When incubated at 4°C for 96 hours, the pathogen showed a gradual decline in the population. There was less than 0.5 log reduction in numbers during the initial 96 hours of incubation at 4°C. At room temperature, however, a sharp decline in numbers takes place after 24 hours of incubation after which that the population remained constant until 72 hours, and then followed by another sharp decline. At 37°C incubation, there was gradual decline in the population until 72 hours of incubation followed by a sharp decline in the population.

Survival of *Salmonella gaminara* on washed orange surface: Figure 8 shows that oranges incubated at 4°C had a significant decrease in pathogens as compared to those incubated at room temperature and at 37°C incubation. At 4°C, about 0.4 log reductions takes place after 96 hours of incubation .At room temperature 0.7 log reductions takes places and at 37 °C a gradual decrease was observed with the reduction in the numbers from the initial concentration of 7.5 log units to 7.3 log units after 96 hours. Statistical analysis of the data shows that, there was a significant difference in the count of the pathogens on oranges when numbers at 4ºC incubation was compared to 37ºC.

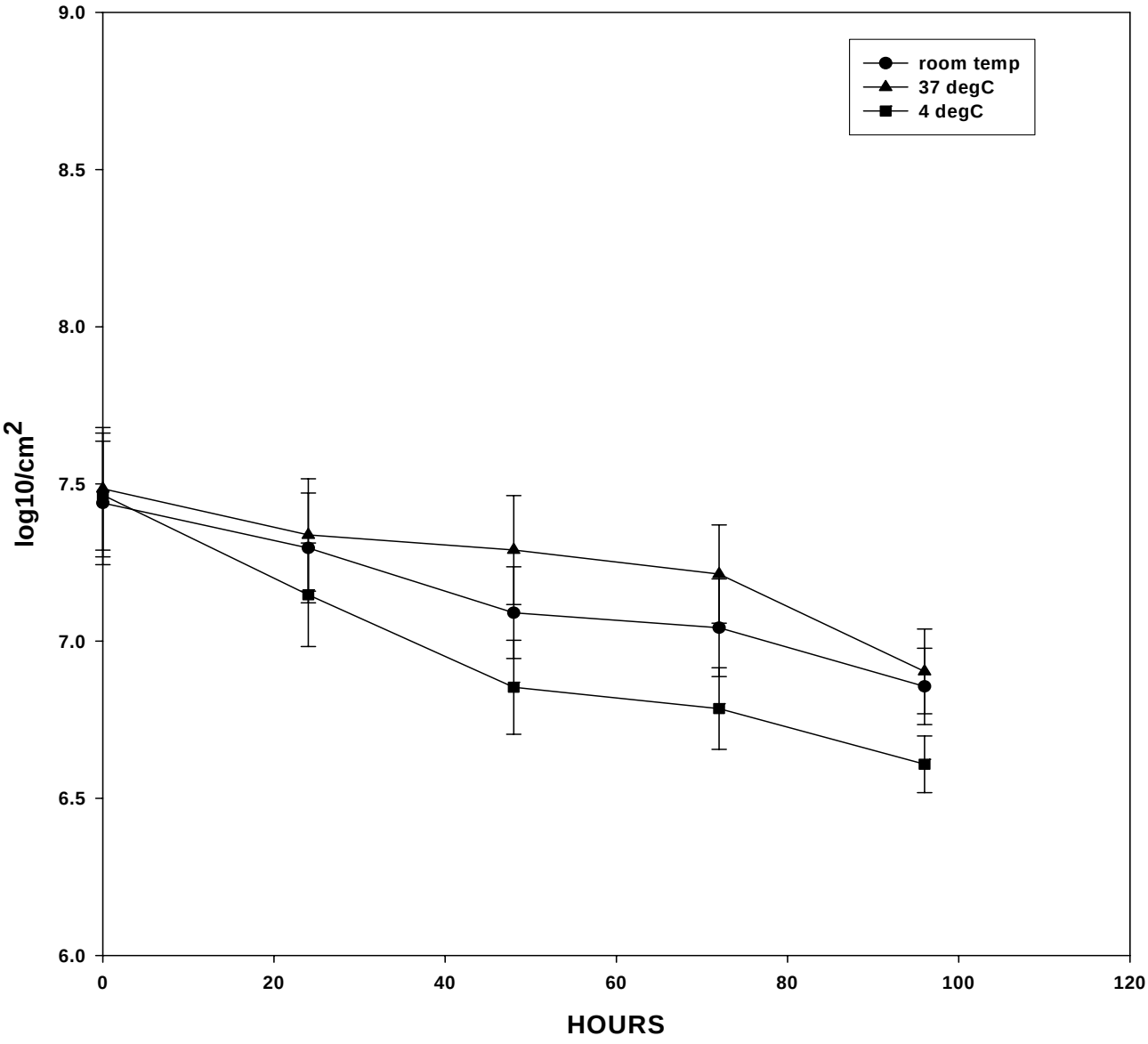

Figure 5: Survival of *Salmonella agona* on unwashed orange surface

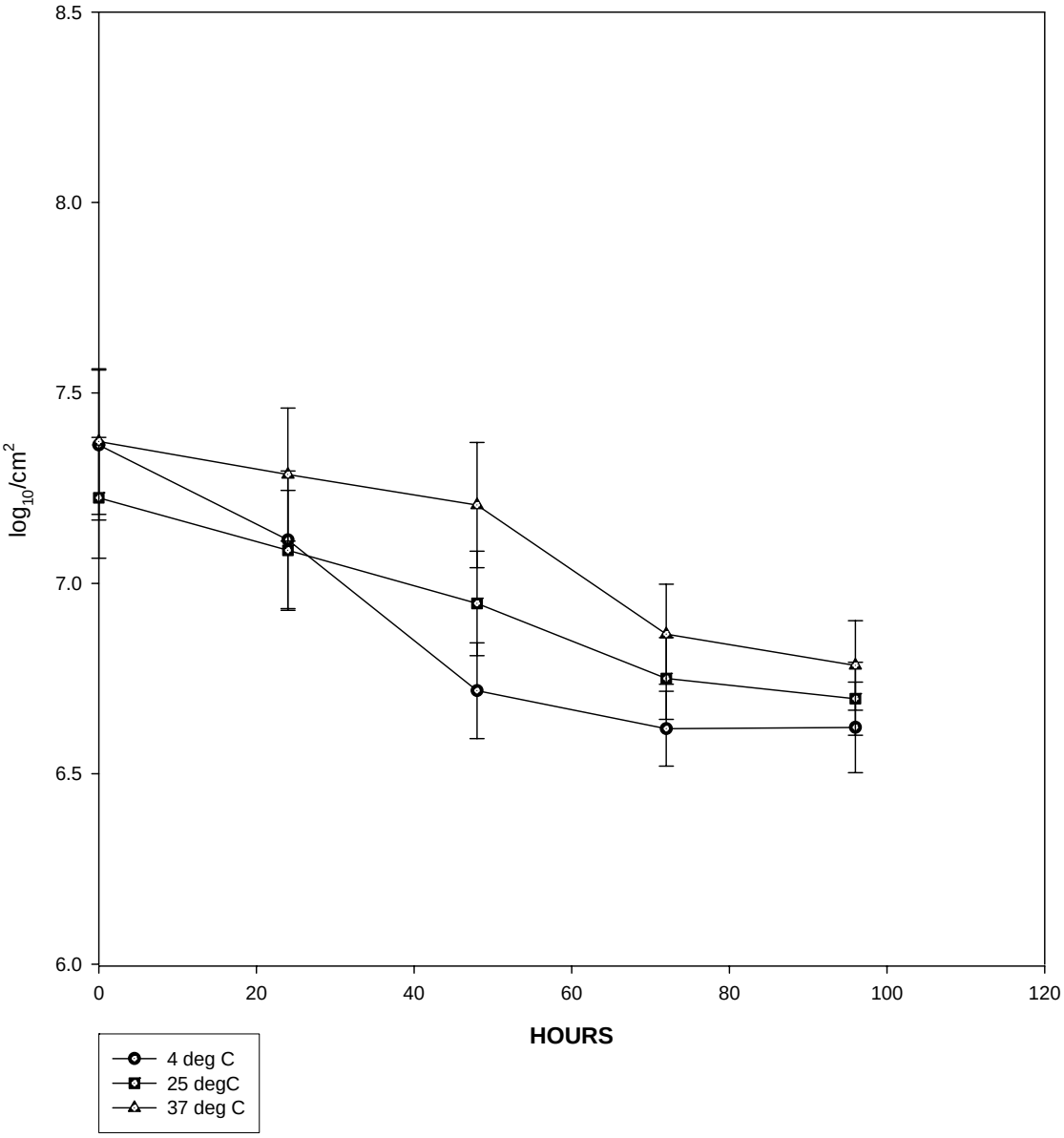

Figure 6: Survival of *Salmonella agona* on washed orange surface

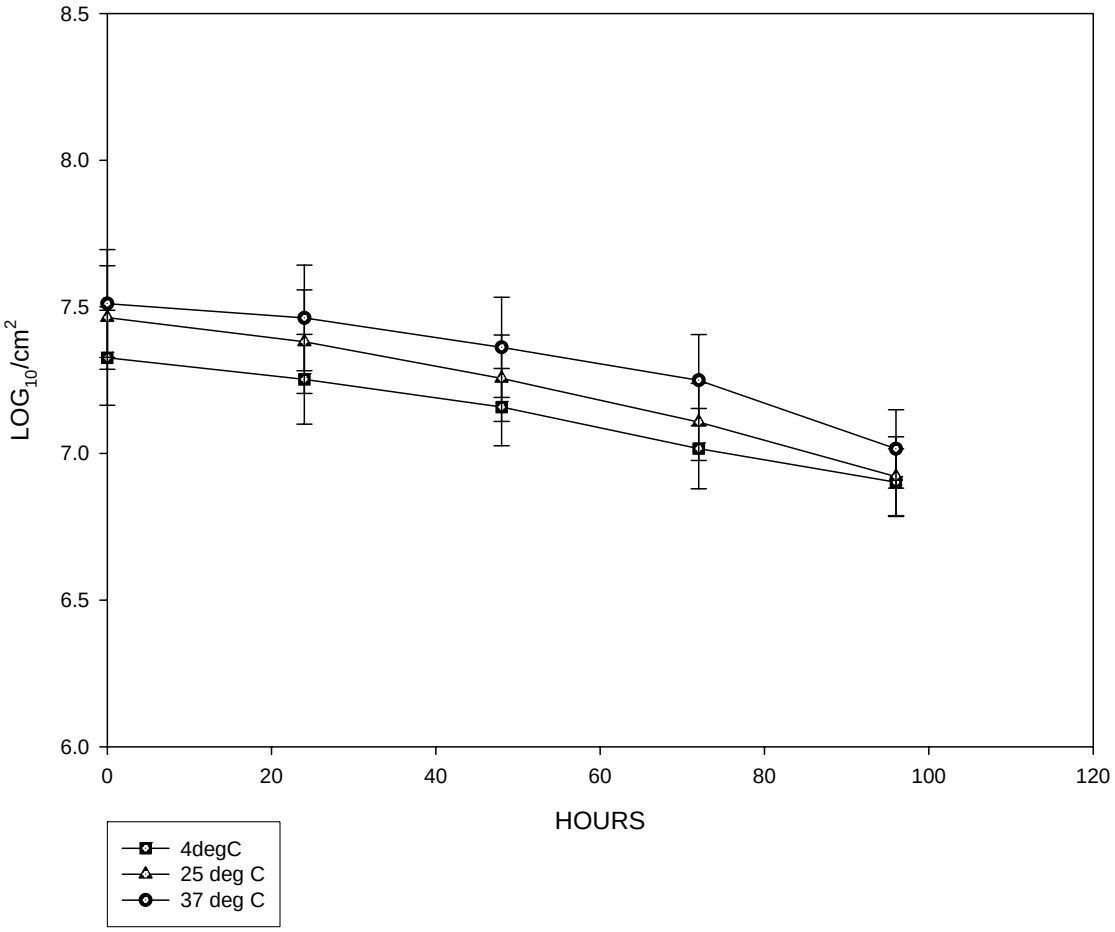

Figure 7: Survival of *Salmonella gaminara* on unwashed orange surface

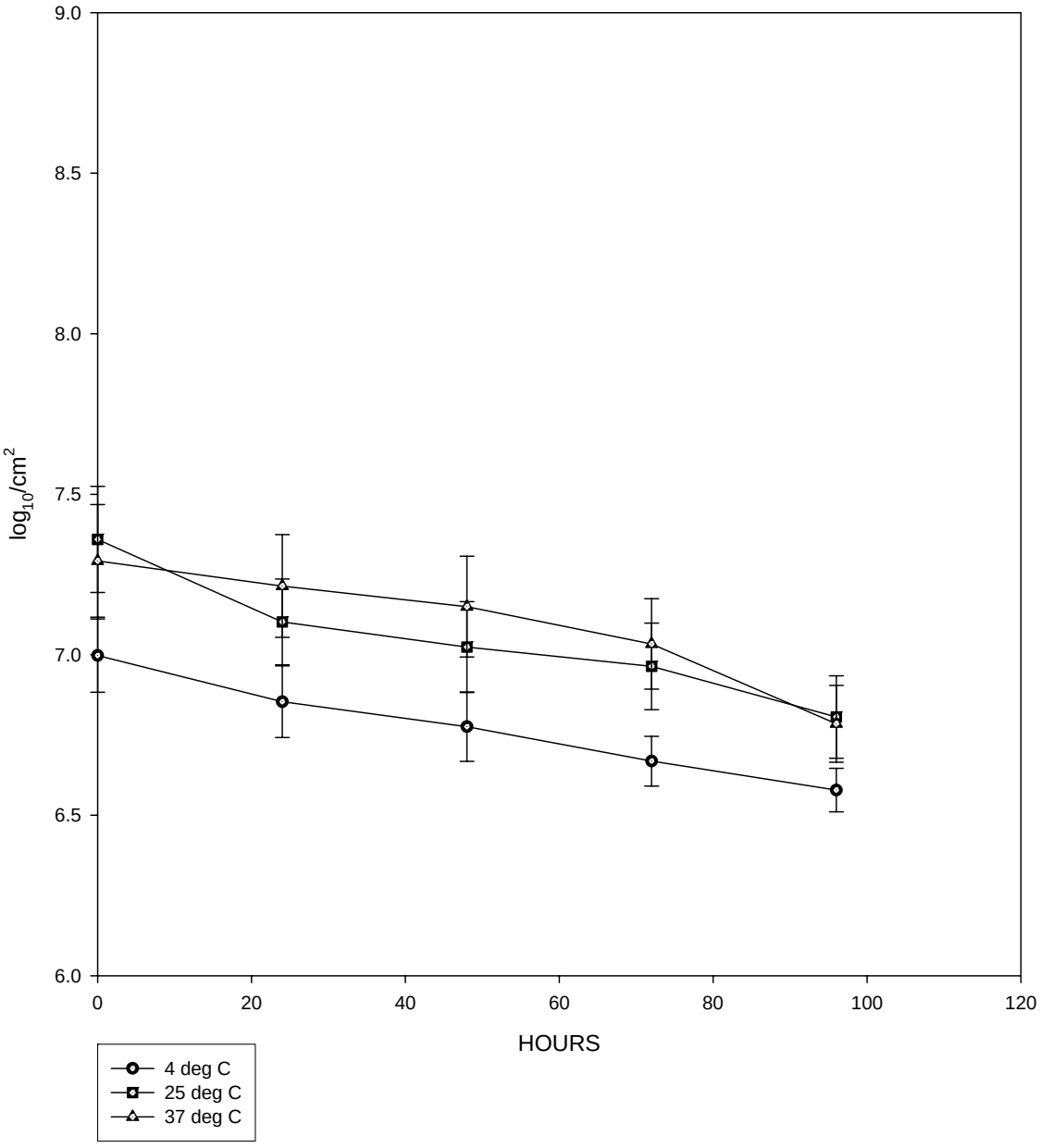

Figure 8: Survival of *Salmonella gaminara* on washed orange surface

It was reported by Goverd *et al.* that survival of *Salmonella* in the apple cider was highest at 22°C and lowest at 4°C (Goverd *et al.,* 1979). At low storage temperature such as -23°C of orange juices, *Salmonella* survived for at least 12 days at detectable level. A 6- log reduction of *Salmonella gaminara, Salmonella hartford* was observed when the strains were stored at 0°C in orange juices. Thus the storage temperature plays an important role in the survival of pathogens in juices (Oyarzabal *et al.,* 2003). Survival of *Salmonella montevideo* on tomatoes was observed by Zhuang *et al.,* and they reported that there wasn't any significant change in the *Salmonella montevideo* population when stored at 10°C but a significant increase in the population of *Salmonella montevideo* was reported at 20°C and 30°C during the 18 day storage period (Zuhang *et al.,* 1995). Results obtained during the survival study show that *Salmonella agona* and *Salmonella gaminara* were able to survive on the orange surface for 144 days at 4°C, room temperature and at 37°C but there was significant decrease in the population of *Salmonella agona* and *Salmonella gaminara* at 4°C incubation as compared to room temperature and 37°C.

Results obtained during the survival study and also the data obtained during other survival studies show that storage temperature influences the survival of *Salmonella.* The growth temperature can modify the bacterial cell structure and composition and thus can give the bacterial cell resistance against the pH, water activity or other stresses. It is stated that bacterial cells has the ability to modify the lipid composition of the cytoplasmic membrane and by doing this they maintain the fluidity in the cell. As a result of this change in the cytoplasmic composition increase in the temperature increases the degree of saturation of the fatty acid and thus increases the survival at higher temperature (Manas *et al.,*2003).

4.3 Role of surface charge on bacterial attachment on the orange surface

Figure 9 shows that when the cells were treated with pH 4.0 buffer, there was significant difference in the cells recovered after 24 hours as compared to cells recovered after 0 hours and 48 hours of incubation. A significant difference was also observed in the cells recovered after 96 hours as compared to 0 hours and 72 hours of incubation. Cells recovered after treatment with pH 7.0 buffer indicates that there was no significant difference in the cell recovery until 72 hours of incubation, but the cells recovered after 96 hours of incubation showed a significant difference as compared to 0 hours and 72 hours of incubation. The cells recovered after treatment with pH 9.5 buffers showed a significant difference after 24 hours of incubation.

Figure 10 shows that when the cells were treated with pH 4.0 buffer, there was not any significant difference in the cells recovered throughout the incubation for 96 hours. Cells recovered after treatment with pH 7.0 buffer indicates that there was no significant difference in the cell recovery and the pH 9.5 data also didn’t show any significant difference in terms of incubation period.

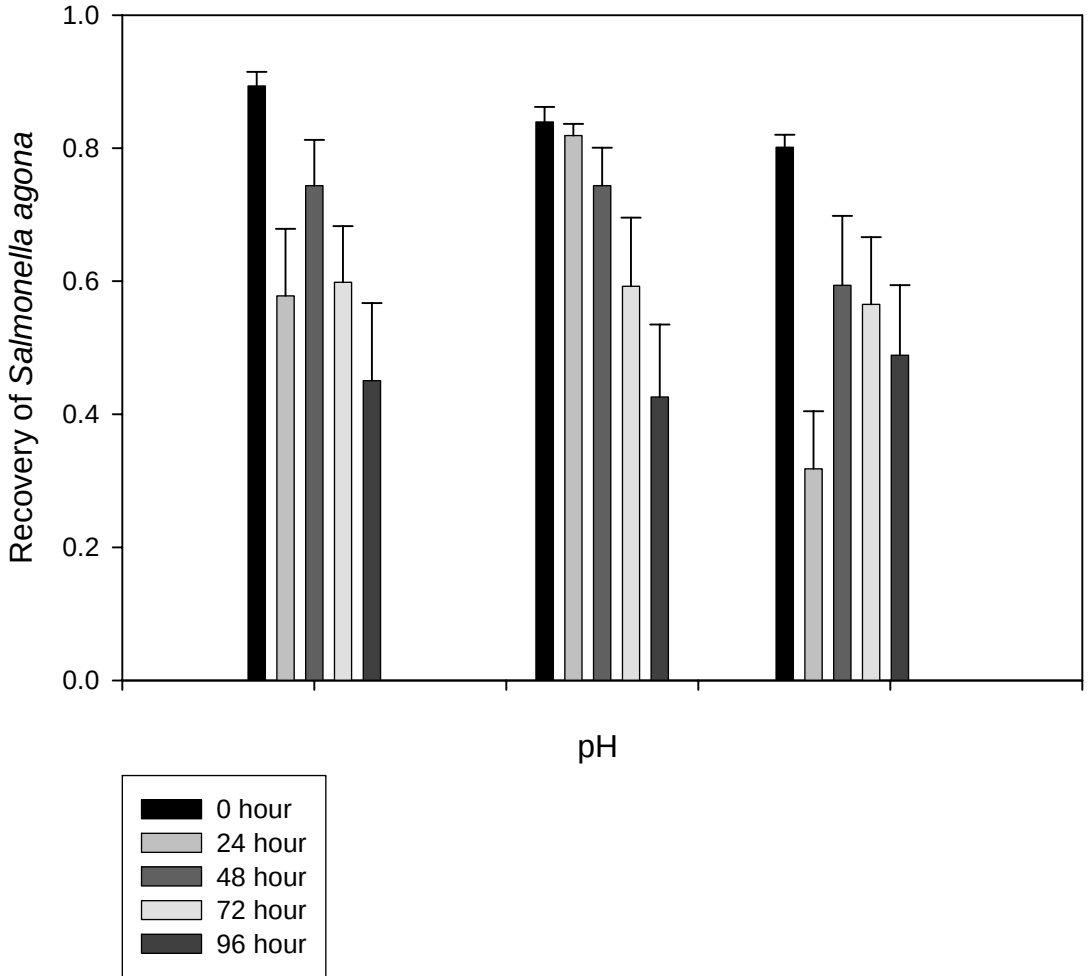

Figure 9: Recovery of *Salmonella agona* after treatment with pH 4.0, 7.0 and 9.5 buffers

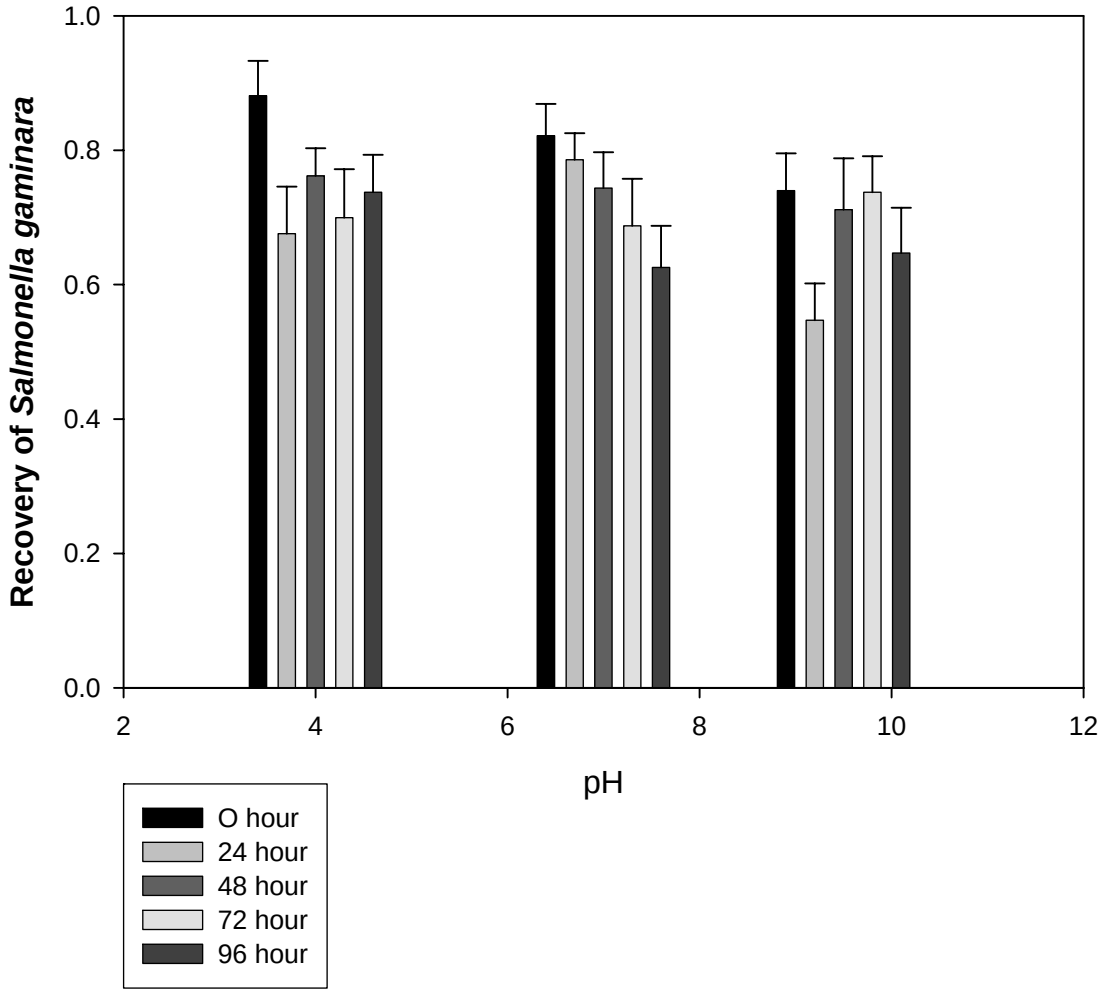

Figure 10: Recovery of *Salmonella gaminara* after treatment with pH 4.0, 7.0 and 9.5 buffers.

Attachment of the bacteria to the surface of the fruits is not only due to the surface charge but also due to the presence of the appendages present on the surface such as flagella and fimbriae (Walker *et al.,* 1999). These appendages and proteins present on the outer surface may help the bacteria to attach to the fruit surfaces. Both plant and the bacterial cell surface are negatively charged and this result in the electrostatic repulsion between them (the major components of the natural waxes present on the plant surface are the fatty acid and alcohol that could give a negative charge to the surface). Appendages such as the pilli present on the surface of the microbes help to build bridge the gap that's formed on the surface due to the electrostatic repulsion (Ukuku *et al.,* 2002). Thus the bacterial cells are able to attach to and possibly colonize the surfaces.

Microbial surface thermodynamics theory is used to explain the microbial attachment to porous media (Strevett *et al.,* 2003). This theory is based on the composition of the outer surface of the bacterial cell membrane and this theory could be used to explain the attachment of *Salmonella* on porous surface of orange (see figures on page 50 and 51).Gram-negative bacteria have high lipid and low peptidoglycan that makes them more resistant to the environment and their surface thermodynamics is very stable in response to change in the surrounding environment. Bacterial attachment or adsorption to the solid surface takes place due to the 'interfacial-attraction' between bacteria and the solid surface. When pathogens attach to the surface and interact with the indigenous microbioata within a certain range known as the range of secondary approach, Van der Waals force of attraction comes in play and thus results in the attachment of the pathogens on the surface (Strevett *et al.,* 2003)

CHAPTER IV

INTERNALIZATION OF *Salmonella* INTO ORANGES

1. Overview

Salmonellosis outbreaks in US have been epidemiologically associated with the orange juices. Oranges are picked from farms and then washed and treated in the processing plants. The oranges from the farms are usually at higher temperature than the water used for cleaning the oranges. This study was conducted to assess whether this difference in the temperature between the oranges and the water used for cleaning the fruits could promote the internalization of a pathogen. During the study oranges maintained at room –temperature and were dipped in the bacterial suspension that was maintained at 4ºC and for 72 hours. *Salmonella* was detected in the oranges after 24 hours of incubation increased after 24 hours of incubation. This study thus confirms that temperature difference between contaminated water and the oranges favors the internalization of the pathogens.

2. Introduction

Outbreaks of Salmonella have been associated with the orange. In 1995, sixty-two cases of salmonellosis were reported and the product associated was orange juice. *Salmonella gaminara* and *Salmonella hartford* were isolated from the stool of the infected persons (Cook *et al.*, 1998).

Acidic foods such as the orange juices are not considered as the vehicle of food-borne illness but there are data that showed that human pathogens have been isolated from the acidic foods. Studies done in the past have shown that if there is a temperature difference between the fruit and a suspension which contains pathogens such as *Salmonella* (with the

suspension being at the lower temperature than the fruits) pathogens can enter fruits and vegetables (Zhuang *et al.,* 1995). The internalization of *Salmonella* has been studied on mangoes, tomatoes, apples and lettuce (Penteado *et al.,* 2004, Buchanan *et al.,* 1999, Zhuang *et al.,* 1995, Reina *et al.,* 2002) and all these studies show that the temperature differential is one of the main reasons for the internalization of the pathogens.

The objective of this study was to study the internalization of *Salmonella* into oranges as a function of a temperature difference between the fruit and a buffer solution containing a defined number of *Salmonella.*

3. Material and Methods

Orange samples and inoculum preparation: The oranges were obtained from the field and from a local grocery store. Orange surfaces are very porous and that could favor the internalization of the pathogen and Environmental Scanning Electron Microscopic (ESEM), of orange surface was taken to show the surface of the oranges. The inoculum was also prepared as mentioned above, however with slight modifications. These strains were grown overnight in TSB supplemented with rifampicin (80µg/ml) at 37º C. The overnight cultures were transferred twice to TSB supplemented with rifampicin (80µg/ml) after 24- hour intervals. Two hundred and fifty microliter volumes of this overnight grown culture were then transferred to 100ml of 0.1M Phosphate buffer (pH 7.0).

Internalization experiment: Since temperature difference between the inoculum and the fruit is considered to be a critical factor for the cells to get internalized (Zuhang *et al.*, 1995)), the oranges were kept at the room temperature while the inoculum was cooled down to 4ºC. The oranges (in triplicates) were then immersed in the bacterial cell suspension for 0 hour, 24 hour, 48 hour, 72 hour and 96 hour of contact times. After the assigned contact times, the

oranges were removed from the inoculum and selected regions of the surface were sampled. Two stem scars and 3 positions on the surface were initially swabbed using sterile cotton swabs. The swabs were placed in 0.1 % peptone. The entire orange was then washed twice with phosphate buffer. The buffer containing the swabs from the scars and puncture wounds, and the buffer that was used to wash the entire orange were then diluted in 0.1% peptone. Dilutions 10^{-4} to dilution 10^{-7} were plated on TSA plates supplemented with rifampicin (80µg/ml). The plates were incubated for five days at 37º C and the *Salmonella* cells were enumerated.

After the oranges were washed, they were carefully peeled under sterile conditions using fresh gloves repeatedly. The peeled oranges were placed in Ziploc® (Whirl-pak) bags and then squeezed to obtain the juice. The juice samples were then diluted in 0.1% peptone and aliquots plated on R2A plates supplemented with rifampicin (80µg/ml). The plates were incubated at 37º C for five days and *Salmonella* cells that had potentially infiltrated into the fruits were enumerated.

Chemical tracer study: To verify the internalization of the bacterial cells into the oranges, the internalization experiment was repeated using the chemical tracer, organic iodide. The experimental conditions were similar to that employed for *Salmonella* internalization studies. Organic iodide (10% and 25 %, College station, TX) was maintained at 4C, and the oranges from the market and field were kept at room temperature to maintain the temperature difference. The oranges were immersed in the organic iodide solution (10% and 25 %) for 24 hours. After 24 hours, autoradiographic images (Large Animal Hospital Texas A&M University) need specific info on film type (Kodak, College Station, TX) of the oranges was taken to detect whether the tracer had migrated into the fruit. The migration of the organic

iodide was detected by a clear zone. Oranges that were not dipped in organic iodide were used as the negative control and as reference samples.

***Statistical analysis*:** The bacterial inoculation experiments were done using triplicate samples and T-test was use to identify the significant difference between the internalization observed in farm and retail oranges.

4. Result and Discussion

The oranges were dipped in the bacterial cell suspension (maintained at 4ºC) and the bacterial count on the stem scars, three wound areas on the oranges, and on the whole orange surface was measured.

The bacterial count of *Salmonella gaminara* and *Salmonella agona* on the stem scar (Figure 11) was observed after the oranges were immersed in the bacterial suspension until 72 hours. The results show that the numbers of *Salmonella agona* declined for up to 24 hours, after which there was a small increase in numbers. *Salmonella gaminara* also showed a declining trend until about 48 hours of incubation. Statistical analysis showed that there was not any significant difference in *Salmonella agona* and *Salmonella gaminara* count on the stem scar.

The bacterial count measured on the puncture wounds (Figure 12) show that after 48 hours of incubation, there was an increase in the number of *Salmonella gaminara*. However, thereafter there was sharp decline in numbers. On the other hand, a gradual decline of *Salmonella agona* was observed. The results show that there was a 0.5 log reduction in the population of the *Salmonella agona* during the 72 hour of incubation. Statistical analysis of the data show that there was not any significant difference in *Salmonella agona* and *Salmonella gaminara* count on the puncture wound.

The bacterial count measured on the orange surface (Figure 13) show that *Salmonella gaminara* show a gradual increase till 48 hours of incubation followed by decline in the numbers. A gradual decline in the numbers of *Salmonella agona* was observed till 48 hours of incubation followed by an increase in the numbers.

The internalization study (Figure 14) results suggest that there was an increase in the *Salmonella* population after 24 hours of incubation. The oranges obtained from the field showed an increase after 48 hours of incubation followed by sharp decline. The market-obtained oranges on the other hand showed a decrease in Salmonella population after 48 hours of incubation. When analyzed statistically, a significant difference was seen in the internalization of *Salmonella* into field and market oranges after 72 hours of inoculation.

The electron microscopic picture of the oranges from market and field (Figures 15-16), shows that the field oranges are very porous as compared to the market oranges. Surface of the oranges from the market are waxed which results in the closure of the pores present on the surface. Presence of high numbers of pores on the field oranges could result in the high internalization into the field oranges.

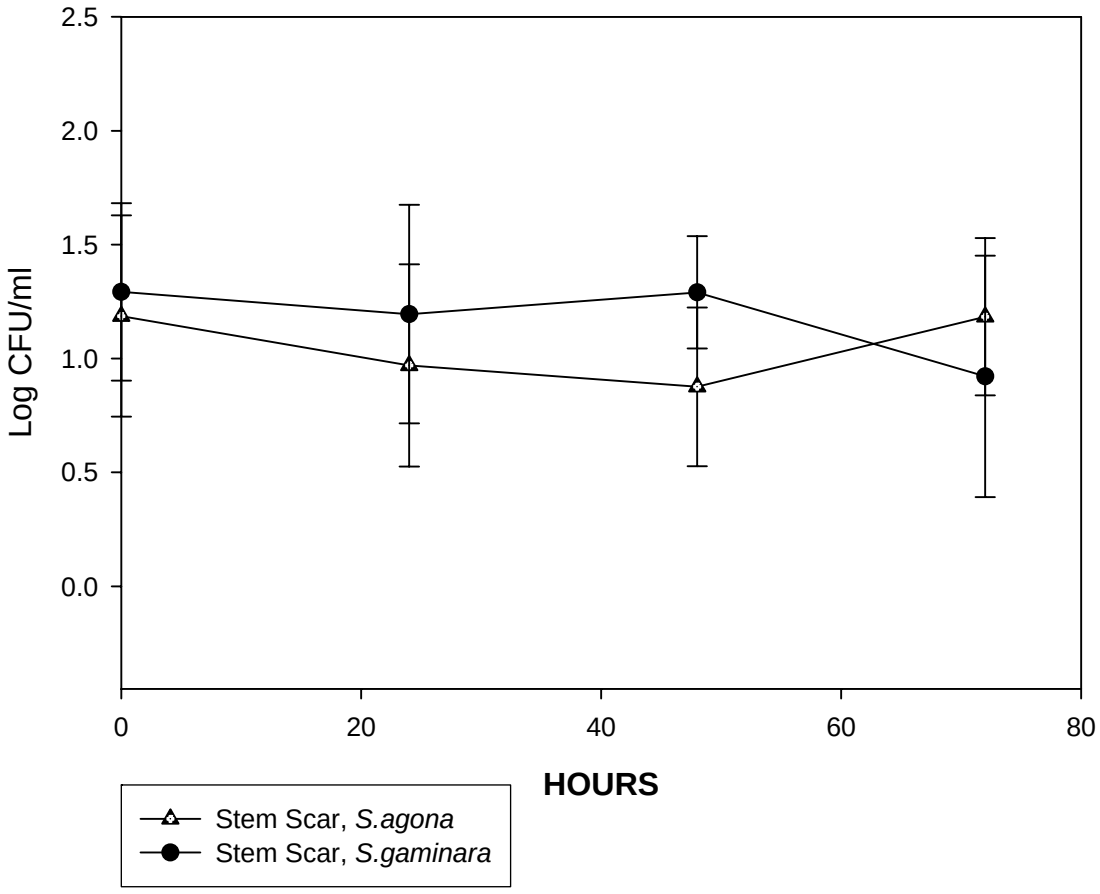

Figure 11 : *Salmonella* persistence on stem scars

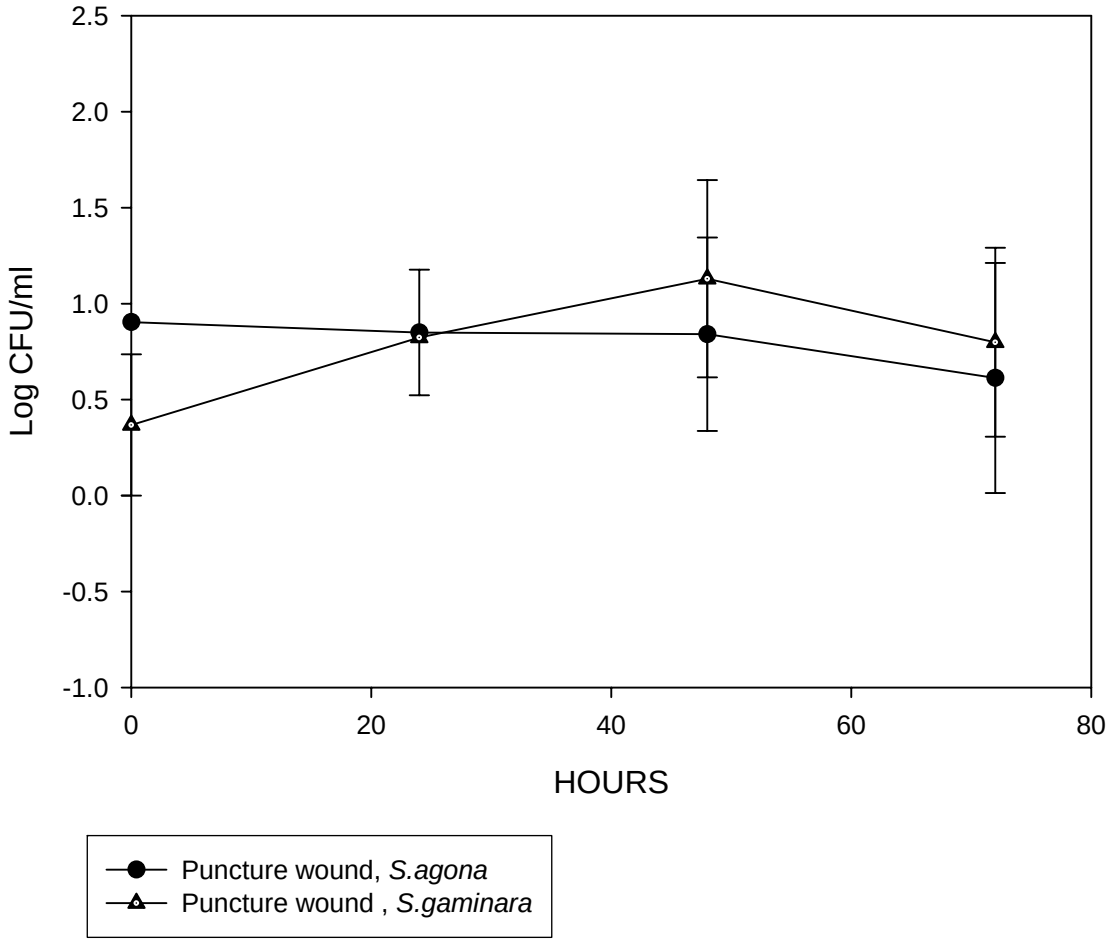

Figure 12: *Salmonella* persistence on surface scars

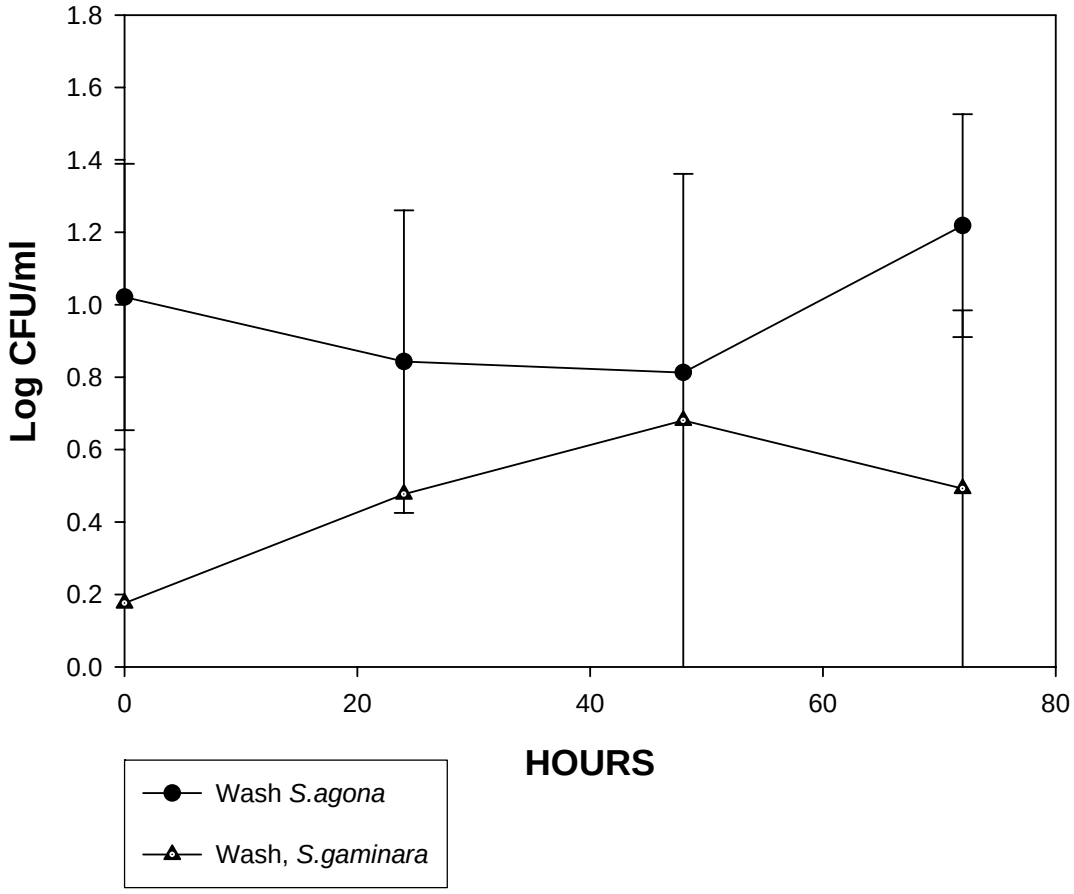

Figure 13: *Salmonella* persistence on orange surface

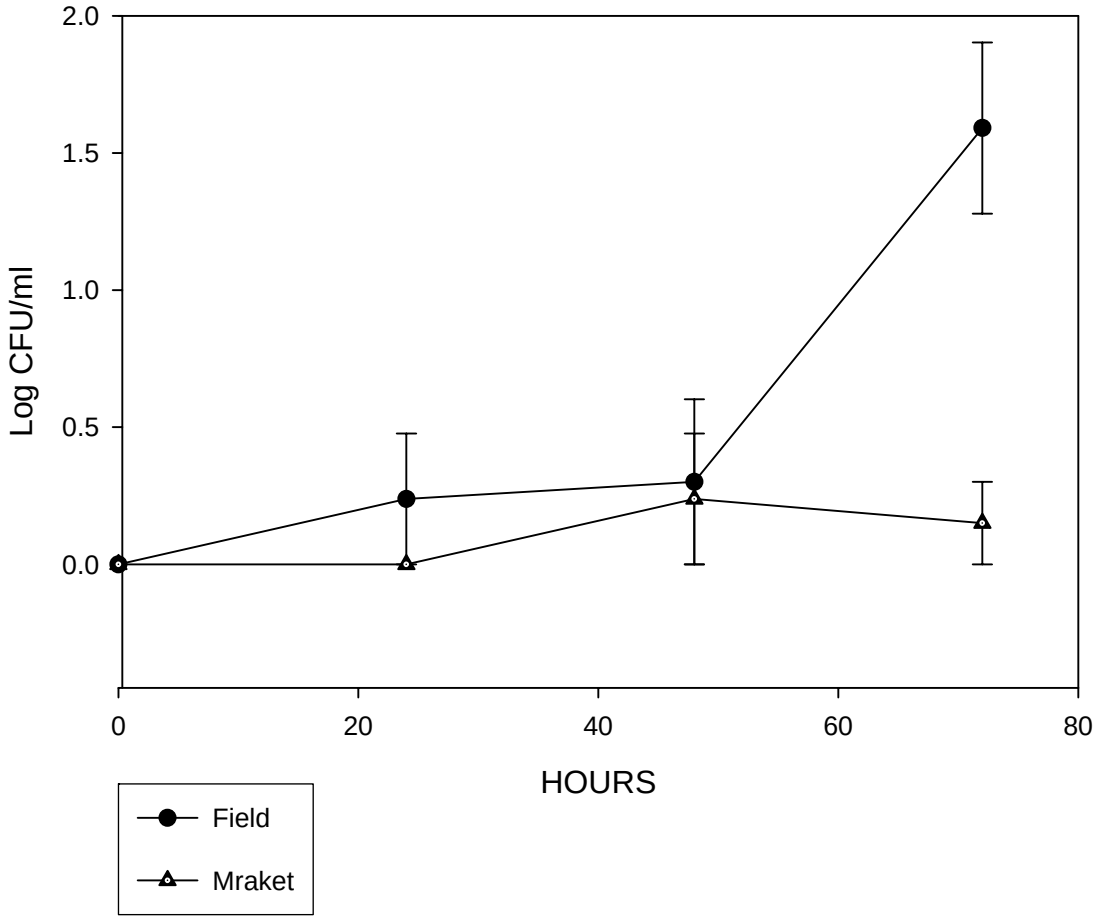

Figure 14: Internalization of *Salmonella*

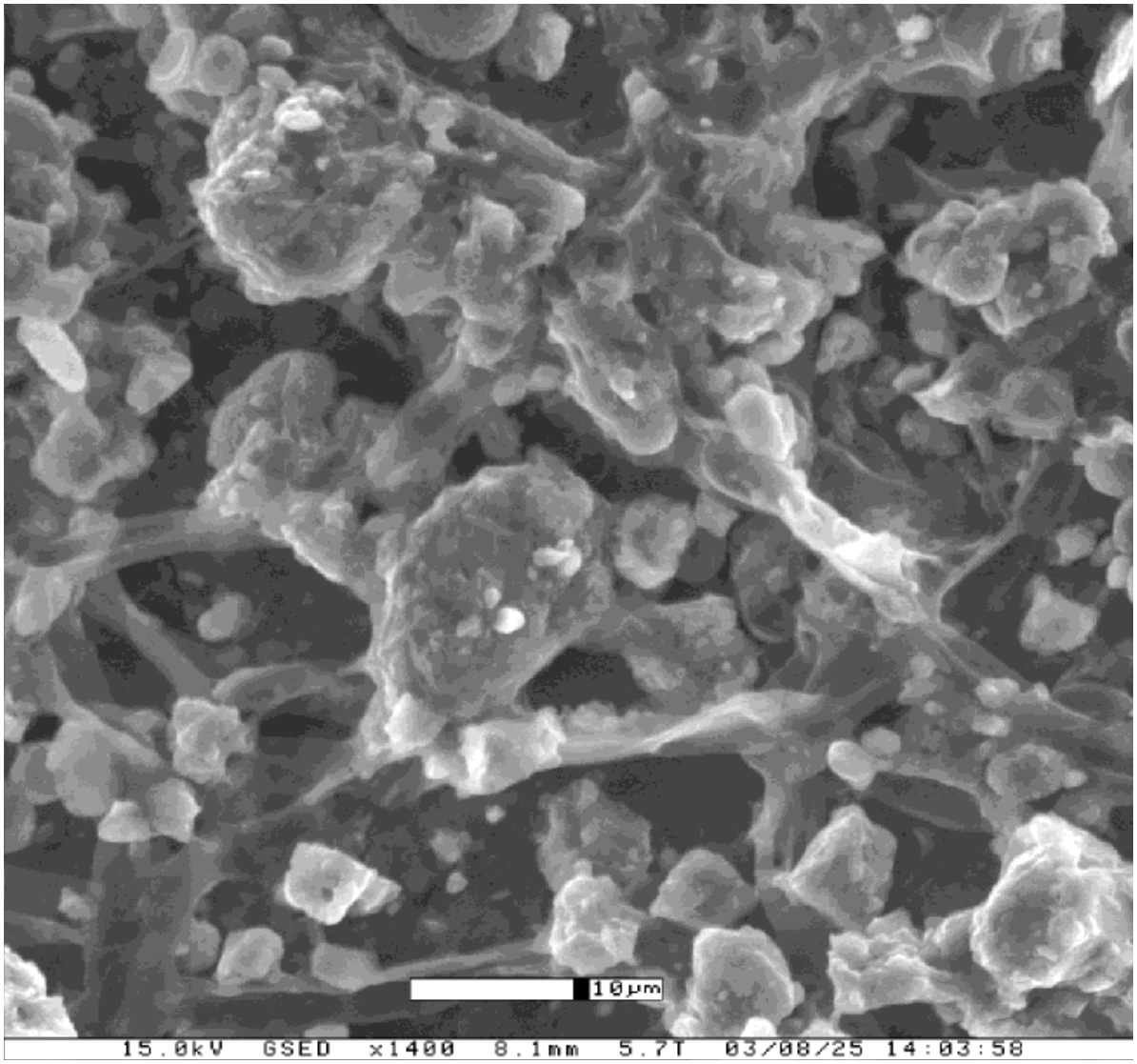

Fig 15: Electron microscopy picture of orange from field (10µm magnification)

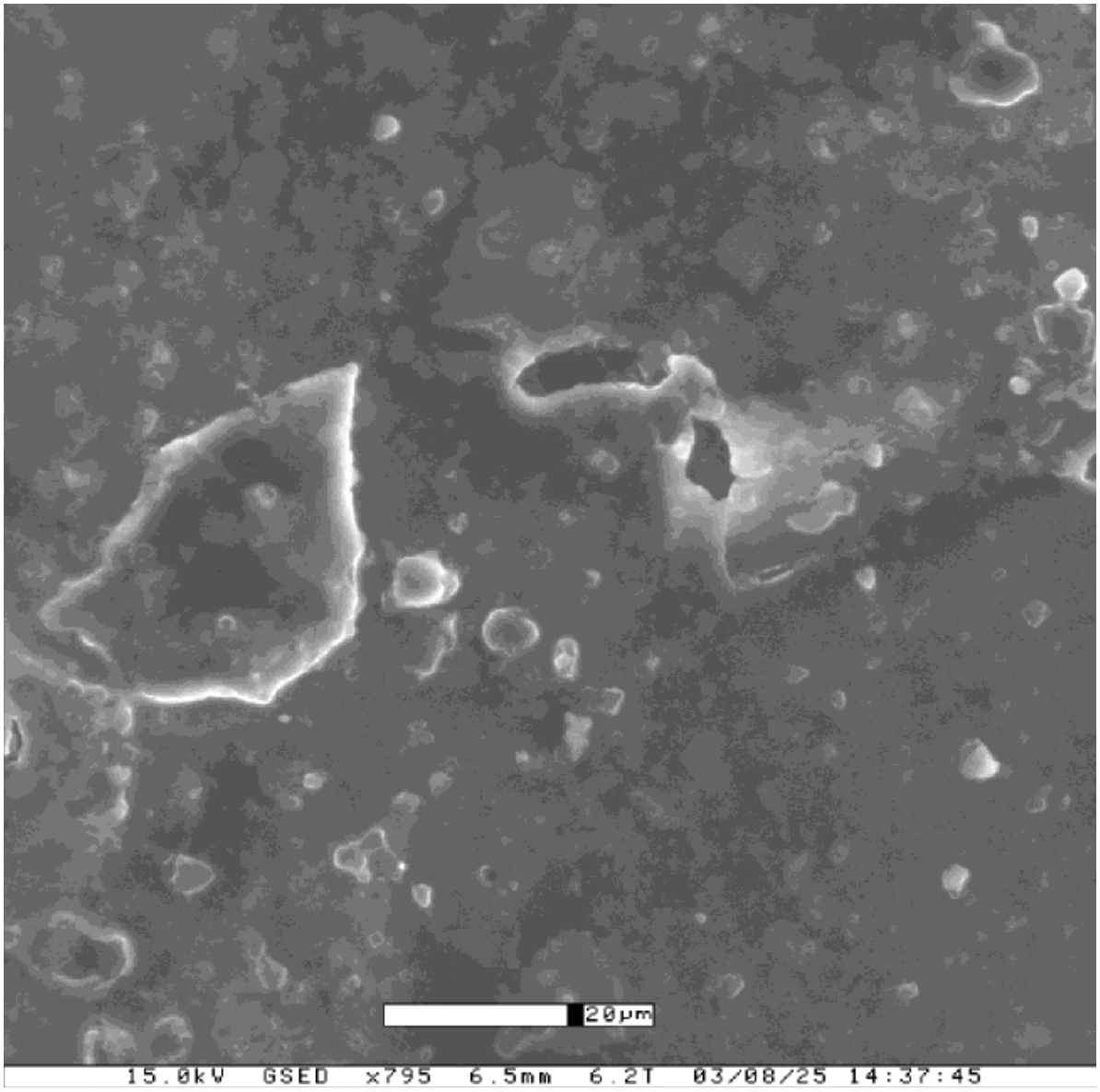

Fig 16: Electron microscopic picture of orange from market (20 µm magnification)

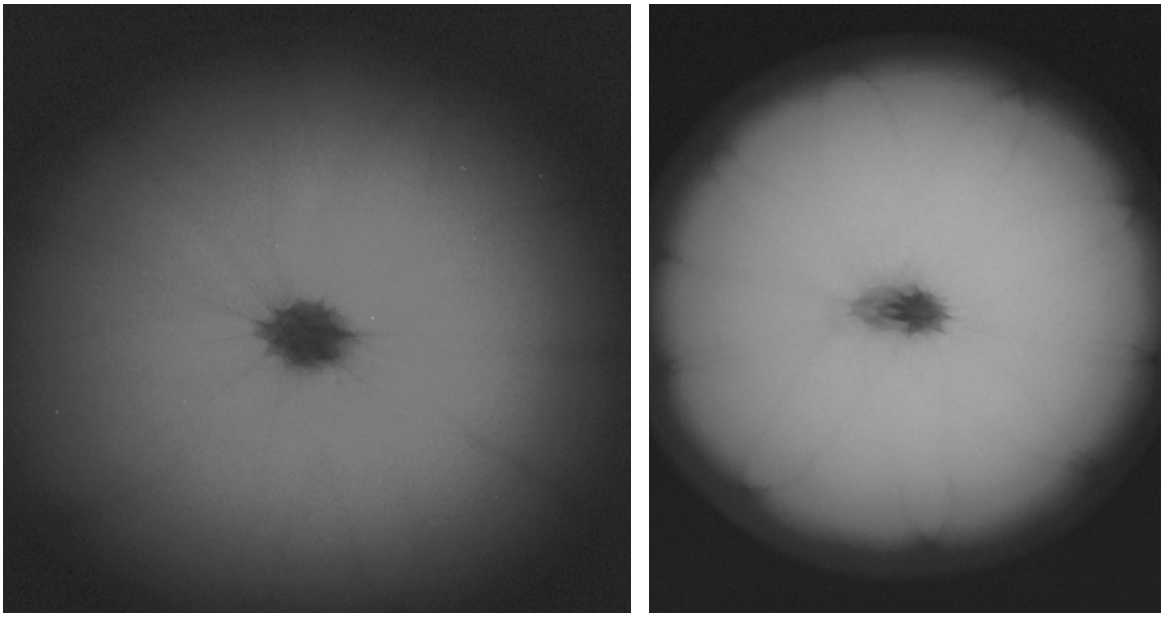

Fig 17 : Autoradiographic images of field and retail oranges (control)

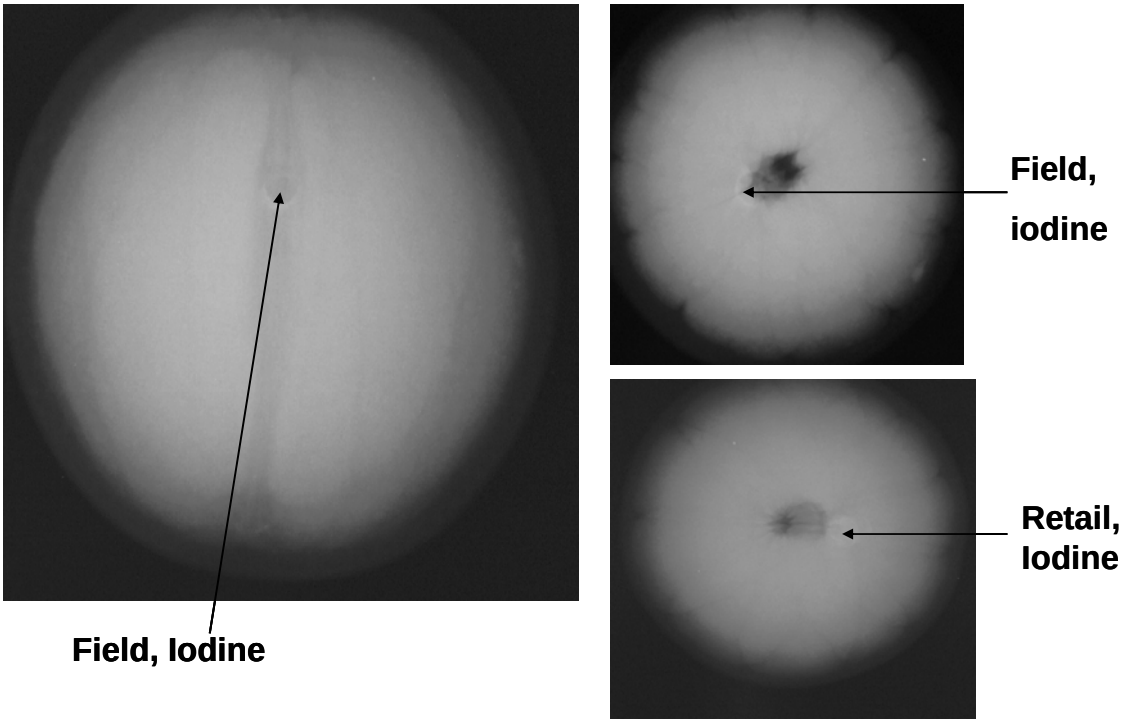

Fig 18 : Autoradiographic images of field and retail orange after 0hr of incubation

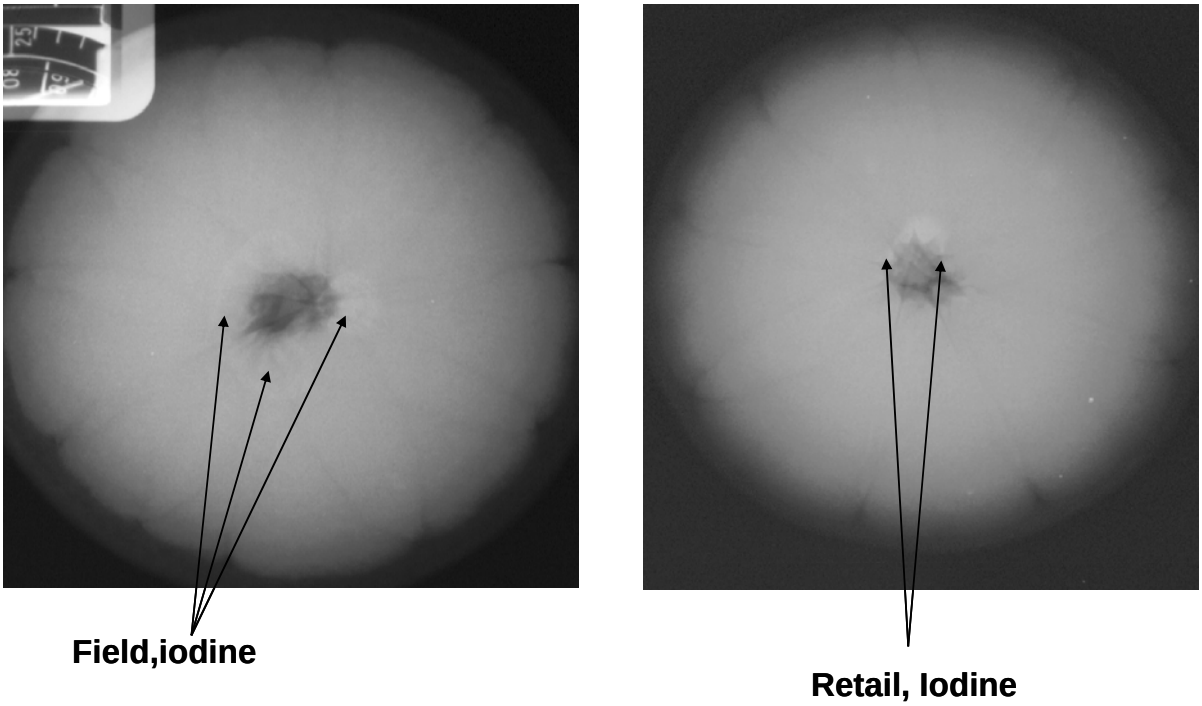

Fig 19 : Autoradiographic images of field and market after 24 hr incubation

The experiment using organic iodide confirmed that stem scar act the potential pathway for the internalization of the *Salmonella* (Figures 17-19). The experimental results obtained when Salmonella was used show that the *Salmonella* can internalize into the oranges and could survive in the oranges for 72 hours despite of the low pH of the oranges.

Water used for the washing of the fruits are contaminated by the pathogens and these act as the source of the internalization of the pathogens and the internalization takes place from the lenticels, stomata and the injured parts (Reina *et al.*,2002). The internalization of the *Salmonella* has been more through the stem scar as compared to the other part of the fruit (Penteado *et al.,* 2004). Internalization of the *Salmonella* into the oranges and then survival poses the dangers of the food- borne outbreak.

Internalization of the pathogens via the water that is used to disinfect the fruit have been reported (Penteado *et al.,* 2004). Studies done on the internalization of pathogens in tomatoes by Bratz *et al.* has shown that when the oranges were immersed in water which was kept at four different temperatures 2ºC, 10ºC, 29 ºC and 41ºC , the increase in the weight of the tomatoes was highest at the 2ºC . They showed in their experiment that the increase in the weight gain was highest when the tomatoes were immersed in the water that was 18 ºC cooler than the fruit. They report that when there is a negative temperature difference between the fruit and the water, internalization takes place from the lower temperature to the higher temperature (Bartz *et al.,* 1980). It was also stated in the experiment done by Bartz that when the fruit was treated with negative temperature differential gained more water as compared to the positive temperature differentials (Bartz *et al.,*1980). The internalization of the *Salmonella Gaminara* and *Salmonella agona* in the present study confirms these findings.

CHAPTER V

CONCLUSIONS

1. The results obtained show that *Salmonella agona* and *Salmonella gaminara* were able to survive on the surface of the oranges at room temperature and at 37º C, this could be the temperature of storage or the temperature when oranges are collected in the warm season and thus could be one of the possible reason for the outbreaks of *Salmonella* in orange juices. Contamination during transportation, storage and the ripening period could lead to prolonged survival of *Salmonella*
2. Outbreaks of *Salmonella* in orange juices indicate that *Salmonella* can survive the low pH of orange juices. Result obtained also indicates the % recovery of *Salmonella* was more at 7.0 pH as compared to 4.0 and 9.5 pH. Although the number of cells obtained after washing the surface with 4.0 pH buffers were less, but it indicates that *Salmonella* can survive the low pH and association of *Salmonella* with the orange surface could be one of the reasons for the attachment at low pH. Internalization of *Salmonella* appears to be controlled by the negative temperature differential. If the *Salmonella* is present on the surface of the oranges and the oranges are treated with cold water during the disinfection procedures, it could lead to the internalization of *Salmonella* into the oranges. Thus the procedures used in the packing houses could be one of the reasons for the outbreaks of the *Salmonella* in oranges.

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VITA

Reema Singh
Dept. of Poultry Sciences
Kleberg, Texas A&M University
College Station, TX 77843
Email- reemasingh_79@neo.tamu.edu

Education: TEXAS A&M UNIVERSITY, College Station, TX
M.S. Food Science and Technology *August 2004*

JAMIA MILLIA ISLAMIA, New Delhi, India
M.S. Bioscience *May 2002*

JAMIA MILLIA ISLAMIA, New Delhi, India
B.S. Bioscience *May 2000*

Experience:

August 2002-2004 **Graduate Research Assistant**
Texas A&M University. Department of Poultry Science. College Station, TX. Advisor: Dr. Suresh Pillai.