

**The prevalence and survival of *Campylobacter*, *Salmonella* and
Listeria species in poultry processing plant**

By

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Submitted in partial fulfillment of the requirements for the degree

Master of Science (MSc)



**UNIVERSITY *of the*
WESTERN CAPE**

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Except where reference is made to the work of others, this thesis is the result of my own original work. No part of this work has already been, or is currently being submitted for any other degree, diploma or other qualification.

Signature.....

Date.....23 January 2003.....

Acknowledgements

I would like to express my sincere gratitude and appreciation to the following people and organizations that contributed to this dissertation:

- I express my gratitude to Professor P.A. Gouws who acted as my supervisor, for his guidance and support throughout my years at the University of the Western Cape
- Mr. Norman Coldrey for his valuable advice on the culturing of *Campylobacter* species.
- I thank all the people who contributed to this work, their fruitful contributions are highly appreciated
- I am grateful to the National Research Foundation for the Financial support.
- I thank my mother Mrs. Makwarela Mabogo, my brothers and sisters, my uncle Mr. Samuel Mathelemusa , for their unwavering support.
- Lusani Sipholi for believing in me
- I express my gratitude to the rest of my family, friends and teachers.

This thesis is dedicated to all those good people whose voices have never been heard and to the loving memory of my father: Frans Mafanedza Mabogo.

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CHAPTER 1

1.1. Introduction

Foods constitute a variety of ecosystem in which microorganisms play important roles. Fruits, vegetables, dairy products and meats contain a variety of microorganisms on their surfaces. This is to be expected because microorganisms are ubiquitous and are especially plentiful in the soil and around animals. Anyone who prepares food has the responsibility of making sure that the numbers and kinds of microorganisms present at the time of its consumption are such that the food is both safe to eat and appetizing.

To ensure that the foods are safe to eat, they must be handled in such a way that the growth of pathogenic microorganisms is eliminated. Illness resulting from microbial growth in food arises chiefly by two mechanisms, the contaminating microorganisms may infect the person who ingests the food or products of microbial growth in the food may be poisonous. Bacteria of the genera *Salmonella*, *Listeria* and *Campylobacter* can be transported by poultry and poultry products to humans. Gastroenteritis, typhoid fever, diarrhea, dysentery may originate from the infection.

Bacteria do not exist as isolated cells, they grow and survive in organized communities in their natural environments. Microorganisms that attach and proliferate on solid surfaces are described as biofilms. Once attached to surface they appear more difficult to remove. Microbial biofilms are attracting attention of scientists in different areas such as the medical field, aquatic environment, food processing industries e.t.c. Microbial biofilms

may be detrimental and undesirable in food processing premises. Biofilms by pathogenic bacteria such as *Salmonella* (Dhir and Dodd 1995), *Pseudomonad* (Brown *et al.* 1995), *Campylobacter* and enteroharmorrhagic *E. coli* (Somers *et al.* 1994) and *Listeria* (Ren 1993) have been reported. Such biofilms could be a continuous source of contamination to foods coming into contact with them when formed on contact surfaces. Biofilm cells have showed increased resistance to antibacterial agents and sanitizers compared to their planktonic cells (Carpentier 1993).

The use of trisodium phosphate (TSP) as a processing aid to eliminate *Salmonella* on raw poultry carcasses was approved by the United States Department of Agriculture (USDA) in 1992 (Giese 1993). TSP has been shown to be active against Gram negative bacteria, including *Salmonella*, coliforms, *Escherichia coli* 0157: H7, *Campylobacter* and *Pseudomonas* on the skin of the chicken carcasses (Colin and Salvat 1997). The use of TSP for poultry meat decontamination is not widely practiced. Within the European Union meat hygiene regulations do not allow any method or product decontamination other than washing with potable water. In order that any processing decontamination method or agent is acceptable, the application can not cause undesirable sensory characteristics in the poultry products. It has been indicated that the treatment of poultry carcasses with 8% to 12% Trisodium phosphate have not any negative effect on the organoleptic quality of the meat (Ellebroek 1997, Hathcox 1995, Hollender 1995)

Even though trisodium phosphate has been shown to be effective against *Salmonella*, there is little published research on the effectiveness of TSP against *Campylobacter*, *Escherichia coli* 0157:H7 and *Listeria* planktonic (suspended) cells or biofilms (attached) cells of all four of these pathogens. It is also very important to know where these pathogens are found in high numbers along the poultry processing plant. This information can be used for their control.

This study was undertaken to achieve the following objectives.

- To determine the incidence of pathogens in poultry processing plant using PCR and conventional tests.
- Determine the formation and survival of biofilm cells of food pathogens in trisodium phosphate.

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CHAPTER 2

Literature review

2.1 INTRODUCTION

Every feed and animal related industry throughout the world has a responsibility to their customers to make every effort to reduce the incidence of chemical, physical and/or microbiological contamination of their products. If the poultry industry provides the consumer with an inferior product, then the consumption of poultry meat and eggs will decline. The mission of the poultry industry today is to produce and market safe and nutritious products for the consuming public.

Risk assessment is a tool used to evaluate the safety of food. Among the classes of hazards associated with food, microbial contamination of the food supply is among the most important. Risk assessment, however, is limited by a lack of data on the incidence of pathogens in foods (Henry 1997). Poultry and poultry products are a common vehicle of foodborne illness. Microbial risks associated with raw poultry products include *Salmonella* spp., *Campylobacter jejuni*, *C. coli* and *Listeria monocytogenes*. *Salmonella* usually causes outbreaks involving large numbers of people. Campylobacteriosis occurs as sporadic cases of illness instead of outbreaks. *Campylobacter* infections are increasing in Europe and other parts of the world (Neiman *et al.* 1998, Schmidt 1998) and are the most common source of diarrhea in England and Wales (Nylen 1998, Smith 1998) and in

the United States (Smith 1998). Eating undercooked chicken is one risk factor associated with sporadic campylobacteriosis (Neiman *et al.* 1998). Worldwide, salmonellae and campylobacters are by far the most important pathogens associated with poultry products (Bryan and Doyle 1995). Case control studies, however, suggest that undercooking raw poultry is involved in human listeriosis among individuals susceptible to the condition. Although sufficient heating will eliminate the above-mentioned pathogens, cross contamination can occur as a result of raw poultry in the kitchen.

With the recent interest and focus on Hazard Analysis and Critical Control Point (HACCP) for reduction of microbial contamination of meat and poultry, critical control point determination at broiler processing has become increasingly important (Rose *et al.* 1996). So it is very important to find where these pathogens and other bacteria are found in high numbers in the poultry processing plant, to make their control a little bit easier.

Biofilms can develop on wet food-processing surfaces that are not completely cleaned or not cleaned often enough. The presence of biofilm reduces the effectiveness of conventional sanitizers such as hypochlorite, quaternary ammonium compounds and acid anionic types. Trisodium phosphate (TSP) has been approved by the United States Department of Agriculture as a post chill antimicrobial treatment for raw poultry.

This chapter will look at the pathogenic bacteria found in the poultry processing environment, the biofilms in the poultry processing environment and lastly it will look at the use of trisodium phosphate in reducing bacterial pathogens.

2.2. Foodborne pathogens

2.2.1. *Campylobacter*

2.2.1.1. History

Awareness of the public health implications of *Campylobacter* infections has evolved over more than a century (Kist 1985). In 1886, Escherich observed organisms resembling campylobacters in stool samples of children with diarrhea. In 1913, McFaydean and Stockman identified campylobacters (called related *Vibrio*) in fetal tissues of aborted sheep (Kist 1985). In 1957, King described the isolation of related *Vibrio* from blood samples of children with diarrhea, and in 1972, clinical microbiologists in Belgium first isolated campylobacters from stool samples of patients with diarrhea (Kist 1985). The development of selective growth media in the 1970s permitted more laboratories to test stool specimens for *Campylobacter*. Some *Campylobacter* spp. were established as common human pathogens. *Campylobacter jejuni* infections are now the leading cause of bacterial gastroenteritis reported in the United States (Tauxe 1992). In 1996, 46% of laboratory-confirmed cases of bacterial gastroenteritis reported in the Centers for Disease Control and Prevention/U.S. Department of Agriculture/Food and Drug Administration Collaborating Sites Foodborne Disease Active Surveillance Network were caused by *Campylobacter* species. Campylobacteriosis was followed in prevalence by salmonellosis (28%), shigellosis (17%), and *Escherichia coli* O157 infection (5%).

2.2.1.2. Disease Prevalence

In the United States, an estimated 2.1 to 2.4 million cases of human campylobacteriosis (illnesses ranging from loose stools to dysentery) occur each year (Tauxe 1992).

Commonly reported symptoms of patients with laboratory-confirmed infections (a small subset of all cases) include diarrhea, fever, and abdominal cramping. In one study, approximately half of the patients with laboratory-confirmed campylobacteriosis reported a history of bloody diarrhea (Blaser *et al.* 1983). Less frequently, *C. jejuni* infections produce bacteremia, septic arthritis, and other extraintestinal symptoms (Peterson 1994).

The incidence of campylobacteriosis in HIV-infected patients is higher than in the general population. For example, in Los Angeles County between 1983 and 1987, the reported incidence of campylobacteriosis in patients with AIDS was 519 cases per 100,000 population, 39 times higher than the rate in complications of campylobacteriosis in HIV-infected patients are recurrent infection and infection with antimicrobial-resistant strains (Perlman *et al.* 1988). Deaths from *C. jejuni* infection are rare and occur primarily in infants, the elderly, and patients with underlying illnesses (Tauxe 1992).

2.2.1.3. Survival in the Environment

Survival of *Campylobacter* outside the gut is poor, and replication does not occur readily (Ketley 1997). *C. jejuni* grows best at 37°C to 42°C (Nachamkin 1995), the approximate body temperature of the chicken (41°C to 42°C). *Campylobacter* grows best in a low oxygen or microaerophilic environment, such as an atmosphere of 5% oxygen, 10%

carbon dioxide, and 85% Nitrogen. The organism is sensitive to freezing, drying, acidic conditions (pH < 5.0), and salinity.

2.2.1.4. Sample Collection and Transport

If possible, stool specimens should be chilled (not frozen) and submitted to a laboratory within 24 hours of collection. Storing specimens in deep, airtight containers minimizes exposure to oxygen and desiccation. If a specimen cannot be processed within 24 hours or is likely to contain small numbers of organisms, a rectal swab placed in a specimen transport medium (e.g., Cary-Blair) should be used (Nachamkin 1995).

Numerous procedures are available for recovering *Campylobacter* from clinical specimens (Nachamkin 1995). Direct plating is cost-effective for testing large numbers of specimens; however, testing sensitivity may be reduced. Pre-enrichment (raising the temperature from 36°C to 42°C over several hours), filtration, or both are used in some laboratories to improve recovery of stressed *Campylobacter* organisms from specimens (e.g., stored foods or swabs exposed to oxygen). Isolation can be facilitated by using selective media containing antimicrobial agents, oxygen quenching agents, or a low oxygen atmosphere, thus decreasing the number of colonies that must be screened (Nachamkin 1995).

The Cape Town protocol is the first to combine both membrane filtration and incubation in an H₂ enhanced microaerophilic atmosphere. The recovery rate of *Campylobacter* from

diarrhoeic stools at the Red Cross Children Hospitaal using the 'Cape Town Protocol' has been 22.4 %, since 1990. (Le Roux and Lastovica 1990)

2.2.1.5. Transmission to Humans

Most cases of human campylobacteriosis are sporadic. Outbreaks have different epidemiologic characteristics from sporadic infections. Many outbreaks occur during the spring and autumn (Tauxe 1992). Consumption of raw milk was implicated as the source of infection in 30 of the 80 outbreaks of human campylobacteriosis reported to CDC between 1973 and 1992. Outbreaks caused by drinking raw milk often involve farm visits (e.g., school field trips) during the temperate seasons. In contrast, sporadic *Campylobacter* isolates peak during the summer months.

A series of case-control studies identified some risk factors for sporadic campylobacteriosis, particularly handling raw poultry and eating undercooked poultry (Kapperud *et al.* 1992). Other risk factors accounting for a smaller proportion of sporadic illnesses include drinking untreated water, traveling abroad, eating barbequed pork or sausage (Kapperud *et al.* 1992), drinking raw milk or milk from bird-pecked bottles (Lighton *et al.* 1991); and contact with dogs (Kapperud *et al.* 1992) and cats, particularly juvenile pets or pets with diarrhea (Saaed *et al.* 1993).

2.2.1.6. Reservoirs

The ecology of *Campylobacter* involves wildlife reservoirs, particularly wild birds.

Species that carry *C. jejuni* include migratory birds, ducks, geese and seagulls (Glunder *et al.* 1992). The organism is also found in other wild and domestic bird species, as well as in rodents (Cabrita *et al.* 1992). Insects can carry the organism on their exoskeleton (Jacobs-Retsma *et al.* 1995). The intestines of poultry are easily colonized with *C. jejuni*. Day-old chicks can be colonized with as few as 35 organisms (Humphrey *et al.* 1993). Most chickens in commercial operations are colonized by 4 weeks (Humphrey *et al.* 1993, Kapperud 1993). Vertical transmission (i.e., from breeder flocks to progeny) has been suggested in one study but is not widely accepted (Pearson *et al.* 1996). Reservoirs in the poultry environment include beetles (Jacobs-Retsma *et al.* 1995), unchlorinated drinking water (Pearson *et al.* 1993), and farm workers (Humphrey *et al.* 1993, Kapperud *et al.* 1993, Kazwala *et al.* 1990). Feeds are an unlikely source of campylobacters since they are dry and campylobacters are sensitive to drying.

Campylobacter is a commensal organism of the intestinal tract of cattle. Young animals are more often colonized than older animals, and feedlot cattle are more likely than grazing animals to carry campylobacters (Giacoboni *et al.* 1993).

2.2.1.7. *Campylobacter* in the Food Supply

C. jejuni is found in many foods of animal origin. Surveys of raw agricultural products support epidemiologic evidence implicating poultry, meat, and raw milk as sources of human infection. Most retail chicken is contaminated with *C. jejuni*; one study reported an isolation rate of 98% for retail chicken meat (Stern and Line 1992). *C. jejuni* counts often exceed 10³ per 100 g. Skin and giblets have particularly high levels of contamination. In one study, 12% of raw milk samples from dairy farms in eastern Tennessee were contaminated with *C. jejuni* (Rohrbach *et al.* 1992). Raw milk is presumed to be contaminated by bovine feces; however, direct contamination of milk as a consequence of mastitis also occurs (Rohrbach *et al.* 1992).

2.2.1.8. Control of *Campylobacter* Infection at processing

Slaughter and processing provide opportunities for reducing *C. jejuni* counts on food-animal carcasses. Bacterial counts on carcasses can increase during slaughter and processing steps. In one study, up to a 1,000-fold increase in bacterial counts on carcasses was reported during transportation to slaughter (Stern *et al.* 1995). In studies of chickens (Izat *et al.* 1988) and turkeys (Acuff *et al.* 1986) at slaughter, bacterial counts increased by approximately 10- to 100-fold during defeathering and reached the highest level after evisceration. However, bacterial counts on carcasses decline during other slaughter and processing steps. In one study, forced-air chilling of swine carcasses caused a 100-fold

reduction in carcass contamination (Oosterom *et al.* 1995). In Texas turkey plants, scalding reduced carcass counts to near or below detectable levels (Acuff *et al.* 1986). Adding sodium chloride or trisodium phosphate to the chiller water in the presence of an electrical current reduced *C. jejuni* contamination of chiller water by 2 log₁₀ units (Li 1995). In a slaughter plant in England, use of chlorinated sprays and maintenance of clean working surfaces resulted in a 10- to 100-fold decrease in carcass contamination . In another study, lactic acid spraying of swine carcasses reduced counts by at least 50% to often undetectable levels (Epling *et al.* 1993). A radiation dose of 2.5 Kgy reduced *C. jejuni* levels on retail poultry by 10 log₁₀ units (Patterson 1995).

2.2.2 Salmonella

2.2.2.1 History

Salmonella typhi, the aetiological agent of the disease was discovered in 1880 by Eberth and isolated in 1884 by Gaffky, *S. cholerae-suis* (the type species) was isolated from swine clinically diagnosed as having hog cholera (Salmon and Smith 1885). The genus name was coined by Lignières in 1900 in honour of Dr Salmon's work. The first laboratory-confirmed outbreak of foodborne salmonellosis involved 57 persons who ate meat from a sick cow. *S. enteritidis* was isolated from the organs of a victim who had not survived and from the meat and blood of the animal. Since then, salmonellae have become recognized as a major cause of enteric fever and gastroenteritis.

2.2.2.2 General characteristics

Salmonella is a genus of the family Enterobacteriaceae (Brenner, 1984). Members of the family are characterized as Gram-negative, facultatively anaerobic, non-spore-forming, rod-shaped bacteria. Motile forms have peritrichous flagella. They produce acid and sometimes gas from glucose, are usually catalase positive and oxidase-negative and reduce nitrates to nitrites. Most members of this family are found in the intestinal tract of man and other animals as either pathogens or commensals.

Primary phenotypic characteristics of the genus are as follows (Le Minor 1984). Salmonellae are usually motile, but *S. pullorum* and *S. gallinarum* and variants of other serovars are non-motile. Most strains, with the exception of *S. typhi*, are aerogenic, use citrate as the sole carbon source, decarboxylate lysine, arginine and ornithine and produce hydrogen sulphide. The methyl-red reaction is positive, the Voges-Proskauer test is negative and indole is negative. Phenylalanine is not delaminated, urea is not hydrolysed, gelatin is not liquified rapidly in nutrient media and neither DNAase nor lipase are produced. The G + C content of DNA is 50-53 mole %. Salmonellae may harbour temperature phages or plasmids that code for metabolic characters used in identification (e.g. H₂S, lactose or sucrose fermentations).

2.3 Symptoms

2.2.2.3.1 Gastroenteritis

The incubation period ranges from 5h to 5 days, but signs and symptoms usually begin 12-36 h after ingestion of a contaminated food. The shorter incubation periods are usually associated with higher doses of the pathogen or highly susceptible persons. Signs and symptoms include diarrhea, nausea, abdominal pain, mild fever and chills. The diarrhea varies from a few, thin, vegetable soup-like stools to massive evacuations with accompanying dehydration. Sometimes vomiting, prostration, anorexia, headache and malaise occur. The syndrome usually lasts 2-5 days. The excreta of infected persons will contain large numbers of salmonellae at the onset of illness. The numbers decrease over time and few persons excrete non-typhi salmonellae after 3 months (Gomez 1997).

2.2.2.3.2. Enteric fever

The incubation period ranges from 7 to 28 days (depending primarily on dose); the averages 14 days. Malaise, headache, high persistent fever, abdominal pain, body aches and weakness occur, commonly with either pea-like diarrhea or constipation. Nausea, vomiting, cough, perspiration, chills and anorexia may occur. Rose spots sometimes appear on trunk, back and chest. A slow heart rate, a tender and distended abdomen, enlarged spleen, and sometimes bleeding from the bowel or nose are observed. The senses are dulled and patients may become delirious. Relapses sometimes occur.

Convalescence is slow (1-8 weeks). The carrier state may be prolonged for several months and extend into years. Long-term human carriers typically harbour the organism in the gall bladder (Muller *et al.* 1995).

2.2.2.3.3. Bacteraemia, focal infections and sequelae

Bacteraemia or septicaemia is caused by the presence of salmonellae in the blood. This may occur metastatically when the initial site of infection is the intestinal tract or other foci. The result is a high, persistent fever, pain in the back abdomen and chest, chills, perspiration, malaise, anorexia and weight loss. The condition may be transient or chronic. Strains of *S. typhimurium*, *S. cholerae-suis* and *S. dublin* are liable to invade the bloodstream and focal infections of various tissues may follow. Although uncommon, identified sequelae include: appendicitis, arthritis, cholecystitis, endocarditis, local abscesses, meningitis, osteomyelitis, osteoarthritis, pericarditis, peritonitis, pleurisy, pneumonia and urinary tract infection (Smith *et al.* 1993).

2.2.2.4 Reservoirs

All *salmonella* are now recognized as belonging to 1 of 2 species. *Salmonella enterica* and *S. bongori*. *S. enterica* is further divided into 6 subspecies, within which more than 2000 serotypes have been identified. Nearly all of the Salmonellae isolated from humans belong to *S. enterica* subsp. *enterica*. The serotypes *typhi* and *paratyphi* colonize only humans and may cause enteric fever or a chronic carrier state (Popoff and Minor 1997).

The main reservoirs hosts of non typhoidal *Salmonella* are animals, including poultry, other fowl, livestock, reptiles and pets (Miller *et al.* 1995, Salmonellosis 1995). Infection in humans frequently occurs through consumption of poultry, eggs, red meat, unpasteurized milk and dairy products (Gomez and Clearly 1997, American Academy of Pediatrics 1997).

Infection has also been associated with consumption of alfalfa sprouts, unpasteurized orange juice, cantaloup, tomatoes and other fresh produce (Gomez and Clearly 1997, Miller *et al.* 1995).

2.2.2.5 *Salmonella* contamination of broiler carcasses

Salmonella contamination of uncooked poultry carcasses and cut up parts continues to be of great concern to the industry because some of these microorganisms are pathogenic and can cause food borne illnesses. In addition the USDA has established Pathogen Reduction Performance Standards of acceptable levels of *Salmonella* on raw poultry that all establishments must meet in order to remain under operation. It is generally thought that *Salmonella* contamination of carcasses during processing originates from bacteria that have colonized the birds ceca or intestinal tract. Another potential, but the frequently overlooked source of contamination results from the bacteria in the bird's crop, which may be spread throughout the body cavity during processing. Crops may become contaminated during the feed withdrawal period prior to processing. During this feed

withdrawal period, birds will consume anything available, including litter and feces which may harbor large numbers of bacteria (Hargis *et al.* 1995).

Research has shown that crops are not only more likely than ceca to be contaminated with *Salmonella*, but they are also more likely than ceca to be raptured during processing (rapture rate of 26 % crops versus 0.3 % ceca) (Hargis *et al.* 1995).

Another research has shown that the length of time the broilers are held without feed before processing may affect *Salmonella* levels in the crop and in the ceca. Research has shown that crops from full fed broilers have low pH (5.3), which minimizes the growth of pathogenic bacteria such as *Salmonella*. Length of feed withdrawal did not change the bacterial levels in the crop, but as the length of feed withdrawal is increased, the crop's environment becomes more favorable for growth of bacteria. After 6 hours of feed withdrawal, crop pH increased to 6.6, which is more conducive for growth of pathogenic bacteria. Ceca from broilers held off feed 12 hours instead of 6 hours had over 100 times more pathogenic bacteria. Bacteria counts in the ceca continued to increase when feed withdrawal times exceeded 12 hours (Hinton *et al.* 1998).

2.2.2.6 Detection and enumeration

Routine detection of salmonellae involves a sequence of pre-enrichment, selective-differential plating, isolation and identification. Pre-enrichment may or may not be done, depending on the degree of likelihood that injured cells are present in the food. It is used

when testing heated, frozen and dried foods, and foods in which cells are expected to be present in low numbers. It reduces any effect the food itself may have on the selectivity of the enrichment broth. Pre-enrichment liquid media (e.g. buffered peptone, nutrient or lactose broths) are non-inhibitory and permit the growth of indigenous flora as well as salmonellae. The media or procedure may be modified for various purposes. Sometime additions are made to the broth to overcome inhibitory materials that may be present in certain foods such as milk powder for cocoa (Poelma *et al.* 1984, Ewing 1986; Silliker and Gabis 1986).

Selective enrichment is designed to inhibit the growth of other organisms while allowing salmonellae to grow. This is accomplished by the use of inhibitory chemicals (e.g. dyes, tetrathionate, selenite), temperature and duration of incubation, permitting the proportion of salmonellae relative to other microorganisms to increase so that they can be recognized on selective-differential plating media or by other identification techniques. Incubation times are usually 16-24 h. Normally, incubation temperatures range from 35 to 43°; incubation at 41 - 43°C often results in increased detection of salmonellae. Commonly used selective broths include tetrathionate with brilliant green, selenite with cystine, Gram-negative (GN) broth and the magnesium chloride-malachite green of Rappaport-Vassiliadis (Vassiliadis 1983). Multiple enrichment media and time and temperature incubation routines are recommended.

The presence of *Salmonella* is determined by plating samples of enrichment broths on selective plating media. Bile salts, deoxycholate, brilliant green, bismuth sulphite and antibiotics are used as selective agents. Differentiation of salmonellae from other

organisms is usually achieved by the colour changes exhibited by pH-indication dyes responding to sucrose or lactose fermentation, but it also relies on the production of H₂S or decarboxylation of lysine. Commonly used selective plating media include brilliant green with or without sulphadiazine or sulphapyridine, xylose lysine deoxycholate, bismuth sulphite, Hektoen enteric agar, MacConkey, deoxycholate citrate and *Salmonella-Shigella* agars. Two or more of these should be used in standard cultural methods in view of the physiological diversity of the *Salmonella* group and the mixed flora present in foods. Salmonellae are enumerated by the most probable number technique. This is a cumbersome procedure and too expensive for use in the examination in a large number of samples. The hydrophobic grid membrane method may be used to enumerate salmonellae in some foods (Warburton *et al.* 1994).

2.2.2.7 Prevention of foodborne Salmonellosis

The prevention of foodborne salmonellosis depends primarily on the careful handling of raw products and finished foods. Contamination of foods may occur at any point from the farm to the table (Slutsker *et al.* 1998). Each stage in the production, storage, processing, distribution and preparation of food may serve as a hazard or as an opportunity for prevention.

In the past, foodborne outbreaks were often local occurrences, affecting people who attended the same social event or who ate at the same restaurant (Slutsker *et al.* 1998).

Today, evidence suggests that outbreaks increasingly cross provincial, territorial and national borders (Tauxe 1996).

Prevention and control of salmonellosis will require enhanced public health surveillance, which includes diagnosis and prompt reporting cases, characterization of isolates and investigation of the sources of infection (Tauxe 1996). Efforts are under way at the local, national and international levels to improve the capacity, coordination and cooperation of public and private laboratories, physicians, hospitals and public health officials (Slutsker *et al.* 1998, Tauxe 1996).

2.2.3 *Listeria*

2.2.3.1 History

The first complete description of this bacterium dates back more than 60 years, when researchers isolated a short, Gram-positive, non-sporing, rod-shaped bacterium causing disease in rabbits and guinea-pigs (Murray *et al.* 1926). The authors named it *Bacterium monocytogenes* because it infected the monocytes (whiter cells) in the blood. Earlier reports of organisms resembling *Listeria monocytogenes* that caused infection in humans and animals date back to the late nineteenth century and are comprehensively reviewed by other researchers (Gray and Killinger 1966). Pirie isolated a similar organism from the livers of sick gerbils in 1930 and called it *Listerella hepatolytica*, after the famous

surgeon Joseph Lister. Since the name ‘Listerella’ had previously been adopted for a group of slime moulds, the name *Listeria monocytogenes* was finally agreed (Pirie 1940).

Infections due to this bacterium have been reported in a wide variety of animals, including cattle, sheep, birds, rodents and fish (Gray and Killinger 1966) as well as in humans. Contaminated animal feed, especially poorly made silage was recognized as a cause of listeriosis, but the existence of a similar cause of human infection was not widely appreciated until the early 1980s, despite a large outbreak in Germany in 1949-51 linked to the consumption of raw milk (Seeliger 1961; Gray and Killinger, 1966).

2.2.3.2 Taxonomy

Listeriae are Gram-positive, short, non-sporing rods, catalase-positive and facultatively anaerobic. They occasionally give rise to coccoid forms of individual cells 10µm in length. They are motile at 25 degrees celsius, showing a characteristic ‘tumbling’ motility, but non-motile at 35 degrees celsius. Colonies have a characteristic bluish-grey appearance, which changes to blue-green when viewed by oblique light (‘Henry illumination’) (Henry 1933). Seven species of *Listeria* were recognized, all closely related. An eighth species, previously named *Listeria denitrificans*, has been reclassified *Jonesia denitrificans*, *L. innocua* and *L. murrayi* (syn. *L. grayi*) are considered non-pathogenic, while *L. seeligeri*, *L. ivanovii* and *L. welshimeri* rarely cause human infection, leaving *monocytogenes* as the most important species.

2.2.3.3 Listeriosis: a typical foodborne disease

Listeriosis is an atypical foodborne disease that most frequently affects pregnant women, new-born infants and children and adults whose immune systems are weakened.

2.2.3.3.1. Severity of symptoms

Listeriosis in pregnancy occurs most frequently in the third trimester. The infection of the mother may be asymptomatic or characterized by a flu-like illness with fever, myalgia or headache (infections of the central nervous system are very rare in pregnant women). It may, however, have more serious consequences for the infant, including spontaneous abortion, foetal death, stillbirth, severe neonatal septicemia and meningitis (Fredriksenn and Samuelsoon 1992). Histories of recurrent foetal loss due to *L. monocytogenes* have been reported in the literature but have never been rigorously documented.

In non-pregnant adults, *L. monocytogenes* has a particular tropism for the central nervous system and meningeal and/or brain parenchymal infections are thus frequent in cases of listeriosis. Gastro-enteritis symptoms (vomiting and diarrhea) during the week before the disease is diagnosed are observed in some cases. Other commonly recognized forms of listeriosis include bacteremia. Infections of the central nervous system are more often diagnosed in patients with no underlying diseases, while bacteremia is more frequent in patients who are severely immunodepressed (Skogberg 1992 and Goulet 1993). While very rarely mentioned in the literature until recently, cases of mild gastrointestinal illness

following the ingestion of food contaminated with *L. monocytogenes* have been documented (Mitchel 1991, Riedo 1994, Dalton 1997 and Schleich 1997). Focal infections, including endocarditis, septic arthritis, osteomyelitis and peritonitis, are rare and usually preceded by septicemia). Rare cases of recurrent listeriosis in adults have been observed and typing of strains isolated during sequential episodes strongly suggest reinfection by the same strain. However, to date, no anatomical site(s) colonized by *L. monocytogenes* for long periods have been identified (McLauchlin *et al.* 1991a).

Sequelae due to invasive listeriosis have been reported sporadically, but their incidence is rarely estimated. Nevertheless, a study in Bristol indicated that a third of the pregnancy-related cases were left with residual symptoms (Jones *et al.* 1994). A report of the cases observed during a Swiss outbreak mentioned that a high proportion of survivors of central nervous system infection suffered from neurological sequelae (Büla *et al.* 1995).

The pathogenesis of human listeriosis is still poorly understood. Two to 6% of healthy individuals are reported to be asymptomatic faecal carriers of *L. monocytogenes* (MacGowan 1991, Mascola 1992 and Schuchat 1992). Association between gastrointestinal symptoms and *L. monocytogenes* carriage has been evidenced rarely and the risk of clinical disease in those harbouring the bacterium in the gut is still unknown. Endogenous infection by *L. monocytogenes* in the gut is plausible, especially in patients receiving immunosuppressive therapy which not only impairs resistance to infection but can also result in alterations of intestinal defense mechanisms favouring listerial invasion.

2.2.3.3.2 High case fatality rate

Infection of pregnant women in the early stages usually leads to spontaneous abortion. Overall lethality is usually around 20-30% for both epidemic and sporadic cases (Gellin 1991, Jurado 1993, Jones 1994 and Goulet 1993). Higher rates have been published, for instance 51% in Barcelona in 1990 (Nolla-Salas *et al.* 1993). Lethality in immunocompromised or elderly patients and among patients suffering from central nervous system infections are higher, at 38-45% (Skogberg 1992).

2.2.3.3.3 A predilection for immunocompromised people

L. monocytogenes more commonly infects individuals with depressed cell-mediated immunity. Populations at risk include pregnant women and neonates, adults with underlying disease (including cancer patients, organ transplantation recipients, people with AIDS, chronic hepatic disorder, diabetes) and the elderly. French data show that patients at higher risk among non-pregnancy related cases are organ-transplantation recipients (200 cases/100,000 organ-transplantation recipients); patients suffering from cancer (13 cases/100,000 patients with cancer) and individuals aged more than 65 years without known underlying diseases (1.4 cases/100,000) (Rebière and Goulet, 1993). A two year study in Atlanta indicates that the incidences of listeriosis among HIV-infected patients and among patients with AIDS are 52 and 115 cases per 100,000 patients, respectively (Jurado *et al.* 1993). Thus, the various populations in decreasing order of

risk are: organ-transplantation recipients, patients with AIDS, HIV-infected patients, patients with cancer and the elderly.

The proportion of patients suffering from a known predisposing disease varies greatly between studies, accounting for 50-66% of cases in some surveys (Skogberg 1992, Nolla Salas 1993, Jensen 1993) to nearly all cases in others (Schuchat *et al.* 1992).

2.2.3.3.4 A limited geographical distribution

Listeriosis is mainly reported from industrialized countries with few or no reports from Africa, Asia and South America. Whether this reflects different consumption patterns, dietary habits, different host susceptibility, or lack of testing facilities is not known (Rocourt 1991, Rocourt and Brosct 1992, and Rocourt *et al.* 1997).

2.2.3.4 *Listeria* on poultry

In recent years, fully cooked, ready to eat products containing poultry meat have been implicated in outbreaks of listeriosis. The poultry industry has recognized the importance of this organism and has recently been proactive in efforts to prevent or reduce product contamination (Berrang *et al.* 2000)

Listeria, including *Listeria monocytogenes* may be isolated from raw poultry products purchased at retail outlets (Cox *et al.* 1997). *Listeria* has also been isolated from raw

poultry carcasses and meat processing plants (Cox *et al.* 1997, Franco *et al.* 1995, Lawrence and Gilmour 1994).

Aside from the possibility of illness due to consumer mishandling of raw poultry, another concern with this pathogen is that it may be carried into a further processing facility where raw product is cooked, making subsequent cross contamination possible. This situation was demonstrated by a study with reported raw turkey necks and breasts among the principal sources of listeriae for a further processing plant (Samelis and Metaxopoulos 1999).

2.2.3.5 The sources of listeriae in poultry slaughter

The sources of listeriae in poultry slaughter establishments are not entirely clear. A low prevalence or complete lack of *Listeria* has been noted in samples drawn from early stages of slaughter and processing (Clouser *et al.* 1995). However it has been reported that a small percentage (0 to 1.3 %) of birds can carry this organism into the plant (Cox *et al.* 1997). Moreover, *Listeria* has been isolated from a low percentage (1 to 6 %) of samples in broiler hatching facilities, a situation which could lead to contaminated broilers (Cox *et al.* 1997).

In reality, the sources of listeriae in a poultry processing plant are probably numerous and varied. Once in the facility, this pathogen can become resident and may be able to survive sanitation procedures (Ojeniyi *et al.* 1996, Fenlon *et al.* 1996).

In general, listeriae become more prevalent on processing equipments and carcasses as they progress through the plant (Cox *et al.* 1997, Franco *et al.* 1995, Clouser *et al.* 1995). Researchers reported an increase in incidence from pre scald carcasses to carcasses after immersion chilling (Cox *et al.* 1997).

2.2.3.6 Investigation of the epidemiology of human listeriosis

Most industrialized countries have developed surveillance systems for listeriosis since 1987 following outbreaks that demonstrated the importance of the foodborne transmission of the disease. The main goals of foodborne disease surveillance by the World Health Organisation (Borgdoff and Motarjemi 1997) are: to evaluate the annual incidence, to identify populations at risk, to determine foods at risk and their source of contamination, to detect outbreaks as soon as they emerge, to monitor foodborne transmission, to identify measures aimed at reducing mortality and morbidity by listeriosis, to evaluate the impact of preventive measures.

2.2.3.7 Control of human listeriosis

The prevention of human listeriosis begins at the farm and continues through processing to the selection and handling of foods by the consumer. *Listeria* is ubiquitous in the agricultural environment and a complete normal diet that is totally free of *Listeria* is therefore impossible to obtain. However, the application of controls can reduce the risk

of foodborne listeriosis. This is a classical case whereby HACCP should be applied from farm to consumer to minimize the risk of foodborne illness.

2.2.3.7.1 Processing

Foods have been placed into four categories (WHO, 1988):

Raw foods (e.g. raw vegetables and meats), processed raw foods not treated listericidally by heating (e.g. coleslaw, fermented sausages, raw-milk cheeses), processed foods treated listericidally by heating but subjected to potential recontamination during subsequent handling (e.g. cheeses and commercially processed meats that are sliced or altered after thermal processing), processed foods treated listericidally by heating while in an intact package (e.g. cooked ham) or which are aseptically packaged immediately after listericidal treatment (e.g. certain dairy products).

Particularly emphasis in control should be placed upon foods identified as of concern through outbreak investigation, prospective epidemiological studies and data demonstrating the multiplication of *L. monocytogenes*. Attention should be given to the conditions of, for example, producing soft cheeses and pâté and to the maintaining of good hygiene during the slicing of meat products (NACMCF 1991).

Processing plants should base their control programme on the HACCP concept. Three major objectives must be pursued. The first is to minimize the multiplication of *L.*

monocytogenes in raw materials, particularly before and during the processing of raw foods (Category 2 above). The second is to use listericidal processes that assure the destruction of *L. monocytogenes* (Categories 3 and 4 above). The third is to minimize the risk of recontamination of ready-to-eat foods that are further processed after receiving listericidal treatment (Category 3 above). Owing to the prevalence of *L. monocytogenes* in raw materials and its ability to multiply in the environment of many food-processing facilities, traditional cleaning and disinfection methods, equipment design and management practices may be inadequate or even impair the control of *L. monocytogenes* (WHO 1988).

2.2.3.7.2. Consumers

The normal population is relatively resistant to infection by the strains of *L. monocytogenes* most commonly found in food. The risk of foodborne listeriosis is much greater among persons with reduced immunity (e.g. pregnant women, persons with malignant disease or AIDS) and patients with certain underlying illnesses (e.g. heart disease, diabetes, renal disease) (Schuchat *et al.*, 1991). The following guidelines are proposed for the selection and handling of foods by these individuals: Avoid eating raw or improperly cooked foods of animal origin (e.g. undercooked poultry).

- Avoid cross-contamination between raw and cooked foods during preparation and storage.
- Reheat leftovers until too hot to touch. Microwave-heated foods should be given

sufficient time for the heat to equilibrate throughout the food before eating.

- Avoid pate and ripened soft cheeses (e.g. Camembert, Brie, red smear cheese) and non-ripened cheeses (e.g. Mexican-style cheeses). Hard cheeses, fresh cheese (e.g. cottage cheese) and processed cheese (e.g. Philadelphia-style cheese) can be eaten without concern for the risk of listeriosis.
- Raw vegetables should be thoroughly washed before eating.
- Maintain a clean refrigerator.
- Store perishable foods in the coldest area of the refrigerator, preferably at or below 5 degrees celsius.
- Avoid keeping perishable foods in the refrigerator for more than 1-3 days.

Prevention and control of foodborne listeriosis, with particular reference to the dairy, meat and seafood industries, is reviewed (Farber 1992). A rationale to assist deciding whether sampling plans and microbiological criteria are appropriate has been proposed (ICMSF 1994).

2.3 Biofilms associated with poultry processing equipment

2.3.1 Introduction

Adhesion to solid surfaces forms an integral part of the physiology of many microbes (Koutzayiotis 1992, Marshall 1992 and Gilbert *et al.* 1993). A biofilm can therefore be described as a collection of attached microorganisms, actively dividing and producing

protective organic polymers which entrap other microbes and nutrients (Koutzayiotis 1992). These polymers protect the attached bacteria from protozoan predators, desiccation, mechanical damage, antibiotics and sanitizers (Mosteller and Bishop 1993).

2.3.2 Biofilm formation

Biofilm formation occurs by a step wise process, which includes conditioning of a solid surface by organic and inorganic nutrients, attachment of primary colonizing microorganisms distinguished in the planktonic population for their ability to adhere to the particular surface, growth and metabolism of attached cells and subsequent biofilm formation, and cell detachment from the biofilm surface (Notermans *et al.* 1991, Koutzayiotis 1992, Costerton *et al.* 1994 and Zotolla 1994).

Individual cells within mature multispecies biofilms live in unique microniches where nutrients are provided by neighbouring cells and by diffusion, and wastes are removed by the same process (Costerton *et al.* 1994).

2.3.3. Disadvantages of biofilms in food processing plant

Microbial cells in biofilms are reportedly more resistant to sanitizers and processing treatments used in the food industries, such as heat (Ronner and Wong 1993, Helke and Wong 1994, Zotolla 1994). Biocide and sanitizer resistance is thought to result from the protection afforded by organic soils and glycocalyx polysaccharides covering the

bacterial cells, either preventing sanitizers from entering the biofilm or by inactivation of the sanitizer, respectively (Notermans *et al.* 1991, Zottola and Sasahara 1994, Zottola 1994).

Biofilm formation on equipment surfaces associated with poultry processing is undesirable since the attachment of spoilage (e.g. *Pseudomonas*) or pathogenic (e.g. *Listeria monocytogenes* and *Salmonella enteritidis*) bacteria to metallic and non metallic equipment surfaces in the abattoir, could result in contamination of carcasses and subsequent economic losses (Notermans *et al.* 1991, Helke *et al.* 1993, Criado *et al.* 1994, Helke and Wong 1994, Zottola 1994).

In vitro development of biofilms on many materials including glass, stainless steel, rubber, cast iron and plastics has been observed (Ronner and Wong 1993) under simulated food processing conditions (Zottola 1994).

In situ biofilm development has been found on food contact equipment associated with meat processing in the United States of America (Zottola 1994) and rubber fingered pluckers in a poultry abattoirs in Great Britain (Notermans *et al.* 1991).

2.4 Methods of reducing bacterial contamination

2.4.1 Introduction

Raw meat, particularly poultry meat, remain an important, and probably the major source of human infection with campylobacters and salmonellas. In spite of decades of effort it has so far proved extremely difficult to raise food animals free of these pathogens. For the foreseeable future, therefore, the most effective approach must be to decontaminate the final raw product. In this way numbers of these pathogens entering kitchens and commercial food processing premises will be reduced substantially, and hence opportunities for cross contamination onto ready to eat foods or for survival during cooking or other will be much lower (Corry *et al.* 1995).

The ideal method of decontamination will method of decontamination will have the following attributes, It will not change appearance, smell, taste or nutritional properties, it will leave no residues, it will pose no threat to the environment, it will encounter no objections from consumers or legislators, it will be cheap and convenient to apply, it will improve the shelf life by inactivating spoilage organisms as well as pathogens (Corry *et al.* 1995).

Numerous disinfectants and treatments have been applied so as to reduce carcass contamination including: acids, bases, halogens, hydrogen peroxide, alcohols, mannose application, ozonation and irradiation. Although all these methods have shown some

degree of bactericidal, each one offers some significant disadvantage(s), such as the cost or adverse sensory changes that have prevented their usage (Bolder 1997, Farkas 1998, Smulders and Greer 1998, Sofos and Smith 1998).

2.4.2 The reduction of pathogens with trisodium phosphate

Trisodium phosphate (TSP) has been approved for use as a food ingredient (Federal Register 1994) and for poultry processing (Anonymous 1993, Federal Register 1994). At 8 to 15 % wt/vol (0.2 to 0.4), it has been demonstrated to kill gram negative organisms artificially inoculated on surfaces of a variety of foodstuffs (Dickson 1994, Hwang 1995, Kim and Slavik 1994, Kim *et al.* 1994, Slavik *et al.* 1994 and Zhuang and Beuchat 1996) and *Listeria monocytogenes* on stainless steel (Somers *et al.* 1992).

2.4.3 Modes of action of TSP

Possible modes of action of TSP include exposure of microorganisms to high pH, which might particularly affect cell membrane components (Mendonca *et al.* 1994), sequestration of metal ions (Lee *et al.* 1994) and its role as a surfactant, enhancing detachment of bacteria from food surfaces (Kim and Slavik 1994).

2.4.4 Disadvantages of TSP food decontamination procedure

The principal disadvantages of TSP food decontamination procedures are the requirement for a high TSP concentration and thus a high pH. This may lead to altered organoleptic food properties, increased wear of industrial equipment, and environmental damage from high phosphate effluents. In addition, TSP killing are aimed primarily at gram negative pathogenic and spoilage bacteria and are relatively ineffective against gram negative spoilage bacteria (Poulenc 1992).

Previous works have indicated that the treatment of poultry carcasses with 8 to 12 % TSP have not any negative effect on the organoleptic quality of meat (Ellebroek *et al.* 1997, Hathcox *et al.* 1995 and Hollender *et al.* 1999).

2.5 Detection methods

Traditional methods for detecting microorganisms have been developed. Molecular methods are used to supplement or confirm traditional methods. Molecular methods do not depend on either growth state nor on environmental influences and are therefore more precise. The most developed in vitro amplification method is the polymerase chain reaction that allows rapid and selective identification of microorganisms (Scheu *et al.* 1998).

2.5.1 Molecular Polymerase Chain Reaction

Rapid and selective identification of microorganisms in different matrices can be achieved through polymerase chain reaction (PCR) technique, which amplifies specific gene fragments.

Hybridization and Southern blotting should follow to confirm the identification of the amplified product and to increase the sensitivity. Specificity and sensitivity of PCR systems are the major criteria for the detection of microorganisms in food. The sequences of primers that are unique for the target microorganisms and the annealing temperature that have to be optimized in order to minimize non specific priming determine specificity. The reaction condition matrix of the food and the DNA extraction method play important role in the sensitivity of a given system (Way *et al.* 1993).

2.5.2 Limits of PCR

PCR techniques developed for detecting viruses and bacteria in food samples are unable to differentiate viable and non viable microorganisms. They only show that the appropriate nucleic acid sequence are present in the sample. Therefore, DNA from dead organisms may lead to false positive results (Allman *et al.* 1995).

Viable but not culturable, and culturable microorganisms can be found in the sample, it is important to have a method which can distinguish between these. The detection of dead

microorganisms can be prevented by propagation step before PCR analysis. In this way it is possible not only to increase the sensitivity of the test, but to restrict the detection of culturable cells (Josephson *et al.* 1993). According to the literature, the selection of living microorganisms with the aid of antibody coated paramagnetic beads should be possible (Fluit *et al.* 1993, Grant *et al.* 1993, Kapperud *et al.* 1993).

2.5.3 Inhibition of PCR

PCR inhibitors found in food can inhibit PCR methods. False negative results occur for various reasons, the presence of substances chelating divalent magnesium ions for PCR, degradation of nucleic acids targets or primers through nucleases (DNAses and RNAses) and direct inhibition of the Taq DNA polymerase (Scheu *et al.* 1998).

2.5.3.1 Substances inhibiting PCR

It has been showed by many reasearchers that many substances can directly ihibit the activity of Taq DNA polymerase (Demeke and Adams 1992, Ahokas and Erkkia 1993, Katcher and Achwarz 1994, Wiedbrauk *et al.* 1995). Ionic detergents (e.g. sodium deoxycholate) hae also been shown to have inhibitory effects (Weyant *et al.* 1990). Calcium ions could be identified as a source of PCR inhibition (Bickley *et al.* 1996). Unspecific (0.4 mg unrelated DNA added to microlitres) is able to completely inhibit the PCR (Rossen *et al.* 1992).

2.5.3.2 Prevention of PCR inhibition

The removal of the substances from DNA is an important step in many applications. Several potential methods of eliminating inhibitory effects have been described. One of the easiest procedures to prevent the inhibition of PCR is diluting the food samples (Wang *et al.* 1992b). Dilution of the samples also decreases sensitivity when the amount of DNA is a limiting factor.

Separating the bacteria from the food matrix before extracting DNA can prevent PCR inhibition. This can be done by preparing subculture of food on selective agar media and using the bacterial colonies for PCR (Furrer *et al.* 1991, Thomas *et al.* 1991).

2.6 Conclusion

Poultry meat constitutes a substantial portion of present day diets, hence the concern to market a safe product of good quality. The presence of pathogenic microorganisms, spoilage microorganisms or both in poultry carcasses is undesirable.

Companies producing processed meat products must assume that all incoming raw meat is potentially contaminated with bacterial pathogens. The following items are very important in the control of microbial contaminants in meat processing facilities:

Every meat processing facility needs to institute and enforce an effective cleaning and sanitizing program that will ensure production of safe products. Floors, drains, walls, ceilings, and each piece of equipment in the plant should be cleaned and sanitized on a regular basis.

Employee movement within a food processing facility also can have a major impact on the microbiological quality of finished products. Traffic patterns that eliminate the movement of workers between raw, processing, filling, packaging and shipping areas need to be developed and enforced.

Good employee hygiene is also a must and plant managers and supervisors need to set a good example for other workers. Employees should always wash their hands thoroughly before starting work, or returning to work and after touching floors, walls, light switches or any unclean surfaces or garbage. Hand washing facility should be properly designed and equipped and be conveniently located near work stations.

Special attention also is needed to assure that street clothes do not enter the processing facility and that factory clothing, including footwear, remain inside the plant.

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Chapter 3

THE INCIDENCE OF PATHOGENS IN A POULTRY PROCESSING PLANT AS DETERMINED BY CONVENTIONAL TESTS AND PCR

3.1 ABSTRACT

Microbial contamination of the food supply is among the classes of hazards associated with food. The incidence of *Campylobacter*, *Salmonella* and *Listeria* species in a poultry processing plant was determined by conventional test and PCR. Chicken samples were collected from different stages along the processing line. *Salmonella*, *Campylobacter* and *Listeria* species were isolated from the collected samples. Proteinase K method was used to for DNA isolation from *Campylobacter* and *Listeria*. Boiling method was used to isolate DNA from *Salmonella* species. The following sets of primers Lis1B and UnilisA for the detection of *Listeria* species, ST11 and ST15 for the detection of *Salmonella* species and CF03 and CF04 for the detection of *Campylobacter* species were used to confirm the results obtained with conventional tests (catalase and oxidase tests as well as Gram Staining). All three pathogens were isolated from some of the stages in the processing plant. *Campylobacter* and *Listeria* were isolated from all the samples collected from bleeding and scalding stages. *Salmonella* was isolated was isolated from 66,7% and 33.3 % of the samples collected from bleeding and scalding. All three pathogens were isolated from 66,7% of the samples collected from plugging. *Campylobacter* and *Listeria* were isolated from 33.3% of the samples collected from

evisceration and *Salmonella* from 66,7% of the samples collected from this stage. The incidences of *Campylobacter*, *Listeria* and *Salmonella* from chlorine wash were 100%, 66,7% and 33,3 % respectively. *Campylobacter* was not collected from all the samples collected from packaging and *Listeria* and *Salmonella* were isolated from 33,3% of the samples collected from this stage. *Campylobacter* and *Listeria* numbers in the poultry processing plant were higher than those of *Salmonella*. *Campylobacter*, *Listeria* and *Salmonella* were isolated from 66,7%, 66,7% and 50% of all products samples respectively. The elimination of these pathogens is very difficult to achieve and a criteria for allowing these pathogens to be present at a reasonable levels in products samples is needed.

3.2 INTRODUCTION

Food borne diseases are an important public health problem in most countries. *Campylobacter* and *Salmonella* are among the most important foodborne pathogens in the world. Poultry and poultry products are the frequent vehicles of these bacterial Pathogens (Dominguez *et al.* 2001). Microbial risks associated with raw poultry products include *Salmonella* species, *Campylobacter jejuni*, *Campylobacter coli* and *Listeria monocytogenes*. *Campylobacteriosis* occurs as sporadic cases of illness instead of outbreaks. Worldwide, *Salmonellae* and *Campylobacters* are by far the most important pathogens associated with poultry products (Bryan *et al.* 1995). Eating undercooked chicken is one risk factor associated with sporadic *Campylobacteriosis* (Neimann *et al.* 1998). Case control studies suggest that undercooking raw poultry is involved in human

Listeriosis among individuals susceptible to the condition. In severe cases, listeriosis may lead to death. Outbreaks have been associated with the consumption of milk (Dalton *et al.*1997), meat and poultry products (Wenger *et al.* 1990) and soft cheeses (Goulet *et al.*1995)

The skin of the poultry is not normally removed during processing. Therefore there is a great concern in poultry processing facilities for microbiological control and product safety because the worker's hands, trimming gloves, knives and equipment surfaces come into contact with the outer surface producing a potential for carcass to carcass contamination.

Traditional morphological and physiological criteria are fundamental parameters contributing to the identification of microorganisms and pathogens in food. These criteria may be influenced by environmental as well as genetic factors and may therefore vary with changes in environmental conditions (Scheu *et al.*1998).

To supplement classical methods, a number of DNA based methods have been developed in the past few years. Identification and detection can be greatly improved through the use of different invitro amplification methods such as Q beta, replicase amplification, ligase chain reaction and polymerase chain reaction. At this stage polymerase chain reaction is a rapid and sensitive method suited to detecting and identifying microorganisms (Scheu *et al.* 1998).

Microbial contamination of poultry can lead to large scale of economic losses to processors, retailers and consumers.

This study was therefore undertaken to determine the incidence of *Campylobacter*, *Salmonella* and *Listeria* species in a poultry processing plant through PCR and biochemical tests.

3.3 MATERIALS AND METHODS

3.3.1 Samples collection

Sampling was performed at a poultry abattoir in the Western Cape. Swab samples from the coacalar vent were collected. Three swab samples for each of the three pathogens from the following stages, bleeding (Ble), scalding (Sca), plugging (Plu), evisceration (Evi), chlorine wash (CHL) and packaging (Pac). Samples were transported to the laboratory at 4 °C for further tests.

3.3.2 Isolation of *Campylobacter* by the Cape Town Protocol

The filter membranes with a pore size of 0.6 µM and a 50mm diameter (Schleicher and Schuell ME 26) were placed onto antibiotic free Tryptose blood agar plates (CM233, Oxoid) containing 10% unlysed horse blood. Few drops of the samples were placed onto the filters and left for 30 min. The filters were then removed and the plates were incubated at 37°C for 48 h in a microaerophilic atmosphere of hydrogen and carbon

dioxide using Oxoid Gas Pack (BR 38) (Le Roux and Lastovica 1998). Gram Staining, catalase and oxidase were performed.

3.3.3 Isolation of *Listeria* from the collected samples

Swab samples were incubated in 10ml of *Listeria* enrichment broth (LEB) medium (CM863, Oxoid) for 24 hours at 37°C. The samples were diluted in 4,5ml of 0.5% KOH and then plated on *Listeria* selective agar plates (LSA) medium (CM856, Oxoid), the plates were incubated at 37°C for 48h and colonies surrounded by black zones were restreaked for further tests, catalase and oxidase. Gram staining was also performed.

3.3.4 Isolation of *Salmonella* from the swab samples

Chicken samples were pre-enriched at 37°C in 1ml buffered peptone water (BPW) (CM 509, Oxoid) for 18 hours in a shaking incubator. For secondary enrichment 1ml of the preenriched suspension was transferred to 9.9 ml of Rappaport Vassiliadis (RV) medium (CM509, Oxoid). The RV medium was then incubated at 42 °C for 24 h. A loopfull of the broth culture was streaked onto xylose lysine deoxycholate (XLD) agar (CM469, Oxoid) and the plates were incubated at 37°C for 48 h. Presumptive positive colonies were confirmed with catalase, oxidase tests and Gram Staining.

3.3.5 Bacterial DNA isolation of *Campylobacter* and *Listeria*

Proteinase K method was used to isolate the DNA from *Campylobacter* and *Listeria* species. Colonies were scraped from the plate and resuspended in 0.5ml 1xTris EDTA buffer, and then centrifuged at 14000rpm for 10 minutes, the supernatant was discarded, 0.5ml of TE was added and the cells were resuspended. Cells were lysed by adding 50µL 10mg lysozyme per ml and mixed gently for cell lysis to take place and incubated at 37°C for 30min. A solution containing 10mg Proteinase K per ml in 1x TE buffer and was added to the above solution and 20 µl of 10%SDS pH 7.2 were added and mixed and then incubated at 56°C for 2 hours. Proteins and cell debris were precipitated with 297 µl of 3M potassium acetate (pH4.8). The tubes were vortexed vigorously and the contents were mixed by inverting the tubes and then centrifuged at 16000rpm for 10min and the supernatant was added to 0.54 volume of 2-propanol and mixed gently. The above solutions were centrifuged at 16000 rpm for 10min and the supernatant was discarded. The pellets were washed twice in ice-cold 600 µl of ethanol and centrifuged at 13000g for 2min, and the supernatant was discarded. The tubes containing the DNA were dried in an oven and resuspended in sterile distilled water (Christensen *et al.* 1999).

3.3.6 DNA Isolation from *Salmonella* cells with boiling method

For DNA isolation 2ml of the broth culture were spun at 13000 rpm for 10 minutes, the supernatant was discarded and the pellet was washed in 1ml phosphate buffered

saline (centrifuged at 13000 rpm for 6 minutes and the supernatant was discarded. The pellet was resuspended in 500ml of sterile distilled water and incubated at 95°C for 10 minutes. This was centrifuged at 13000 rpm for 6 minutes and the supernatant was transferred into a clean tube.

3.3.7 PCR protocol for the detection of *Campylobacter* species

The following primers were checked against a gene bank database, BLAST program was used, to check if they have any homology to other organisms except *Campylobacter* and used for PCR under its optimal conditions, CF03, CF04 (Wegmuller et al. 1993).

3.3.7.1 Oligonucleotides

Oligonucleotides were defined by comparison of published *flaA* and *flaB* sequences. The sequences of the nucleotides are as follows:

Table 3.1. The sequences of the primers for the detection of *Campylobacter* species.

CF03	5'-GCTCAAAGTGGTTCTTATGCN ATGG-3'
CF04	5'-GCTGCGG AGTTCATTCTAAGACC- 3'

3.3.7.2 PCR reaction

Optimized concentrations for PCR (100 µl total volume) were 4mM magnesium chloride; 0.25µM primers (CF03 and CF04); 200µM (each) dATP, dCTP, dGTP and dTTP (Promega); 1x reaction buffer(Promega); 0.2µg of bovine serum albumin per 100µl; 1µl DNA, sterile distilled water and 2U of Taq DNA polymerase. These were subjected to PCR with the following temperature programme: denaturation at 94°C for 4min; 40-50 cycles at 95°C for 5s, 53°C for 30s, and 72°C for 40s; and a final extension at 72°C for 5 min. The amplified products were stored at 4 °C.

3.3.8 PCR protocol for the detection of *Listeria* species

The following oligonucleotide primers derived from the *iap*- related gene sequences of the different *Listeria* species were used to carry out polymerase chain reaction (Bubert *et al.* 1992).

Table 3.2. The sequences of the primers for the detection of *Listeria* species.

UnilisA	5'-GCTACGCTGGGATTGCGG -3'
Lis1B	5'-TTATACGCGACCGAAGCCAA -3'

3.3.8.1 PCR reaction

Optimized concentrations for PCR (50µl total volume) were 1,5mM magnesium chloride; 5µM primers (UnilisA and Lis1B); 150µM (each) dATP, dCTP, dGTP and dTTP(Promega); 1x reaction buffer(Promega), 1µl DNA, sterile distilled water and 0,5 units of Taq DNA polymerase. PCR conditions were as follows: 30 cycles each at 94°C for 2min, 94°C for 30s, 50°C for 45s, and 72°C for 2min; and a final extension at 72°C for 5 min. The amplified products were stored at 4 °C.

3.3.9 PCR protocol for the detection of *Salmonella* species

The Salmonella specific primers ST11 and ST15 (Aabo *et al.* 1993) were used to successfully detect Salmonella species from chicken samples, in this study.

Table 3.3 The sequences of the primers for the detection of *Salmonella* species.

ST 11	5'- AGCCAACCATTGCTAAA TTGGCGCA -3'
ST 15	5'-GGTAGAAATTCCCAG CGGGTACTG -3'

3.3.9.1 PCR conditions

The extracted products (1,5 µl) were added to a reaction mixture (23.5 µl) consisting of 2,5mM Magnesium chloride, 0.625 mM (each) dATP, dCTP, dTTP, dGCT, 1x reaction

buffer, 03 µg/µl each of oligonucleotide primers ST11 and ST15, sterile distilled water and 1U of Taq DNA polymerase. Samples were denatured at 94°C for 2 min, 35 cycles of amplification were run at 95°C for 30s, 60°C for 30s and 72°C for 30s. The reaction was completed by a final extension at 72°C for 10 min (Gouws *et al.* 1998).

3.3.10 VISUALIZATION

A total of 15 µl (5 µl of loading buffer and 10 µl of PCR product)was analyzed by agarose gel electrophoresis(1.2 % agarose gel in 1x Tris-borate EDTA (TBE)) . This was run at 75 volts and made visible by ethidium bromide staining and UV transillumination.

3.4 RESULTS AND DISCUSSION

Table 3.4. Phenotypical and genotypical results of *Salmonella* and *Listeria* species isolated from chickens

Isolates no.	Source	Phenotypic		PCR	
		<i>Salmonella</i>	<i>Listeria</i>	<i>Salmonella</i>	<i>Listeria</i>
1	Ble1c	+	+	+	+
2	Ble2c	+	+	+	+
3	Ble3c	-	+	-	+
4	Sca1c	-	+	-	+
5	Sca2c	+	+	-	+
6	Sca3c	+	+	+	+

7	Plu1c	+	+	+	+
8	Plu2c	+	+	+	-
9	Plu3c	+	+	-	+
10	Evi1c	-	+	Not tested	+
11	Evi2c	+	+	+	+
12	Evi3c	+	+	+	-
13	CH1c	+	+	+	+
14	CH2c	+	+	-	+
15	CH3c	+	+	-	-
16	Pac1c	+	+	-	-
17	Pac2c	+	+	-	-
18	Pac3c	+	+	+	+

The results of *Salmonella* and *Listeria* isolation from chicken samples appear in table 3.4. Of the 18 samples only 15 yielded gram negative and produced *Salmonella* like colonies on XLD plates. All 15 samples were phenotypically confirmed to be *Salmonella* and only 9 of the 15 samples were genotypically confirmed. The other samples did not yield a PCR products (figure 3.2).

The contamination rate for *Listeria* reported in this study was 66,7 %. All 18 samples yielded a gram positive and produced a *Listeria* like colonies on Listeria (LSA) selective agar plates. All of the 18 isolates were phenotypically confirmed to be *Listeria*. However of the 18 isolates only 12 were genotypically confirmed. The other isolates did not yield a PCR product (figure 3.3).

Of the 18 samples only 12 yielded a gram negative and produced a *Campylobacter* like colonies on blood agar plates. All of the 12 isolates were phenotypically and

genotypically confirmed to be *Campylobacter* (Table 3.2). All samples yielded PCR products, 350 bp in length (figure 3.1).

The phenotypic results used to identify *Campylobacter* gave results which corresponds positively with those obtained through PCR. Molecular tests remain indispensable since some tests yielded contradictory results in other bacteria, *Salmonella* and *Listeria*. Some isolates were found being positive through phenotypic tests and proved to be negative with PCR while others were confirmed to be positive.

1 2 3 4 5 6 7 8 9 10 11 12 13



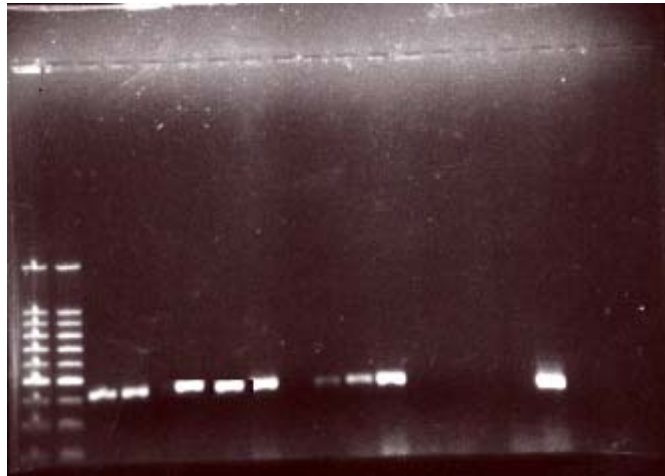
350bp PCR products

Figure 3.1 Agarose gel electrophoresis of PCR products of *Campylobacter* species isolated from a poultry processing plant with CF03 and CF04 primers. Lanes 1, 100 bp

DNA ladder; 2, Ble1c; 3, Ble2c; 4, Ble3c; 5, Sca1c; 5, Sca2c; 6, Sca3c; 7,Plu1c; 8, Plu2c; 9, Evi1c; 10, CH1c; 11, CH2c; 12, CH3c

It was also found from this study that *Campylobacter* was not isolated where either *Salmonella* or *Listeria* existed, (Table 3.1). This may be due to its inability to co-exist with other bacteria in the same host. *Campylobacter* is widely thought as an environmentally fragile organism. It is sensitive to drying, acidity, freezing, salting, osmotic stress, chemical rinses and disinfectants and it does not compete well with other microorganisms (Nachamin 1997, Solomon and Hoover 1999).

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18



429bp PCR products

Figure 3.2 Agarose gel electrophoresis of PCR products of *Salmonella* species isolated from a poultry processing plant with ST11 and ST15 primers. Lanes 1, 100 bp DNA ladder; 2, DNA ladder; 3, Ble1s; 4, Ble2s; 5, Sca2s; 6, Sca3s; 7, Plu1s; 8, Plu2s; 9, Plu3s; 10, Evi2s; 11, Evi3s; 12, CH1s; 13, CH2s; 14, CH3s; 15, Pac1s; 16, Pac2s; 17, Pac3s; 18, Negative control.

Table 3.5 Phenotypical and genotypical results of *Campylobacter* species isolated from chickens

Isolates no.	Source	Phenotypic	PCR
1	Ble1c	+	+
2	Ble2c	+	+
3	Ble3c	+	+
4	Sca1c	+	+
5	Sca2c	+	+
6	Sca3c	+	+
7	Plu1c	+	+
8	Plu2c	+	+
9	Plu3c	-	Not tested
10	Evi1c	+	+
11	Evi2c	-	Not tested
12	Evi3c	-	Not tested
13	CH1c	+	+
14	CH2c	+	+
15	CH3c	+	+
16	Pac1c	-	Not tested
17	Pac2c	-	Not tested
18	Pac3c	-	Not tested

It was shown in this study that *Salmonella* contamination was reduced during chlorine wash after evisceration, but recontamination occurred during packaging.

Table 3.2 shows that the level of *Campylobacter* which was supposed to be reduced during during chlorine wash increased. This might have been caused by the following

reason, *Campylobacter* is a natural constituent of poultry faeces and during the visceral removal and picking procedures, it can be transferred from the intestines to the surface of the chickens and come into contact with equipment surfaces, worker's hands and trimming gloves producing the potential for carcass to carcass cross contamination.

Campylobacter was not isolated from the final stage (packaging), this is because the chickens are thoroughly washed and then rapidly chilled with chilled water to preserve quality and spoilage by food pathogens and spoilage microorganisms. The chickens are also drained of excess moisture after chilling. The absence of *Campylobacter* from packaging may be attributed to a combination of exposure to oxygen, decreased humidity and suppression of the organism by competitive microflora.

One study indicated that *Listeria* frequently enters the processing plant on broiler hens (Cox *et al.* 1997). The organism appears to take up residence in the plant, leading to cross contamination during processing (Ojeniyi *et al.* 1996). This is also indicated by the higher rate of contamination on the samples.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19



1.5 kb PCR products

Figure 4.3 Agarose gel electrophoresis of PCR products of *Listeria* species isolated from a poultry processing plant with Lis1B and UnilisA primers. Lanes 1, 100 bp DNA ladder; 2, Ble1L; 3, Ble2L; 4, Ble3L; 5, Sca1L; 6, Sca2L; 7, Sca3L; 8, Plu1L; 9, Plu2L; 10, Plu3L; 11, Evi1L; 12, Evi2L; 13, Evi3L; 14, CH1L; 15, CH12L; 16, CH13L; 17, Pac1L; 18, Pac2L; 19, Pac3L

Salmonella is brought into the processing plant by chickens because of the way they are reared and transported. *Salmonella* cells are initially entrapped in a water film on the skin and then migrate to the skin, where they are entrapped in ridges and crevices that become more pronounced in the skin after immersion in water (Lillard 1989). This could explain the presence of *Salmonella* from the collected samples, even those collected from the first stage (bleeding).

The hygiene rules of slaughter and meat processing must rigorously observed in order to lower the contamination rates of *Campylobacter*, *Salmonella* and *Listeria*. The number of *Campylobacter* on poultry can be lowered by improved hygiene control and improving abattoir hygiene may reduce the incidence of *Listeria* (Mead *et al.* 1995).

This study shows that the elimination of all pathogens cannot realistically be achieved; therefore there is a need to come up with a way of allowing these pathogens to be present at reasonable levels in poultry. The presence of *Salmonella* and *Listeria* from the final stage of the processing plant shows us the importance of that good cooking techniques, good kitchen and personal hygiene during preparation.

3.5. Acknowledgements

I wish to thank the National Research Foundation for financial support during this study.

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Chapter 4

The formation and survival of biofilms cells of food pathogens in trisodium phosphate.

4.1 Abstract

The survival of biofilm cells of *Campylobacter*, *Listeria* and *Salmonella* in trisodium phosphate was determined. The use of TSP as a post chill antimicrobial treatment for raw poultry has been approved by the United States Department of Agriculture (USDA). For biofilm development 15ml of the cultures (*Salmonella* and *Listeria*) were added to Tryptic soy broth and *Campylobacter* in Brucella broth, containing 5 stainless steels and the jars were incubated in a shaking incubator at 37°C. After seven days of incubation stainless steels were rinsed and dipped in different concentrations of TSP for 2 and 30 minutes. Both *Listeria* and *Salmonella* cells formed biofilms at 37°C. *Campylobacter* did not form biofilms at 37°C under aerobic conditions. Under microaerophilic conditions, biofilm cells of *Campylobacter* were formed. Biofilm cells were reduced in all applications. Eventhough the application of 1% TSP for 2 minutes gave a decrease in the number of biofilm cells, the cells were highly reduced when treated with 12% TSP for 30 minutes. *Listeria* and *Campylobacter* cells were not detected even when treated with the lowest concentration, 1% TSP for 30 minutes. It was seen from this study that an increase in the concentration of TSP and contact time causes an increase in the reduction of

biofilm cells. These results suggested that trisodium phosphate has the ability to be used in the reduction of bacterial cells on poultry and food contact surfaces.

4.2 Introduction

The most common pathogens of concern to the meat, poultry and dairy industry include *Campylobacter jejuni*, *Campylobacter coli*, *Salmonella typhimurium* and *Listeria monocytogenes* (D' Aoust 1989, Farber and Peterkin 1991, Padhye and Doyle 1992)

When microorganisms are isolated from processed food, sometimes it is because of the post process contamination of the product. The food becomes recontaminated during further handling of the food with bacteria present in contaminated food processing equipments, especially in places, which are inaccessible to ordinary cleaning procedures.

Over the past few years there has been a growing awareness of the role that biofilms play in our lives. Biofilms can easily formed in stainless steel, rubber and other surfaces in the processing environment (Somers *et al.* 1994).

When bacteria are attached and growing on food contact surfaces, the effect of the antimicrobial agents is neutralized and this increases resistance to disinfection (Arizcun *et al.* 1998). Microorganisms attached to food contact surfaces may be difficult to remove and stronger physical force may be needed to remove biofilm (Helke *et al.* 1993, Saurez *et al.* 1992). It has been showed that unattached cells of *Listeria monocytogenes*

and *Salmonella typhimurium* were more sensitive to the effect of sanitizers when compared to attached cells (Ronner and Wong 1993)

The microbial load of fresh meat can be reduced by the application of decontaminants. Numerous disinfectants and treatments have been applied so as to reduce carcass contamination including irradiation, acid, bases, halogens, and hydrogen peroxide. Although all these methods have shown some degree of bactericidal efficacy, each one offers some significant disadvantage(s), such as cost or sensory changes that have prevented their usage (Bolder 1997; Farkas 1998; Smulders 1998; Sofos and Smith 1998).

Food and Safety Inspection Service (FSIS) division of the United States Department of Agriculture has approved trisodium phosphate as a post chill anti microbial treatment for raw poultry (Anon 1992). It has been shown to be effective in reducing the numbers of *Salmonella* and other Enterobacteriaceae on poultry (Blender 1992) without affecting the flavour of the product (Holender *et al.* 1993). It is used as a dip immediately after water chilling or before air chilling. Previous works have indicated that the treatment of poultry carcasses with 8% to 12% trisodium phosphate have not any negative effect on the organoleptic quality of the meat (Hollender 1999).

This study was undertaken to determine the survival of biofilm cells of *Campylobacter*, *Listeria* and *Salmonella* in trisodium phosphate.

4.3 Materials and Method

4.3.1 Cultures

Isolates obtained from poultry processing environments were used. *Salmonella* and *Listeria* were grown in Tryptic soy broth (CM129, Oxoid) and *Campylobacter* in brucella broth (249520, Difco) for 18 hours. Incubation was at room temperature for all the cultures. All cultures were shaken during incubation.

4.3.2 Preparation of stainless steel

Stainless steels, 6 by 2 cm, were used as surfaces to form the biofilms. All slides were cleaned to remove grease. They were then rinsed in distilled water, air dried, placed in jars containing 500ml of Tryptic soy broth (TSB) (CM129, Oxoid), for *Salmonella* and *Listeria*, brucella broth (249520, Difco) for *Campylobacter*. The jars containing 5 stainless steels were autoclaved.

4.3.3 Biofilm formation

Biofilms were formed according to the protocol used by Jeong and Frank (1994). For biofilm formation 15 ml of the cultures at the concentrations of about 7,81, 6,57 and 6,18 log counts of *Salmonella*, *Listeria* and *Campylobacter* were added to growth medium contained in jars respectively. The jars were incubated for 5 days at 37°C. Stainless steel

were aseptically removed from the medium and rinsed with Phosphate Buffered Saline (PBS) to remove unattached cells. One steel from each jar was used as a control and the other four were exposed to the biofilm removal solution (TSP).

4.3.4 Effect of trisodium phosphate on cells in biofilm

Test solutions of 1%, 4%, 8% and 12 % TSP were made in sterile distilled water for the survival of biofilm cells. Stainless steels were placed in TSP solutions. Control slides were placed in sterile distilled water and incubated for 2 and 30 minutes at 10°C. 10°C meets the requirement approved for post chill treatment of raw poultry. After treatment stainless steels were placed in 0.1% Peptone bacteriological with 1 % hexametaphosphate. Cells were vortexed.

Samples were enumerated by plating the appropriate dilutions on *Listeria* selective agar plates (CM856, Oxoid) for *Listeria*, Mac Conkey agar (1.05463, Biolab Merck) for *Salmonella* and Tryptose blood agar plates (CM233, Oxoid) with 10 % horse blood for *Campylobacter*. Plates were incubated at 37°C for *Salmonella* and *Listeria* for 48 hours except for tryptose blood agar plates, which were incubated at 42°C under a microaerobic atmosphere.

4.4 Results and discussion

It has been showed by other researchers that food borne microorganisms adhere to food contact surfaces (Helke *et al.* 1993). One of the most common surfaces found in food processing plants is stainless steel. It was expected from this study that biofilm cells will be formed. Biofilm formation was only seen in some cases. The results indicate that both *Salmonella* and *Listeria* will easily form biofilms when exposed to a solid surface (Stainless steel) under aerobic conditions at 37°C and they can survive

Table 4. 1 Counts of *Salmonella* isolates in biofilms after being exposed to trisodium phosphate.

TSP Concentration	<i>Salmonella</i> (Log counts)		Log reduction	
	2min	30 min	2 min	30min
1%	7,7	7,25	1,08	1,53
4%	6,88	6,58	1,9	2,2
8%	6,78	4,46	2	4,32
12%	4,77	3,20	4,01	5,58
Control	8,78	8,78		

for extended time periods. Therefore it appears that a seven day period is a sufficient time for *Listeria* and *Salmonella* biofilm development, even under low nutrient conditions (diluted Tryptic Soya Broth) used in the experiment.

Table 4.2 Counts of *Listeria* isolates in biofilms after being exposed to trisodium phosphate.

TSP Concentration	<i>Listeria</i> (Log counts)		Log reduction	
	2min	30 min	2min	30 min
1%	6,21	0	2,11	8.32
4%	5,34	0	2,98	8.32
8%	3,38	0	4,94	8.32
12%	3,34	0	4,98	8,32
Control	8,32	8,32	0	0

Salmonella formed biofilm on stainless steel with a cell density of 6.08×10^8 cfu/ml (Table 4.1) and *Listeria* with a cell density of 2.07×10^8 cfu/ml (table 3.2).

These results show that *Listeria* and *Salmonella* will acquire an extreme resistance to antimicrobial agents when exposed to a solid surface because cells in biofilms are more

resistant to sanitizers, chemicals and heat, they are much more difficult to kill than the same cells in a free floating state. They can also enter a viable but non culturable (VBNC) form of life (Zechowski and Benner 1992 and Hood and Zotolla 1995). This means that they will not grow in the media usually used for their detection.

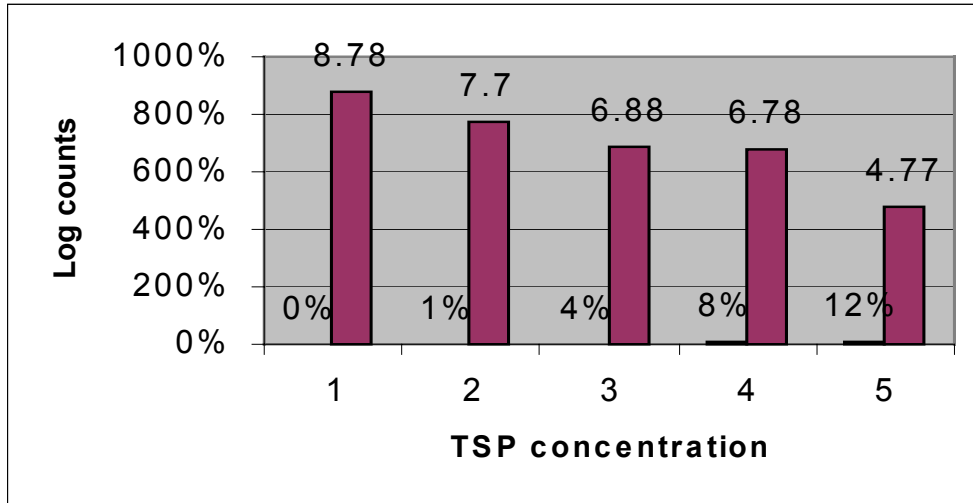


Figure 4.1. Effect of TSP applied at 10°C for 2 min on *Salmonella* isolates growing in biofilms.

Throughout the time period of seven days, *Campylobacter* species did not form biofilms at 37°C when incubated aerobically. *Campylobacter* is a microaerophilic organism, which means that it has a requirement for reduced levels of oxygen. It is relatively fragile and sensitive to environmental stresses (e.g. 21% oxygen, drying, heating and disinfectants) and because of its microaerophilic characteristics it requires 3 to 5 % oxygen and 2 to 10% Carbon dioxide for optimal growth conditions. This can explain why it did not form biofilms in this experiment.

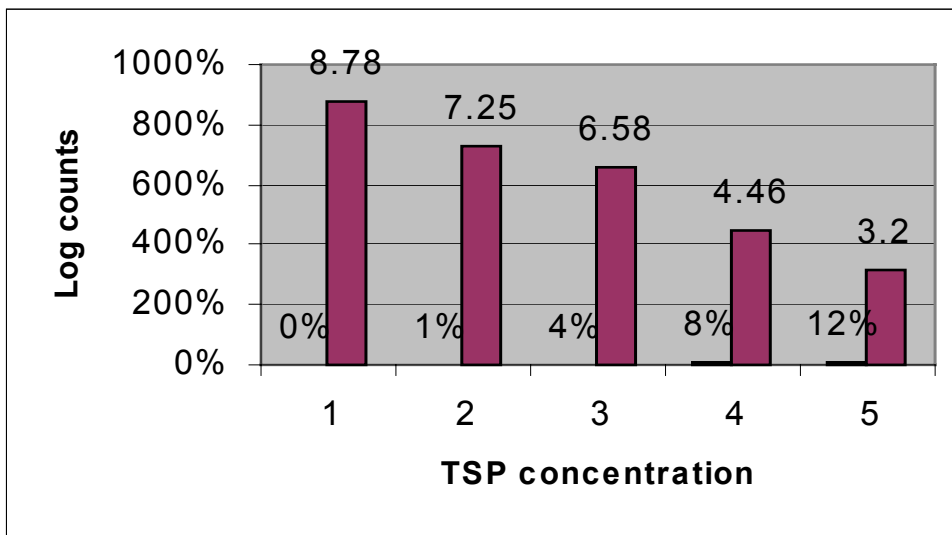


Figure 4.2 Effect of TSP applied at 10°C for 30 min on *Salmonella* isolates growing in biofilms.

When *Salmonella* cells were treated with different concentrations of trisodium phosphate for 2 minutes the following results were obtained, a decrease of 1.08 log counts with 1% TSP and 1.9 log counts with 4% TSP. The highest concentrations of TSP used in this experiment were 8% and 12% and they gave a log reduction of 2 and 4.98 log counts respectively when *Salmonella* cells were incubated at 10°C for 2 minutes (Figure 4.1). These concentrations do not have any effect on the organoleptic quality of meat (Capita *et al.* 1992). The contact time was increased to 30 minutes and the following was observed. A decrease of 1.53 log counts with 1% TSP. Treatment with 4% decrease the cells with 2.2 log counts and a 4.3 log counts reduction was obtained when cells were treated with 8% TSP. A decrease of 5.58 log counts was obtained when cells were treated with 12% TSP (Figure 4.2).

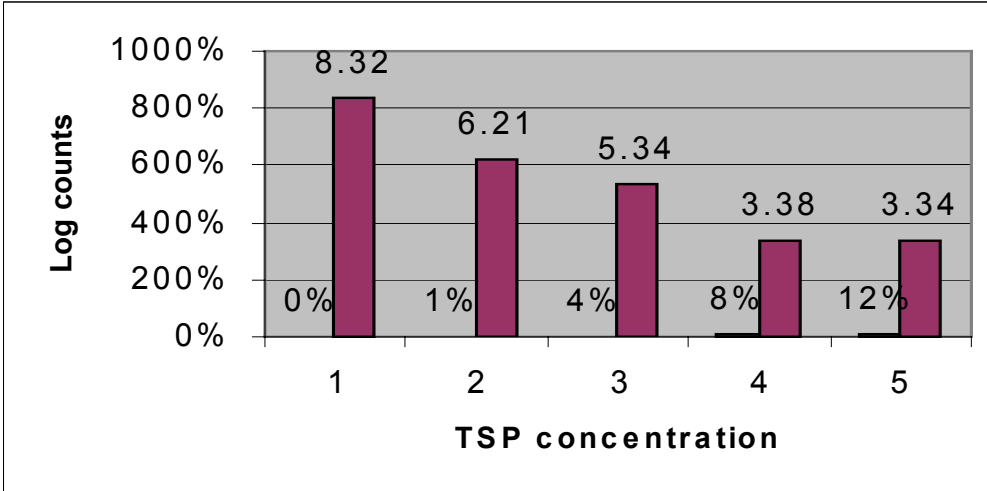


Figure 4.3 Effect of TSP applied at 10°C for 2 min on *Listeria* isolates growing in biofilms.

When *Listeria* cells were treated with the lowest concentrations of TSP 1% and 4% for 2 minutes the following results were obtained, a decrease of 2.11 and 2.98 log counts respectively. A decrease of 4.94 log counts was obtained when TSP concentration was increased to 8% and the highest concentration (12%) gave a reduction of 4.98 log counts (Figure 4.3) All these results were obtained when cells were incubated at 10°C for 2 minutes. *Listeria* cells were not detected at all when contact time was increased to 30 minutes.

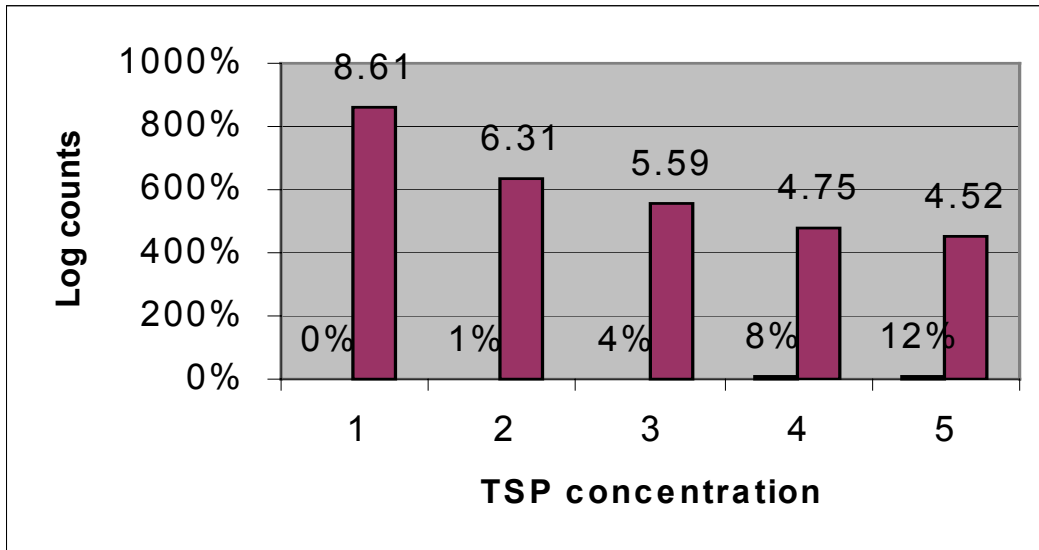


Figure 4.4. The effect of TSP applied at 10°C for 2 minutes in *Campylobacter* cells growing on biofilms under microaerophilic conditions

Campylobacter formed biofilms under microaerophilic conditions with a cell density of 8,61 log counts. Close to half of the cells 4,09 were not recovered when *Campylobacter* cells were treated with 12% TSP at 10°C for 2 minutes. The lowest concentration of 1% reduced the cells by 2,3 log counts. *Campylobacter* cells treated for 2 minutes with 4% and 8% were reduced by 3,02 and 3,86 log counts respectively (Figure 4.4).

Campylobacter cells were not recovered when treated TSP solutions at 10°C for 30 minutes. This suggests that both *Salmonella* and *Campylobacter* cells may have been injured or sensitized by the presence of trisodium phosphate, rather than acquiring resistance when treated with TSP for 30 minutes.

It was expected from this study that an increase in contact time as well as the concentration of trisodium phosphate will cause a decrease in the number of cells, and

this was seen in all the applications. This study confirms the ability of trisodium phosphate treatment to reduce the biofilm cells, of *Listeria*, *Salmonella* and *Campylobacter*. It also show that the time of exposure and the concentration of TSP are very important and would need to be defined for each application in the processing environment.

The consequences of biofilm formation in food plants can be serious leading to the contamination of the product compromising shelf life and food safety. Because of these problems, it is extremely important that proper measures be taken to prevent the formation of biofilms and eliminate them. Trisodium phosphate is not corrosive and can be used in cleaning food systems. Proper cleaning followed by sanitizing with an effective disinfectant is required to keep food surfaces from the effects of bacterial attachments.

4.5 Acknowledgements

I wish to thank the National Research Foundation for financial support during this study.

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Chapter 5

5.1 Summary and Conclusion

A number of organisms were chosen for use in the study due to their association with foods and their potential as food borne pathogens. Food borne diseases are an important public health problem in most countries. *Campylobacter* and *Salmonella* are among the most important food borne pathogens in the world. Poultry and poultry products are the frequent vehicles of these bacterial species. *Listeria* is a organism that has recently received attention among food processors and consumers because of its ability to cause listeriosis, a potentially fatal food borne illness. Undercooking chicken may cause human listeriosis.

Foodborne disease is often spread by cross contamination between foods and unclean surfaces including packaging materials, holding vessels or tanks and conveying equipments. Microorganisms that cause diseases and food spoilage can adhere to these food contact surfaces and become much more resistant to sanitizers than when they are not adhered. Together with entrapped organic matter, adhered bacteria form biofilms, which can resist the action of antimicrobials. In a food processing facility, bacterial adhesion to food and food contact surfaces is significant in that it may transmit diseases or cause product spoilage losses.

At this time there is no material inherently to biofilm attachment. However, true biofilms take several days to reach equilibrium. Proper cleaning ensures that the cells in an unstable biofilm can be reached by chemical cleaners and sanitizers. The design of equipment with smooth, highly polished surfaces may impede biofilm formation by making the initial adsorption step more difficult.

Ways of preventing biofilm formation

- Ensure that cleaning and sanitizing solutions reach all parts of the equipments effectively.
- Rigorously follow the temperature, contact time, chemical concentration outlined by chemical supplier.
- Adhere to all the cleaning steps: pre rinse, wash, post rinse and sanitize every time.
- Inspect equipment after cleaning visually and microbiologically.
- Do not run several short cleaning shifts in a row.
- Do not run several very long production runs in a row.

It was found from this study that it is not easy to eliminate all pathogens in the processing environment and that the time of exposure and the concentration of TSP are very important and would need to be defined for each application.

Appendix A

POLYMERASE CHAIN REACTION

CF03 AND CF04

Reagents	1 Reaction	5 Reactions	10 Reactions	15 Reactions	Reagents
Int. Conc.	volume in μ l	volume μ l	Volume μ l	Volume μ l	Final Conc.
25mM Mgcl ₂	16	80	160	240	4mM
25 μ M CF03	1	5	10	15	0.25 μ M
25 μ M CF04	1	5	10	15	0.25 μ M
2.5mM dNTPs	8	40	80	120	200 μ M each
10X Buffer	10	50	100	150	1X
100 μ g/1ml BSA	0.2	1	2	3	0.2 μ g/ 100 μ l
Taq	0.4	2	4	6	2U
S. d H ₂ O	62.4	312	624	936	
<hr/>					
DNA	1	1	1	1	
Cocktail	99	99	99	99	

Lis1B and UnilisA

Reagents	1 Reaction	5 Reactions	10 Reactions	15 Reactions
Conc.	volume in μ l	volume μ l	Volume μ l	Volume μ l
1.5Mm Mgcl ₂	3	15	30	45
5 μ M Lis1B	2	10	20	30
5 μ M UnilisA	2	10	20	30
150 μ M dNTPs	4	20	40	60
1X Buffer	5	25	50	75
Taq(U)	0.2	1	2	6
S. d H ₂ O	33.8	169	338	507
<hr/>				
DNA	1	1	1	1
Cocktail	49	49	49	49

ST 11 and ST 15

Reagents	1 Reaction	5 Reactions	10 Reactions	15 Reactions
Conc.	volume in μ l	volume μ l	Volume μ l	Volume μ l
2.5mM Mgcl ₂	2.5	12,5	25	37.5
5 μ M ST15	1	5	10	15
5 μ M ST11	1	5	10	15
2.5mM dNTPs	1	4	10	15
10X Buffer	3	15	30	45
Taq(U)	0.2	1	2	3
S. d H ₂ O	14.8	74	148	222
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DNA	1	1	1	1
Cocktail	49	49	49	49

Appendix B

Preparation of:

0.85% Physiological Saline Water

Add 8.5g of NaCl in 1 litre of d.H₂O and autoclave

Blood agar plates

Add 18g of Tryptose Blood agar Base(TBA)(oxoid CM 233) in 600ml of distilled water and autoclave to dissolve. Allow the sample to cool down and squat 60ml of fresh horse blood into the bottle. Mix well and pour plates.

0.8% agarose gel

Add 0.8g of agarose into a conical flask. Add 100ml of 1X TBE. Dissolve the agarose by heating in a microwave. Allow it to cool to about 55°C and add 2 microliters of Ethidium Bromide. Transfer the sample into a gel tray and leave it until it forms a gelly like substance.

1.6% agarose gel

Add 16g of agarose into a conical flask. Add 100ml of 1X TBE. Dissolve the agarose by heating in a microwave. Allow it to cool to about 55°C and add 2 microliters of Ethidium Bromide. Transfer the sample into a gel tray and leave it until it forms a gelly like substance.

10 X TBE (*1L*)

108g of Tris

55g of Boric acid

93g of EDTA

Make the volume up to 1L s. dH₂O

Check if the pH is 8.3

1 X TBE (*1L*)--Add 900ml of s. dH₂O into 100ml of 10 X TBE and mix well