INACTIVATION OF *Listeria monocytogenes* ATCC 7644 ON TOMATOES USING SODIUM DODECYL SULPHATE, LEVULINIC ACID AND SODIUM HYPOCHLORITE SOLUTION

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ABSTRACT

Listeria monocytogenes have been implicated as a public health concern worldwide. The study explored the survival of non-adapted, heat adapted and chlorine adapted *L. monocytogenes* on tomatoes; as well as the survival of non-adapted, heat adapted and chlorine adapted biofilms after exposure to sodium dodecyl sulphate (SDS), levulinc acid, sodium hypochlorite solution. Contact time of 1, 3 and 5 minutes was used. The survival of *L. monocytogenes* was monitored at 0, 24, 48 and 72 hours. The sanitizers were used individually or combined as follows; 1% sodium dodecyl sulphate individually; 0.5% levulinic acid individually; 200 ppm sodium hypochlorite solution individually and 0.5% levulinic acid/0.05% sodium dodecyl sulphate in combination (mixture). The samples were kept at 4 $^{\circ}$C throughout the period of assessment. The effect of these sanitizers on pH, total soluble solids (TSS) and titratable acidity (TA) was also determined. Furthermore, the attachment of *L. monocytogenes* on tomatoes was investigated using a scanning electron microscope.

Highest log reduction of non-adapted *L. monocytogenes* were observed on tomatoes treated with 1% SDS and least log reduction was achieved when tomatoes were treated with sodium hypochlorite solution. Though the log reduction achieved by 0.5% levulinic acid was higher that sodium hypochlorite solution, it was lower than log reduction achieved when 0.05% SDS / 0.5% levulinic acid mixture was used for all contact times. Using non-adapted *L. monocytogenes*, SDS was able to destroy all *L. monocytogenes* at 1, 3 and 5 minutes contact time. The trend was the same when heat adapted and chlorine adapted *L. monocytogenes* were used. There was no significant log reduction observed with biofilms. More favourable results were observed as contact time was increased from 1 to 5 minutes. Though there was a decrease in surviving bacteria from 1 to 3 minutes contact time, this decrease was not significant.

The study investigated if exposure to sanitizer has an effect on pH, titratable acidity (TA) and total soluble solids (TSS) of the tomatoes. It was revealed that levulinic acid and mixture can have detrimental effect on pH, TA and TSS of tomatoes. The TA and TSS of samples treated with levulinic acid and mixture varied significantly ($P \leq 0.05$) compared to the control sample. Although the TA and TSS of samples treated with SDS and sodium hypochlorite solution were different from the control, the differences were not significant.

As much as sanitizers have the potential to reduce the bacterial population in fresh produce they may not completely destroy pathogens. Chlorine based sanitizers such as sodium

hypochlorite though frequently used in the fresh produce industry, are not the best sanitizer to be used against food borne pathogens. Other sanitizers such as SDS used alone or in combination with another sanitizer can achieve better results than the widely used sodium hypochlorite solution as observed in this study. Stress adapted pathogens become less responsive to sanitizers during subsequent treatments. Through this research, it was established that biofilms are resistant to sanitizers. Though application of sanitizers in fresh produce is cheaper and simpler to apply, there is need to monitor varying concentrations of sanitizers, contact time and minimise contact with sub-surfaces as this could lead to sensory quality losses.

Keywords: adapted biofilms, heat adapted *L. monocytogenes,* chlorine adapted *L. monocytogenes*, food borne illnesses, fresh produce

DECLARATION

I, **ELIZABETH MNYANDU** hereby certify that I am the sole author of this thesis. I certify that, to the best of my knowledge, my thesis does not infringe upon anyone's copyright nor violate any proprietary rights and that any ideas, techniques, quotations, or any other material from the work of other people included in my thesis, published or otherwise, are fully acknowledged in accordance with the standard referencing practices.

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Signed……. Date……..

As Research Supervisor, I agree to submission of this thesis for examination.

Signed…… Date……

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As Research Co-supervisor, I agree to the submission of this thesis for examination.

Signed…… Date……..

Prof. Suren Singh

DEDICATION

To Hloniphani Lifa Mnyandu and Mpumelelo Mziwandile Mnyandu

Luke 18:27

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ACRONYMS

ANOVA	Analysis of variance
AEW	Acidified electrolysed water
ClO_2	Chlorine dioxide
EW	Electrolysed water
HPP	High pressure pasteurisation
HSP	Heat shock proteins
Levulinic	Levulinic acid
LM	*Listeria monocytogenes*
Mixture	A combination of 0.5% levulinic acid and 0.05% sodium dodecyl sulphate
NaClO	Sodium hypochlorite solution
NAOH	Sodium hydroxide
PB	Phosphate buffer
PEF	Pulsed Electric field
ppm	Parts per million
QAC	Quaternary ammonium compound
RTE	Ready to eat foods
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS/Lev	A mixture of levulinic acid and SDS
SEM	Scanning electron microscope
SPSS	Statistical package for social sciences
TA	Titratable acidity
TSS	Total soluble solids
UV	Ultra violet light

LIST OF PUBLICATIONS AND CONFERENCES

1. MNYANDU, E., IJABADENIYI, O, A. & SINGH, S. 2014. Inactivation of *Listeria monocytogenes* ATCC 7644 biofilms using sodium dodecyl sulphate, levulinic acid and sodium hypochlorite solution. *Journal of pure and applied microbiology*, 8**,** 1973-1980.

2. MNYANDU, E., IJABADENIYI, O, A. & SINGH, S. 2014. Inactivation of *Listeria monocytogenes* ATCC 7644 using sodium dodecyl sulphate, levulinic acid and sodium hypochlorite solution. *Italian Journal of Food Science*, doi:10.1080/02652030110028353 [Accepted].

3. MNYANDU, E., IJABADENIYI, O, A. & SINGH, S. *20th SAAFOST Biennial International Congress & Exhibition.* CSIR Convention Centre, Pretoria. 7 – 9 October 2013.

CHAPTER ONE: GENERAL INTRODUCTION

1.1 Problem statement

Food borne illnesses are on the increase despite measures being taken to reduce them (CDC., 2011). High ranking food borne illnesses are caused by food borne pathogens such as *Escherichia coli, Listeria monocytogenes* and S*almonella* spp, among others. These pathogens naturally exist in the environment and are capable of getting in contact with foods by various means. They are also responsible for the increasing cases of food borne illnesses associated with fresh produce (Erickson, 2012). This places the consumers at risk as consumer's demands have shifted towards consumption of minimally processed foods for health and convenience reasons (Fernandez *et al.*, 2012, CDC., 2011). Consumption of fresh produce and minimally processed foods is also encouraged among children, pregnant women and the immune compromised populations particularly those with HIV/ AIDS (Beuchat, 2012, Trevejo *et al.*, 2005). It is paramount for this population to be protected against food borne illnesses.

Food borne pathogens can exist as single bacteria or in form of biofilms in a three dimensional structure (Bae *et al.*, 2012). Biofilms are difficult to penetrate hence any form of treatment may not be sufficient to eradicate them on food surfaces (da Silva Meira *et al.*, 2012). Recent research has also shown that food borne pathogens can adapt to several forms of stress such as extreme temperatures, pH changes, antibiotics and sanitizers (Bibek and Arun, 2013). When the pathogens develop stress adaptation they become resistant to that same stress in subsequent treatments (Bridier *et al.*, 2011). It is because of this and their ability to aggregate into biofilms that contribute to the challenges being faced in the food industry.

Efforts have been made to come up with methods that can totally destroy these pathogens of concern. Current research shows that several methods such as the use of sanitizers, high pressure pasteurisation, irradiation and high temperature long time processing have been employed on several foods against these pathogens. On fresh produce, quaternary based compounds, chlorine based compounds, organic acids and chemical preservatives have also been tested (Mani-López *et al.*, 2012). Some of these methods have detrimental effects to the final quality of the processed produce (Alexandre *et al.*, 2012, Mukhopadhyay and Ramaswamy, 2012) and hence can be used at minimal concentrations or short contact times to achieve high log reduction and minimal sensory quality damage.

Most fruits and vegetables are consumed raw or minimally processed. Due to their sources, they create a suitable habitat for growth of a wide range of pathogens some which are not destroyed by subsequent processing. Contamination of fresh produce can occur at different points along the food chain; from harvesting, transportation, processing, post-processing handling up to consumption (Zhao *et al.*, 2009). It is important to find means of decontaminating fresh produce before consumption. *Listeria monocytogenes* have been cited to be among the food borne pathogens of concern (Muñoz *et al.*, 2012). Though commonly associated with dairy products, current research has shown that they are also associated with fresh produce (Olaimat and Holley, 2012). They have also been detected in commonly consumed vegetables such as tomatoes (McCarthy and Burkhardt, 2012).

Tomatoes are among the popularly consumed vegetables (García-Valverde *et al.*, 2013). They are used as part of other dishes or they can be consumed as fruits. Tomatoes can be consumed raw or cooked. They are very rich in antioxidant compounds such as carotenoids, lycopene and flavonoids (Reif *et al.*, 2013). They are also rich in vitamin A, vitamin C and mineral elements (Hermsdorff *et al.*, 2012). The study tested *L. monocytogenes* ATCC 7644, on tomatoes and on biofilm slides. From non-adapted *L. monocytogenes* ATCC 7644 heat adapted and chlorine adapted *L. monocytogenes* were produced. Heat adapted and chlorine adapted biofilms were also prepared and subjected to the action of above mentioned sanitizers.

1.2 Research question

Incidence of fresh produce related food borne illnesses are on the increase despite several intervention methods being deployed to reduce their occurrences.

1.3 Aim and objectives

1.3.1 Aim

To determine the effectiveness of sodium dodecyl sulphate, levulinic acid and sodium hypochlorite in reducing the survival of *L. monocytogenes* ATCC 7644 on tomatoes

1.3.2 Specific objectives

i. To determine the survival of *L. monocytogenes* ATCC 7644 on tomatoes after exposure to sodium dodecyl sulphate, levulinic acid and sodium hypochlorite solution

ii. To determine the survival of heat adapted and chlorine adapted *L. monocytogenes* ATCC 7644 on tomatoes after exposure to sodium dodecyl sulphate, levulinic acid and sodium hypochlorite solution

iii. To determine the survival of non-adapted, heat adapted and chlorine adapted *L. monocytogenes* ATCC 7644 biofilms after exposure to sodium dodecyl sulphate, levulinic acid and sodium hypochlorite solution

iv. To determine the effect of sodium dodecyl sulphate, levulinic acid and sodium hypochlorite solution on the physicochemical properties of fresh tomatoes (pH, titratable acidity and total soluble solids)

v. To determine the attachment of *L. monocytogenes* ATCC 7644 on tomatoes using a scanning electron microscope

1.4 Study limits and assumptions

This study was done at Durban University of Technology, South Africa. The heat adapted, chlorine adapted bacteria as well as the biofilms were prepared in Durban University of Technology Research Laboratory by the researcher, using non adapted *L. monocytogenes* ATCC7644 purchased from Merck South Africa.

1.5 Outline of the thesis

Chapter 1 presents the background to the problem and the objectives addressed in this research. Chapter 2 reviews the literature on food borne illnesses occurrences, common food borne pathogens, novel methods of food preservation and the sanitizers used in food processing plants to reduce microbial load. The importance of tomatoes as food, benefit of fresh produce and minimally processed foods is also addressed.

Chapter three, four and five that follow present papers that cover the five objectives of this study. Chapter three reports on the investigation of action of sodium dodecyl sulphate, levulinic acid and sodium hypochlorite on non-adapted *L. monocytogenes.* The effects of these sanitizers on physicochemical properties of tomatoes and results from scanning electron microscope are reported in the same chapter. Chapter four reports on the

investigation of action of sodium dodecyl sulphate, levulinic acid and sodium hypochlorite on heat adapted and chlorine adapted *L. monocytogenes.* Chapter five reports on the investigation of action of sodium dodecyl sulphate, levulinic acid and sodium hypochlorite on non-adapted, heat adapted and chlorine adapted *L. monocytogenes* ATCC 7644 biofilms. General discussion, conclusions and recommendations are presented in Chapter six.

CHAPTER TWO: LITERATURE REVIEW

2.1 Background and occurrence

Listeria monocytogenes is a food borne pathogen that is responsible for food related listeriosis (Chen *et al.*, 2012, Swaminathan and Gerner-Smidt, 2007). It is a slender, short, gram-positive rod, with peritrichous flagella (Chaturongakul *et al.*, 2008). The organism is psychrophilic and facultative, and grows in a wide range of pH (Moltz, 2005). Though *L. monocytogenes* is associated with dairy products, it has been detected in a variety of foods including raw meat, cheese (Gunasena *et al.*, 2013, Chavant *et al.*, 2004), different pasteurised products and even in fruits and vegetables (Khelef *et al.*, 2006, Ooi and Lorber, 2005); as well as in ready to eat (RTE) meats and hot dogs (Guenther *et al.*, 2009). *L. monocytogenes* has also been detected in sausages (Thévenot *et al.*, 2005).

There are six species currently recognized which are *Listeria monocytogenes*, *Listeria innocua*, *Listeria ivanovii*, *Listeria seeligeri*, *Listeria welshimeri* and *Listeria grayi*. Only two species of the genus are generally considered to be pathogenic, *L. monocytogenes* in humans and *L. ivanovii* in other mammals. In 1981 Canada experienced a first time an outbreak of listeriosis which was linked to a contaminated food source (Gasanov *et al.*, 2005). The first isolation of a multiresistant strain was in France in 1988 (Wang *et al.*, 2013).

L. monocytogenes is considered a pathogen that causes serious threat to public health by several researchers (CDC., 2011, Gilmour *et al.*, 2010, Guenther *et al.*, 2009, Chaturongakul *et al.*, 2008, Denny *et al.*, 2007). With regards to fresh produce safety, this pathogen has also been implicated as a high focus point (CDC., 2011, Foulds, 2011, Berger *et al.*, 2010, Fretz *et al.*, 2010a, Chaturongakul *et al.*, 2008, Goulet *et al.*, 2008, Akbas and Ölmez, 2007, Denny *et al.*, 2007). It can multiply rapidly at intracellular level and on food substances (Liu, 2006). Laboratory experiments have shown that the number of *L. monocytogenes* can increase to 10^7 organisms per gram of product without visible deterioration (Ooi and Lorber, 2005, Doyle, 2001) and it thrives better on cut vegetables, including tomatoes (Huff *et al.*, 2012, Moreno *et al.*, 2012). Sauders and Wiedmann (2007) report that this pathogen is capable of surviving harsh natural environment such as soils, streams and within food processing environments. Due to the properties exhibited by this pathogen, fruits and vegetables may therefore be inadvertently contaminated before or after processing. Table 2.1 below shows conditions that promote the growth of *L. monocytogenes*.

Table 2.1 Factors identified to have an impact on the growth and survival of *L. monocytogenes* (Ágoston, 2009)

Parameter	**Minimum**	**Maximum**
Temperature	0 – 2.5 ºC	43 ºC
Water activity (aw)	0.89	0.9
pH	4.5 (Optimum pH 6.5 – 7.5)	9
Salt concentration (%)	<0.5 (optimal 0.7)	12
Atmosphere	Facultative anaerobe, psychrophilic	
Heat resistance	Heat resistance	Heat D63,0 (milk) 62 sec

Listeria monocytogenes can enter food at any stage along the food chain from harvesting, cooking and packaging (Khelef *et al.*, 2006). Handa *et al.* (2005) investigated the incidence of *L. monocytogenes* in raw fish, shellfish and fish roe collected from randomly selected retail stores in and around Tokyo, Japan. Out of the 280 samples examined 10 were positive for *L. monocytogenes* of which 7 were fish roe (cod, salmon) and 3 were minced tuna. *L. monocytogenes* was also detected in turkey meat in Denmark in 2001 (Olsen *et al.*, 2005). Studies on seafood salad using different storage temperatures and varying pH showed that *L. monocytogenes* can grow in seafood salad under aerobic and vacuum conditions (Hwang and Tamplin, 2005). Another study in Northern Spain revealed that ready to eat (RTE) smoked fish was the most frequently contaminated food category (Garrido *et al.*, 2009).

In a study undertaken in UK on RTE meat and seafood, overall contamination of *Listeria* spp. and *L. monocytogenes* found in samples of mixed salads was 10.8 and 4.8%, respectively. Salad samples with meat ingredients were contaminated with *Listeria* spp. and *L. monocytogenes* twice as much compared to samples with seafood ingredients (Little *et al.*, 2007). Hwang and Marmer (2007) investigated and modelled the behaviour of *L. monocytogenes* in egg salad and pasta salad as affected by mayonnaise pH and storage temperature. *L. monocytogenes* was able to grow in both salads regardless of the mayonnaise pH at each storage temperature. Rapid growth was observed in egg salad than in pasta salad. In another recent study, salads also had the highest prevalence of *L. monocytogenes* (Jamali *et al.*, 2013). Despite the fact that fruit and vegetables are associated with contamination with *L. monocytogenes*, they are still an important part of human diet.

2.2 Importance of fresh and minimally processed fruits and vegetables.

Vegetables are an important source of minerals and vitamins (Stipanuk and Caudill, 2013). Minimal processing preserves delicate nutrients that would be lost due to extended processing and preserves sensory attribute (Brennan and Grandison, 2012). Minimally processed fruits and vegetables are important components of a balanced diet. Their consumption is encouraged to protect against a range of illnesses such as cancers (Rao and Rao, 2007) and cardiovascular diseases (Willcox *et al.*, 2003). Improving technologies together with increased consumer awareness has increased the consumption of minimally processed foods, as consumers have shifted their eating habits. Consumers are becoming sensitive to prolonged processing methods, the use of synthetic additives used to preserve food or enhance attributes such as colour, appearance and flavour. Minimal processing techniques have emerged to replace traditional preservation methods while retaining nutritional and sensory quality. Vegetables are convenient to prepare, can be consumed raw and when fresh they have a high nutrient content, (Corbo *et al.*, 2005). Among other vegetables, tomatoes are also considered an important element of diet.

2.2.1 Importance of tomatoes in diet

Tomatoes are the widely consumed vegetables around the world and a third leading contributor of vitamin C. They are also a fourth leading source of vitamin A (Sanchez-Moreno *et al.*, 2004). Tomatoes are consumed in their raw state and also used for the preparation of a wide range of food products. They are encouraged in the diets of pregnant women (Strobel *et al.*, 2007), infants and the immune compromised population as well as physically fit population due to their high nutritional value (Rao and Rao, 2007).

Tomatoes and tomato products provide a convenient matrix by which nutrients and other health related food components are supplied to human beings because they are rich sources of folate, vitamin C, and potassium (Willcox *et al.*, 2003); as well as vitamin E, trace elements, flavonoids, phytosterols (Frusciante *et al.*, 2007). Tomatoes have a high levels of antioxidants such as polyphenols (including flavonoids) and carotenoids (such as lycopene and β-carotene) (Hallmann, 2012). Their consumption has been correlated to a reduction in risk of cancers and cardiovascular diseases (Borguini and Ferraz Da Silva Torres, 2009).

2.3 The history of outbreaks of listeriosis

As the demand for consumption of fresh produce increases, it also poses a health risk to the consumers concerned because they provide a good growth condition for pathogenic micro-organisms (Hamon *et al.*, 2006). According to Centre for Disease Control CDC. (2011) an increasing consumption of fresh produce is attributed to promotion of fruits and vegetables as an important part of a nutritive diet and also because of their convenience in preparation (Akbas and Ölmez, 2007). There is also a rise in popularity of salad bars which thereby promote consumption of foods prepared away from home.

Food borne outbreaks have been traced to minimally processed vegetables and this has resulted in increased desire to know more about the microbiological safety of fresh whole and cut produce. The occurrence of food borne illnesses is also caused by global trade which facilitates movement of commodities from one country to another, thereby increasing human exposure to a variety of food borne pathogens, including *L. monocytogenes* (CDC., 2011). Recent studies estimated that food borne illnesses associated with fresh produce account for 24% of total illnesses; salads, vegetables and fruits were linked to 345, 228 and 111 illness outbreaks, respectively (Olaimat and Holley, 2012). In South Africa a high percentage of the population is susceptible to food borne diseases but there is little reliable data and statistics on the subject. Cases of food borne illnesses have been reported to have doubled in some developing countries in recent years (Foulds, 2011).

Cases of listeriosis infection traced to a processing plant in New York resulted in 4 deaths and 3 miscarriages in year 2000 (Olsen *et al.*, 2005). Another outbreak associated with turkey meat was reported in 9 states in the United States; out of the 54 patients identified, 8 died and 3 pregnant women had foetal deaths (Gottlieb *et al.*, 2006). The researcher also claims that 2500 Americans are affected by listeriosis annually. In France, the incidence of listeriosis increased to 4.7 cases per million persons in 2006 (Goulet *et al.*, 2008).

Mead *et al.* (2006) reported an outbreak of listeriosis associated with contaminated frankfurters and deli meats in the United States. This outbreak was reported as the second largest outbreak of listeriosis in US history, with over 100 reported cases and 14 fatalities. Denny *et al.* (2007) reported a mortality rate of up to 14% in European Union in 2006. In 2008, an outbreak associated with RTE meat products was reported in multiple provinces of Canada (Gilmour *et*

al., 2010). Other outbreaks have also been reported in North America, Europe and Japan (Swaminathan and Gerner-Smidt, 2007).

Germany and Austria suffered another outbreak in 2009 which was associated with acid cheese which comprised 14 cases 5 of which were fatalities caused by *L. monocytogenes* (Fretz *et al.*, 2010b). Fretz *et al.* (2010a) reported another multinational outbreak in 2009 which involved 34 cases of invasive listeriosis in which 25 cases originated from 7 Austrian provinces; of these; 4 patients had meningitis and 8 had a fatal outcome. Of the affected patients, 1 patient was from the Czech Republic while 8 patients were from 4 German federal states. In South Africa *L. monocytogenes* were successfully isolated from avocados (Strydom *et al.*, 2013) and also in Botswana (Morobe, 2009). Measures should be taken to decontaminate them before or during processing and if they are to be consumed raw so that their safety may be assured.

2.4 Other pathogens associated with food borne illnesses

Apart from *L. monocytogenes* many bacterial pathogens are associated with food borne illnesses. However some occur in rare cases than others. Table 2.2 below shows a list of food pathogens that have been of greater concern to the 21st century food safety arena.

Table 2.2 Recent multinational foodborne outbreaks associated with fresh produce (Lynch *et al.*, 2009)

Year	Pathogen	No. of cases	No. of countries	Affected regions	Implicated food
2008	*Salmonella Saintpaul*	1442	2	North America	Fresh peppers, tomatoes
2007	*Salmonella Senftenberg*	51	5	Europe, North America	Fresh basil
2007	*Shigella sonnei*	175	2	Australia, Europe	Raw baby corn
2006	*E. coli O157:H7*	206	2	North America	Fresh spinach
2006	*Salmonella thompson*	20+	3	Europe	Ruccola

In addition to the pathogens listed above, research shows that *Salmonella enteritidis* and *Campylobacter* outbreaks linked to poultry and poultry products are often in the EU. *Salmonella typhiumurium* outbreaks are common in Australia and New Zealand (Greig and Ravel, 2009). There are also other pathogens that cause food borne illnesses but have not been

of major concern to food safety as they rarely cause food borne illnesses. These are listed in the Table 2.3 below;

Table 2.3 List of pathogens that cause food borne illnesses (FDA, 2012)

Pathogen	Sources	Associated food
Bacillus cereus	Cause of illness: large molecular weight protein (diarrheal type) or highly heat-stable toxin (emetic type)	Meats, milk, vegetables, fish, rice, potatoes, pasta, and cheese
Campylobacter jejuni	Infection, even with low numbers	Raw milk, eggs, poultry, raw beef, cake icing, water
Clostridium botulinum	Toxin produced by Clostridium botulinum	Low-acid canned foods, meats, sausage, fish
Clostridium perfringens	Undercooked meats and gravies	Meats and gravies
Cryptosporidium parvum	Drinking contaminated water; eating raw or undercooked food; putting something in the mouth that has been contaminated with the stool of an infected person or animal; direct contact with the droppings of infected animals.	Contaminated water or milk, person-to-person transmission (especially in child day care settings). Contaminated food can also cause infections.
Giardia lamblia	Strain of *Giardia lamblia*	*Giardia* is found in soil, food, water, or surfaces that have been contaminated with the feces from infected humans or animals.
Hepatitis A	Hepatitis A Virus Symptoms: fever, malaise, nausea, abdominal discomfort	Water, fruits, vegetables, iced drinks, shellfish, and salads
Norovirus	Infection with Norwalk virus	Raw oysters/ shellfish, water and ice, salads, frosting, person-to-person contact
Oxoplasma gondii	Parasitic infection	Cat, rodent or bird faeces, raw or undercooked food.
Vibro cholerae	Excretion of toxin from infected fish and shellfish	Fish and shellfish and other foods; water
Yersiniosis	Infection with *Yersinia enterocolitica*	Raw milk, chocolate milk, water, pork, other raw meats

Pathogens get into contact with food through accidental contamination in food chain. Some of the sources of contamination are indicated below.

2.5 Sources of contamination

As much as minimally processed fruits and vegetables have credit due to their nutritional value, they are also important vehicles for transmission of food borne pathogens which are commonly associated with foods of animal origin (Corbo *et al.*, 2005). Many incidences of cross contamination have been reported and contamination occurs through various means. Sofos (2008) implied cross contamination from other ingredients such as food additives such as food colourants and preservatives, or from work surfaces such as cutting boards. Other studies indicate that direct contact of produce or seeds with contaminated manure and irrigation water leads to contaminated crops (Hanning *et al.*, 2009).

Significant contamination is also caused by the use of animal waste as manure, during farming (Mukherjee *et al.*, 2007). Lynch *et al.* (2009) suggested that subsequent processing after harvesting such as cutting, coring, slicing and handling during shipping expose inner tissues of produce to microbiological contact. Proximity of irrigation wells and water bodies exposed to faeces from animals as well as exposure of fields to animal waste materials have also been implicated (Ijabadeniyi *et al.*, 2011b, Doyle and Erickson, 2008). Table 2.4 shows sources of pre harvest and post-harvest contamination.

Table 2.4 Pre-harvest and post – harvest sources of contamination (Beuchat, 2006)

Pre harvest	**Postharvest**
Faeces	Faeces
Soil	Human handling (workers, consumers)
Irrigation water	Harvesting equipment
Green or inadequately composted manure	Wild and domestic animals (including fowl and reptiles)
Air (dust)	Insects
Wild and domestic animals (including fowl and reptiles)	Air (dust)
Insects	Wash and rinse water
Human handling	i) Sorting, packing, cutting, and further processing equipment, ii) Ice, iii) Transport vehicles iv) Improper storage (temperature, physical environment), v) Improper packaging (including new packaging technologies) vi) Cross-contamination (other foods in storage, preparation, and display areas) vii) Improper display temperature

Fruit and vegetable handling should be monitored through processing to minimize contamination that may lead to increased food borne outbreaks.

2.6 Reasons for increased food borne illnesses

Despite efforts being made to curb food borne illnesses, the levels of outbreaks are still high (CDC., 2011). These are attributed to several factors among which are changes in eating habits, and international trade (Oliver *et al.,* 2005). It is also attributed to evolving pathogenic microorganisms or resistance to antibiotics (Sofos, 2008, Angulo and Mølbak, 2005). According to Sofos (2008), food borne illnesses are also increasing due to cross-contamination of foods and water with enteric pathogens of animal origin. This contamination could occur during harvesting and transportation. Contamination can also be acquired from food additives and chemical residues that come into contact with fresh produce or foods being processed. Lack of improved test methodologies for pathogen detection can also result in continued and increased contamination. Outbreaks are also increasing due to tendencies of eating foods raw in modern societies. Oliver *et al.* (2009) found that many people still consume milk raw due to enhanced nutritional qualities, taste, and health benefits. Mitchell *et al.* (2007) also attributed the increase to unsafe food handling practices in food service establishments as a major contributor to the transmission of food-borne illness.

Other reasons for increasing outbreaks as identified by Newell *et al.* (2010) are rapid population growth and a demographic shift towards an ageing population; improved transport logistics and conditions, which enable agents to survive on food products and reach the consumer in a viable form; an increasingly transient human population carrying its intestinal flora worldwide; higher proportions of immunologically compromised individuals either as a consequence of changing demographics producing an increasingly elderly population or the generation of highly susceptible groups with immunosuppressive diseases or treatments; changing farming practices for example, intensification to produce cheaper food or a shift to free-range/organic animal production to respond to consumer welfare concerns; climate change for example, bringing novel vectors into temperate regions or temperature-associated changes in contamination levels. Increased outbreaks could also be attributed to development of bacterial resistance, making it difficult to reduce or effectively combat the pathogens associated with food borne illness.

2.7 Development of bacterial resistance

Bacterial resistance can develop due to continued exposure to one chemical, biofilm formation or stress adaptation.

2.7.1 Antibiotic resistance

Bacteria are highly adaptable organisms; capable of developing resistance to antibiotics (Mathur and Singh, 2005). This can occur due to various mechanisms such as chromosomal mutations and transfer of resistance determinants borne on genetic elements (Walsh and Fanning, 2008). Mechanism of resistance is attributed to production of enzymes that inactivate antimicrobial agents through degradation or structural modification thereby, changing the pathogen to a slightly different or more adapted strain. Reduction of bacterial cell permeability to antibiotics can also result in resistance. Some bacteria have the capability to activate antimicrobial efflux pumps and modify cellular drug targets (Hur *et al.*, 2012). Antibiotic resistance determinants can be facilitated by food. Among others, *E. coli, L. monocytogenes, Salmonella, Campylobacter*, *Shigella, Vibrio* spp., *methicillin, Staphylcoccus aureas* and *Enterococci* have been implicated as developing antibiotic resistance (Newell *et al.*, 2010). *Listeria monocytogenes* was found to show resistance to clindamycin, linezolid, ciprofloxacin, ampicillin, rifampicin, trimethoprim/sulphamethoxazole and vancomycin and tetracycline (Conter *et al.*, 2009). It is because of antibiotic resistance that food borne illnesses are on the increase as most food borne pathogens can easily develop resistance and the once effective antibiotics can no longer destroy the pathogen of concern. Apart from antibiotic resistance is also stress adaptation.

2.7.2 Stress adaptation

Yousef and Courtney (2003) define stress adaptation as any deleterious physical, chemical or biological factor that adversely affects microbial growth or survival. Exposure of micro-organisms to temperatures above optimal growth temperatures for short periods of time results in unique physiological responses being triggered within the cells along with the synthesis of heat shock proteins (Rodriguez-Romo *et al.*, 2006). This will cause extended tolerances to multiple stressors. Stressors also cause microorganisms to exhibit cross-protection mechanisms against other food preservation techniques (Rodriguez-Romo *et al.*, 2005). *Listeria monocytogenes* and other bacteria can survive under low temperature, acid stress and or osmotic stress through several mechanisms. Some of the mechanisms include change in membrane composition, changes in gene expression and induction of proteins synthesis (Gandhi and Chikindas, 2007).

Previous studies established that gradual exposure to acidic conditions of *Salmonella* spp caused cross protection against lethal heat treatments (Bacon *et al.*, 2003). Skandamis *et al.* (2008) evaluated the adaptive responses to heat (52, 57 and 63 °C) or lactic acid (pH 3.5) by a 10-strain composite of *L. monocytogenes* meat and human isolates at stationary phase, following exposure to combinations of osmotic (10% NaCl), acidic (pH 5.0 with HCl) and thermal (T; 46 °C) stresses within 1.5 hours. Adaptive responses were observed on *L. monocytogenes* at 57 °C for all treatments and no cross-protection was observed at 52 and 63 °C.

In another study, the growth of stationary phase *L. monocytogenes* was enhanced in cells grown on glucose containing media when exposed to various stresses (Koutsoumanis *et al.*, 2003). A study by Chen *et al.* (2009) revealed that the gene *lmo 0038* belonging to the peptidylarginine deiminase family, grows optimally under stress conditions such as low pH and heat shock (52 °C). The ability of *L. monocytogenes* to survive in low pH environments therefore can significantly influence survival and growth in foods as well as subsequent pathogenesis.

2.7.3 Biofilm formation and resistance

Also of concern in food manufacturing are biofilms. Microorganisms on wet surfaces have the ability to aggregate and grow to form micro colonies forming a biofilm. Their formation starts with motility to the surface, attachment, formation of clusters, development of differentiated structures, and dispersal (Wood *et al.*, 2011). Biofilms attach via appendages, such as fimbriae and flagella, and micro-colonies are formed by the production of microbial products including polysaccharides, proteins, lipids, and DNA and these play a structure-stabilizing and protective role in biofilm (Renier *et al.*, 2011). Environmental factors such as pH, water activity, temperature and nutrient composition of the matrix is important for the phenotypic transition of planktonic cells to sessile form (Belessi *et al.*, 2011). The resultant body exhibit different characteristics to a singular bacterium from which they were made and can either be mixed species or one type of bacteria (Bridier *et al.*, 2011).

Biofilm growth in food processing environments increases the opportunity for microbial contamination of the processed product (Joshua *et al.*, 2006). Formation of biofilms may be through the aggregation of spoilage and pathogenic bacteria which increases post-processing contamination and risk to public health. The phenotypic modification as a result of biofilm development into a three dimensional structure may lead to impaired activity of sanitizers as

sanitizers may not be able to penetrate into the biofilm. Continued use of the sanitizer on the biofilm may lead to adaptive response due to repeated exposure to sub-lethal concentrations (Bisbiroulas *et al.*, 2011, Chorianopoulos *et al.*, 2011). According to Stewart and Franklin (2008) resistance may be due to physiological heterogeneity created by uneven distribution of nutrients and oxygen during biofilm formation leading to expression of stress adaptive genes that will increase biofilm resistance.

Biofilm forming strains of *S. enterica*, *E. coli* O157:H7, *P. aeruginosa*, *K. pneumoniae*, and *A. baumannii* were found resistant when tested on four antibiotics (Kim and Wei, 2007). *Salmonella* biofilms increased adaptation to benzalkonium chloride after repeated exposure (Mangalappalli-Illathu and Korber, 2006). There could also be presence of oxidative response genes that protect biofilms against oxidising agents (Pham *et al.*, 2010) and the existence of efflux pumps in biofilms as their protective measure against toxins (Kvist *et al.*, 2008).

Elhanafi *et al.* (2010) and Gillings *et al.* (2008) identified quaternary ammonium compound resistant genes carried by transferrable genetic elements. These findings indicate that lateral transfer of genetic material such as plasmids, transposons or interogons may lead to the development of environmental adaptation. Biofilm resistance may also occur due to presence of multiple species in a biofilm as some strains may be protected from the sanitizer by their aggregation with others within the 3 dimensional structures (Van der Veen and Abee, 2011). Lapidot *et al.* (2006) noted that the ability (or inability) to penetrate plant tissues or the pre-existing biofilms and production of different polysaccharides other than cellulose provide the protection.

2.8 Use of chemical sanitizers to combat food borne bacterial pathogens

Spoilage and pathogenic micro-organisms should be critically controlled throughout the production chain of growing, processing, distribution and consumption (Riazi and Matthews, 2011). Washing of fresh fruits and vegetables using sanitizers is necessary as it improves food quality and microbial safety (Lynch *et al.*, 2009). Sanitizers have the ability to minimize the growth of food borne pathogens in fruits and vegetables. Several researchers have tested a number of sanitizers for their efficacy in reducing or totally inactivating pathogenic bacteria, such as *E. coli* O157:H7, *Salmonella* spp. and *L. monocytogenes*, on fresh produce. Various chemicals such as chlorine dioxide (ClO_2), hydrogen peroxide (H_2O_2), organic acids, trisodium phosphate, ozone and calcinated calcium solution among others, have been evaluated for their

action against foodborne pathogens on fresh produce (Koseki and Isobe, 2005). Further studies in the use of sanitizers are still being done as there are still challenges associated with food borne pathogens and use of sanitizers.

2.8.1 Use of oxidising agents and organic acids

Chlorinated water is the most frequently and widely used disinfectant method in food processing plants (Ruiz-Cruz *et al.*, 2007). Chlorine washing has a minimal sanitising effect and results in microbial reduction by a small margin as indicated by several researchers. Lin *et al.* (1996) cited in Gonçalves *et al.* (2005) observed that *L. monocytogenes* washed off the fish cubes at high level inoculation was not completely eliminated by the three chlorinating solutions used (aqueous chlorine, chlorine dioxide obtained in situ and commercial chlorine dioxide) up to 400 ppm. In another study it was found that chlorine worked better on the surfaces than on sub surfaces when used on spinach and tomatoes (Ijabadeniyi *et al.*, 2011b).

Stopforth *et al.* (2008) using acidified sodium chlorite found reduction in populations of *L. monocytogenes* (3 to 3.8 log CFU/g) which was more effective than chlorinated water (2.1 to 2.8 log CFU/g reduction). Hellstrom *et al.* (2006) investigated the efficacy of water, chlorinated water (100 ppm), acetic acid solution (0.05%) and commercial citric acid-based produce wash (0.25%) to reduce the population of *L. monocytogenes* on pre-cut lettuce, *L. monocytogenes* were reduced at maximum 1.7 log CFU/g and number of *L. monocytogenes* reached the inoculation level during 6 days of storage. This showed that acidified sodium chlorite does not eliminate *L. monocytogenes* on pre-cut lettuce and cannot be solely relied upon in producing pre-cut lettuce safely.

Treatment of vegetables with solutions of Enterocin AS-48 plus lactic acid, sodium lactate, sodium nitrite, sodium nitrate, trisodium phosphate, trisodium trimetaphosphate, sodium thiosulphate, *n*-propyl *p*-hydroxybenzoate, *p*-hydoxybenzoic acid methyl ester, hexadecylpyridinium chloride, peracetic acid, or sodium hypochlorite reduced viable counts of *L. monocytogenes* below detection limits (by approximately 2.6 to 2.7 log CFU/g) using the immersion treatment or further storage for 24 hours, depending on the chemical preservative concentration (Molinos *et al.*, 2005). In other studies using shredded carrots, the effectiveness of chlorine treatment was significantly higher than peroxy acetic acid with reductions of 2–3 logCFU/g, but lower than acidified sodium chlorite (ASC) (Ruiz-Cruz *et al.*, 2007). Acidified sodium chlorate was the most effective treatment in reducing pathogens at all concentrations

evaluated reducing *E. coli*, *Salmonella* and *L. monocytogenes* populations to undetectable levels, to reductions of 4.81, 4.84 and 2.5 log CFU/g, respectively. In another study on iceberg lettuce using organic acids and chlorine solution, log reductions of 1.0 and 2.0 log CFU/g were achieved for *L. monocytogenes* and *E. coli*, respectively (Akbas and Ölmez, 2007). Maximum reduction of these food borne pathogens was found when using organic acids.

While evaluating the resistance of biofilms of *L. monocytogenes* to peroxides, quaternary ammonium compounds and chlorine, Pan *et al.* (2006) found that the cells from Teflon were inactivated to a lesser extent (at most 0.3 log CFU/ cm^2) than those on stainless steel by all tested sanitizing agents. The effects of ozone (O_3) treatment on fresh strawberry and shredded lettuce quality were tested by varying ozone concentration, contact time, pH and temperature. Doses below 10 mg/L were not effective in eradicating micro flora grown on produce surfaces. Ozone treatments increased lettuce browning and firmness deterioration after 21 days of storage as compared to chlorine treatments. For strawberry, no significant difference in food quality was observed between ozone and chlorine treatments (Wei *et al.*, 2007).

The mesophiles, psychrotrophes and fungal populations of the fresh-cut peppers were reduced to 2.5, 3.3 and 1.8 log units, respectively, after exposure of pepper to gaseous ozone for three minutes (Horvitz and Cantalejo, 2012). Greene *et al.*(2005) cited in Tang *et al.* (2010) reported that ozonated water and chlorine have equivalent decontamination efficacies, however ozone does not require heating on application and its contact time is likely to be less than that of chlorine due to ozone being a more powerful oxidizing substance than chlorine.

Using blueberries and chlorine dioxide (ClO_2), ClO_2 was more effective in reducing *L. monocytogenes* as compared to *Pseudomonas aeruginosa*, *Salmonella Typhimurium*, *S. aureus* and *Y. enterocolitica* (Wu and Kim, 2007). (Mahmoud *et al.*, 2007) reported that approximately a 4.3–4.7 log CFU reduction per strawberry of all examined bacteria was achieved by treatment with 5 mg/l ClO_2 for 10 minutes. Other researchers have considered chlorine dioxide as a powerful oxidizing substance that does not form significant amounts of chlorinated by-products like chlorine (Gómez-López *et al.*, 2009).

2.8.2 Electrolysed water

Electrolysed water (EW) is a chemical sanitizer produced using water and sodium chloride. It is easy to use, relatively inexpensive and environmentally friendly (Huang *et al.,* 2008). Acidic electrolyzed water (AEW) is a strong bactericide against most pathogenic bacteria in lettuce, alfalfa seeds, sprouts and tomato (Koseki and Isobe, 2005). It is produced by the electrolysis of an aqueous sodium chloride solution. It has been used on various food substances including fresh produce.

Electrolysed water was able to significantly reduce populations of *Escherichia coli* O157:H7, *Salmonella typhimurium* and *L. monocytogenes* from the surfaces of spot-inoculated green onions and tomatoes and higher reductions were achieved with increasing exposure time (Park *et al.,* 2008). Studies suggest that alkaline EW alone was not effective on *L. monocytogenes* biofilms while treatment with acidic EW only for 30 to 120 seconds reduced the viable counts in the biofilms by 4.3 to 5.2 log CFU per coupon. Reductions increased significantly by increasing exposure time. A combined treatment of alkaline EW followed by acidic EW produced an additional 0.3 to 1.2 log CFU per coupon reduction (Ayebah *et al.*, 2005). Electrolysed water has a potential to be used for decontamination of raw fish (Ozer and Demirci, 2006). Other studies by Fabrizio and Cutter (2005) however, did not produce favourable results when evaluating efficacy of acidic EW in RTE meat as increased contact time treatments did not attain regulatory requirements for control of *L. monocytogenes*.

2.9 Mode of action of sanitizers

Sanitizers have been used effectively against food borne pathogens. They interact with cell membrane causing irreversible damage to the membrane and genetic material (Gandhi and Chikindas, 2007). Earlier studies cited in Gómez-López *et al.* (2009) indicated that the lethal lesion produced by ClO_2 to microbial cells is related to protein synthesis. The destruction of the trans-membrane ionic gradient is caused by loss of permeability control at the physiological level of ClO_2 on bacterial cells and that of non-specific oxidative damage of the outer membrane. When cells of *Bacillus cereus* were treated with ClO_2 they were elongated, exhibited surface roughness and indentations while control cells remained uniform, with smooth surfaces (Peta *et al.,* 2003). Elongation of cells result from the inhibition of cell division and associated metabolic damage (López-Gálvez *et al.,* 2010). It was also reported that sodium

hypochlorite interferes with the germination of *B. subtilis* spores, possibly by damaging the spore's inner membrane (Young and Setlow, 2003).

The antimicrobial activity of hydrogen peroxide or sodium hypochlorite is attributed to the generation of hydroxyl radicals. They work through radical-mediated reactions oxidizing organic material. Hydrogen peroxide or hypochlorite solution can cause disruption and extraction of coat material of pathogens facilitating sanitizer penetration to the cortex and protoplast of bacterial spores (Chapman, 2003). Hypochlorous acid reacts rapidly with proteins, DNA, lipids, thiols and disulfides. High concentrations of hydroxyl chlorides have the ability to damage nucleic acids, proteins and lipids (Hawkins *et al.*, 2003). According to DeQueiroz and Day (2008), the action of hydrogen peroxide and sodium hypochlorite are a function of concentration, light, pH, temperature, heavy metals and organic matter.

Quaternary compounds (QAC) are used widely as surface and topical antimicrobials in general hygiene delivery due to their surfactant properties. The list includes benzalkonium chloride, cetrimide, barquat among others. The QACs are amphoteric surfactants containing one quaternary nitrogen associated with major hydrophobic substituents (Gilbert and Moore, 2005). The mode of action of QAC against bacterial cells involves a perturbation of lipid bilayer membranes and the outer-membrane of Gram-negative bacteria, leading to leakage of cytoplasmic material (Gilbert and Moore, 2005). Quaternary ammonium compounds a bind firmly to anionic sites on the membrane surface causing cells to lose osmo-regulatory capability and releasing potassium ions and protons. This interruption occurs even at low concentrations. This interrupts the membrane-located physiological activities such as respiration, solute transport and cell wall biosynthesis (López-Gálvez *et al.*, 2010). According to Gilbert and Moore (2005), action involves an association of the positively charged quaternary nitrogen with the head groups of acidic-phospholipids within the membrane causing the hydrophobic tail to inter-digitate into the hydrophobic membrane core at a molecular level. This interaction increases the surface pressure in the exposed membrane to decrease membrane fluidity and phase transition temperature. The membrane undergoes a transition from fluid to liquid crystalline state losing osmo regulatory and physiological functions.

2.10 Factors influencing action of sanitizers

Chemical sanitizers for washing fresh-cut produce are very promising tools used to reduce microbial risk. However, there are a number of factors that influence the action of the sanitizers. These are contact time, selectivity, pH of sanitizer, concentration and temperature of sanitizer solution; i.e., action of sodium hypochlorite is affected by pH, concentration, temperature and exposure time (Artés *et al.*, 2009).

For effective application of sanitizer, contact time should be monitored. Thus, the sanitizer has to be in contact with the affected surface or product over a specific period of time, depending on the type of product and extent of contamination. The longer the contact time the more effective the sanitizer (Park *et al.*, 2011). Surfaces need to be properly pre-cleaned to facilitate maximal action of sanitizers, surfaces with grease or foreign matter will impair the action of sanitizers. When chlorine based sanitizer is to be used, the presence of organic matter also needs to be considered as it impairs the action of chlorine (Artés *et al.*, 2009).

With regards to concentration, high concentration of chemical sanitizer proportionately leads to high rate of microbial destruction (Stebbins *et al.*, 2011). However, beyond a certain concentration the effectiveness level drops, thus further increase in concentration will have no advantage in destroying the microbes. Gómez-López *et al.* (2009) found that increasing the concentration of chlorine dioxide and the contact time increased their effectiveness against Bacillus *thuringiensis*. Zhao *et al.* (2009) reported similar results using SDS and levulinic acid on lettuce and poultry skin. When using chlorine, too high concentrations may lead to tainted products (Artés *et al.*, 2009). Quaternary ammonium compounds are effective against Gram positive, Gram negative and vegetative microorganisms and studies have shown that they are bacteriostatic at low concentrations and bactericidal at high concentrations (Velázquez *et al.*, 2009).

Selectivity and pH of sanitizer affect their effectiveness against bacteria. Chlorine is relatively non-selective and can destroy microorganisms at a wide range of pH (Estrela *et al.*, 2002). Iodophors and quaternary compounds have a limited activity due to narrower range and selectivity of these compounds; hence their application may be limited. The effectiveness of range of chlorine and iodine compounds decrease in increase in pH (McDonnell and Russell, 1999). High pH will lead to their decomposition, hence it is important to ensure that their pH is controlled. Most soap and detergents are alkaline with a pH between 10 and 12 and will work

well in these pH ranges. Chlorine is effective at pH around 6 to 7.5 to avoid corrosion (Artés *et al.*, 2009). Activity of sanitizer also depends on the temperature of solution. The activity of chemical sanitizers increases as the solution temperature increases. Recommended ranges of temperatures are 24 to 49 °C. Water temperature higher than 49 °C should be avoided when using chlorine and iodine compounds as their effectiveness will be reduced as they being evaporate (McDonnell and Russell, 1999).

2.11 Effect of sanitizers on sensory quality of fresh produce

Apart from being effective against food borne pathogens, sanitizers can have detrimental effects on the sensory quality of fresh produce. Several studies showed that sanitizers play an important role in determining the final sensory quality of fresh produce. Chlorine, the widely used sanitizer is known to be associated with reduced microbiological efficiency coupled with organoleptic properties as well as quality changes and the formation of carcinogenic chlorinated by-products (Rahman *et al.*, 2011). For effective results using sanitizers, concentrations and contact times should be strictly monitored.

The effects of levulinic acid in combination with SDS on the inactivation of *E. coli* O157:H7 and sensory quality of fresh-cut Iceberg lettuce in modified atmospheric packages during storage at 4 °C was investigated. Results showed that levulinic acid (0.5 to 3%), with 0.05% SDS caused detrimental effects on visual quality and texture of lettuce. Levulinic acid treated samples were organoleptically unacceptable due to the development of sogginess and softening after 7 and 14 days of storage (Guan *et al.*, 2010). In other studies using neutral and acidic electrolysed water on mizuna baby leaves at 5 °C, all treatments showed a slight decrease in the overall sensory quality after 7 days with no noticeable differences on visual appearance, browning, dehydration, off-odours and off-flavours. Further degradation and unacceptability at 5 °C was reached at 11 days (Tomás-Callejas *et al.*, 2011).

Studies using Tsunami and SDS revealed that tissue damage expressed as electrolyte leakage, total colour difference, firmness, and total aerobic plate counts were not significantly different among treatments on two types of lettuce samples. Treatment of Iceberg lettuce with sonication in combination with Tsunami or Tsunami + SDS did not degrade quality. For Romaine lettuce, chlorine-treated samples had a significantly higher overall quality score when compared to other treatments (Salgado *et al.*, 2013). Li and Wu (2013) found no significant difference in pH and total anthocyanin value between untreated and treated blueberries. Studies using

shredded carrots, combinations of alkaline electrolyzed water and citric acid did not affect sensory and microbial quality of the fresh-cut carrots but enhanced the overall shelf-life of the produce (Rahman *et al.*, 2011).

With regards to tomatoes pH, titratable acidity and total soluble solids affect its sensory quality of tomatoes. Interactions between sugar, organic acids, free amino acids, salts and volatile compounds contribute significantly to the perceptible flavour of fresh tomatoes (Yilmaz, 2009). Citric acid is abundantly present in tomatoes and contributes to titratable acidity. A pH of 4.4 has been indicated as maximum desirable for safety. The acidity of tomatoes is also important as a contributor to the flavour of its products (Anthon *et al.*, 2011). The pH and acidity of tomatoes should be monitored during sanitizer washing as increase or decrease in these parameters is detrimental to the quality of its product.

2.12 Novel methods used to combat food borne bacterial pathogens

Increasing numbers of food borne outbreaks of listeriosis have led to *L. monocytogenes* gaining increasing attention as a pathogen of public health concern (Scharff, 2012). Because of changing consumer perception towards chemical preservatives, attention is shifting towards natural alternatives to preserve foods and or minimal processing. While every effort should be made to prevent contamination of fruits and vegetables along the food chain, much improvement in processing methods is still needed to obtain products that satisfy consumer's needs (WHO., 2011). A number of novel methods have been applied in food processing in order to combat food borne pathogens. These include pulsed electric field, hydrostatic pressure and ionisation radiation technology. These methods are reviewed subsequently.

2.12.1 Pulsed electric field

In Pulsed electric field (PEF), food is processed by placing between electrodes with high voltage pulses in the order of 20-80 kV. Microbial inactivation arises from high voltage applied in the form of exponentially decaying, square wave, bipolar or oscillatory pulses at ambient, sub-ambient or slightly above-ambient temperatures (Aronsson *et al.*, 2005). After treatment, the food is aseptically packaged and refrigerated (Ramaswamy *et al.*, 2005).

Vegetative microorganisms in liquid media are damaged by a series of short high voltage pulses by expansion of existing or new pores (Castro *et al.*, 1993). Depending on the electric field intensity, pulse duration and number of pulses, the damage can be reversible or

irreversible (Wan *et al.*, 2009). Pulsed electric field treatment causes the membranes to be permeable to small molecules which causes swelling and then rupture of the cell membrane (Toepfl *et al.*, 2007).

Mosqueda-Melgar *et al.* (2007) successfully used PEF against *Salmonella enteritidis, E. coli* and *L. monocytogenes* populations inoculated in melon and watermelon juices. In that study, higher reduction of *L. monocytogenes* were observed when treatment time increased. A study on the effect of square-wave pulsed electric fields (PEF) on the inactivation of *L. monocytogenes* in mcIlvaine buffer at varying pH conditions showed that *L. monocytoges* was more PEF sensitive at higher electric field strengths and in media of low pH (Gómez *et al.*, 2005). Permanent loss on membrane integrity was also achieved in *L. monocytogenes* cells using PEF (García *et al.*, 2007). Pulsed electric field technology has successfully been used successfully in the pasteurization of foods such as juices, milk, yogurt, soups, and liquid eggs (Ramaswamy *et al.*, 2005).

2.12.2 High pressure processing

High-pressure processing (HPP) has been used successfully to improve microbiological safety of ready-to-eat foods. Due to technological development, high pressure processing has received increased attention in food processing (Sanchez-Moreno, *et al.*, 2004). The process causes damage to the cell membrane of microorganism causing changes in cell morphology, biochemical reactions and genetic material (Hogan *et al.*, 2005). The use of pressures within ranges of 300–700 MPa has been adopted for selective commercial applications by the food industry. High pressure pasteurisation has been used effectively in controlling *L. monocytogenes* in ready to eat meats (Quaranta *et al.*, 2007). It has been used successfully against *L. monocytogenes* and *Salmonella enterica* in cooked ham (Aymerich *et al.*, 2005). Treatment of Iberian and Serrano hams at 450 MPa for 10 minutes significantly reduced the population of *L. monocytogenes* by 1.50 and 1.16 log CFU/g, respectively without any detrimental effect on the sensory characteristics of the ham (Morales *et al.*, 2006). The major disadvantage regarding the application of HPP is its high cost (Patterson *et al.*, 2006). High pressure pasteurisation also presents several implementation challenges such as the modelling of food temperature, the determination of inactivation kinetics particularly for bacterial spores, and the prediction of chemical changes including the potential formation of toxic compounds (Mújica-Paz *et al.*, 2011).

2.12.3 Ionisation radiation

Food irradiation is a process exposing food to ionising radiations such as gamma rays emitted from the radioisotopes or high energy electrons and X-rays produced by machine sources, ultra violet and ultra sound (Christopher and Glenn, 2005). It is effective in controlling the growth of food spoilage and foodborne pathogenic bacteria on meat and poultry, fruits and vegetables, seafood and RTE meat products (Sommers and Boyd, 2006). Mahapatra *et al.* (2005) reported that ionising radiation is likely to be effective against all food spoilage and food poisoning microorganisms in all food matrices. Zhu *et al.* (2005) suggested that irradiation is an effective technology to eliminate *L. monocytogenes* but can negatively influence the quality of RTE meat products significantly. According to Mintier and Foley (2006) low-dose irradiation can effectively reduce or eliminate *L. monocytogenes* thereby improving the safety of RTE salads. Various effects can be achieved resulting in reduced storage losses, extended shelf life, improved microbiological and parasitological safety of foods depending on the absorbed radiation dose (Farkas, 2006).

Irradiation of broccoli and bean sprouts at 1.0 kGy resulted in reductions of approximately 4.88 and 4.57 log CFU/g, respectively, of a five-strain cocktail of *L. monocytogenes*; reductions of approximately 5.25 and 4.14 log CFU/g were achieved with cabbage and tomato respectively, at a similar dose (Bari *et al.*, 2005). In another study, *Listeria monocytogenes and S. typhimurium* could not be recovered from 2-kGy dose radiation-processed samples of cucumber and carrot after storage for up to 8 days (Dhokane *et al.*, 2006). Irradiation was also used successfully in meat sausages (Samelis *et al.*, 2005).

Though irradiation has been shown to be effective against food pathogens, Morris *et al.* (2007) indicated that irradiation is associated with undesirable sensory quality changes such as lipid oxidation, off-flavour and pink/red colour that affect product quality and acceptability by consumers. Irradiation also has a negative impact on fat soluble and water soluble vitamins (Dionísio *et al.*, 2009). For these reasons irradiation may be not suitable for a wide variety of foods.

2.13 Summary

Fresh produce is a high health risk food, hence control of food borne pathogens is paramount to promote the consumer's confidence and reduce incidence of food borne outbreaks associated with fresh produce. Decontamination methods that are easy to implement while preserving sensory qualities and nutrients of fresh produce are encouraged. Sensory attributes should be preserved because they are the primary factors that determine the acceptability of a product, hence they should be monitored to enhance consumption of fresh produce. To succeed in reducing the occurrence of food borne illnesses, an understanding of the complexity of food borne pathogens and most appropriate method to combat them at any level along the food chain is required. This can only be achieved through studies aimed at identifying suitable decontamination methods in particular to each food borne pathogen, as well as determining appropriate levels and exposure times for any chosen decontaminants.

CHAPTER THREE: INACTIVATION OF *Listeria monocytogenes* ATCC 7644 ON TOMATOES USING SODIUM DODECYL SULPHATE, LEVULINIC ACID AND SODIUM HYPOCHLORITE SOLUTION

3.1 Abstract

The human pathogen *Listeria monocytogenes* poses a serious threat to public health. A study was carried out to evaluate the effectiveness of four sanitizers, used individually or in combination on *L. monocytogenes*. The contact times for bacteria and sanitizer were varied to 1, 3 and 5 minutes. Levulinic acid, sodium dodecyl sulphate (SDS), sodium hypochlorite solution (NaClO) and a combination of SDS and levulinic acid were tested. Results revealed that 0.5% levulinic acid when used individually is capable of reducing the surviving LM by 3.63, 4.05 and 6.71 log CFU/ml after exposure for 1, 3 and 5 minutes, respectively. SDS resulted in an 8 log CFU/ml reduction after 1, 3, 5 minutes. A combination of 0.5% levulinic acid and 0.05% SDS caused a 3.69, 4.4 log and 7.97 log CFU/ml reduction for 1, 3, 5 minutes, respectively. Sodium hypochlorite solution was the least effective with 2.93, 3.16 and 4.53 log CFU/ ml reduction, respectively. When stored for up to 72 hours at 4 °C the surviving LM remained viable and decreased in number significantly ($P \leq 0.05$). Exposure to tomato sub-surfaces to sanitizers showed significant changes in pH and titratable acidity for mixture and levulinic acid. SDS can be used to replace the routine hypochlorite washing that has been implicated as not completely effective in reducing viability of food borne enteric pathogens, while monitoring concentrations and contact times to prevent loss in quality attributes of fresh produce.

Keywords: sanitizers, *L. monocytogenes*, foodborne illnesses, foodborne pathogens

3.2 Introduction

The increase in fresh produce consumption has caused a rapid evolution to the fresh fruits and vegetable industry (Johnston *et al.*, 2005). This, coupled with recommendations to eat minimally processed foods led to an increase in consumption of fresh fruits and vegetables among consumers (Berger *et al.*, 2010). Consumption of minimally processed foods and fresh produce has also been encouraged among children, pregnant women and the immune compromised populations, such as those infected with HIV/AIDS (Berger *et al.*, 2010, Gandhi

and Chikindas, 2007). Consumer demands and habits have also shifted with many consumers in the busy world preferring to eat ready to eat foods and eating from salad bars (Oms-Oliu *et al.*, 2010, Berdegué *et al.*, 2005).

A variety of fresh produce such as lettuce, cantaloupes, peppers, tomatoes, herbs and green leafy vegetables, among others are linked to food borne illnesses associated with either *Salmonella, Escherichia coli O157:H7 and Listeria monocytogenes* contamination (Tauxe *et al.*, 2010). Contamination of fresh produces by these pathogens occurs by various means. Ijabadeniyi *et al.* (2011a) reported irrigation water as a major pre-harvest source of contamination for fresh produce in South Africa. Other factors as cited by Johnston *et al.* (2005) include the use of biocides as fertiliser, poor worker hygiene and poor sanitation.

Listeria monocytogenes among other food borne pathogens, have been implicated as a public health threat (Velusamy *et al.*, 2010) estimated to cause about 1600 illness, more than 1400 hospitalisations and about 250 deaths per year in the United States (Kyle, 2012). They are responsible for carrying food borne listeriosis. They can grow in the soil, drains and food preparation surfaces (Gálvez *et al.*, 2010, Pan *et al.*, 2006, Djordjevic *et al.*, 2002). They have been largely associated with dairy products but recent research has also shown their increasing association with fresh produce (Gandhi and Chikindas, 2007) including tomatoes.

Tomatoes are widely consumed and can be eaten raw, partially cooked or processed into other products. They are a very rich source of carotenoids, folate, vitamin C, mineral elements and phenolic compounds (Frusciante *et al.*, 2007). Of major importance are the antioxidants (carotenoids). Epidemiological research has shown that the antioxidants are capable of preventing chances of developing cancers and cardio vascular diseases (Leonardi *et al.*, 2000). Tomatoes also provide a dietary source of soluble and insoluble fibres such as pectin, hemicellulose, and cellulose. Due to their nutritional value, they form an important part of the human diet. Elimination of food borne pathogen that can contaminate tomatoes is essential for preventing food borne illnesses that may be associated with the consumption of tomatoes.

Many methods are being used to attempt and eliminate the food borne pathogens. Use of phage or phage products in food production has been considered as a novel method for the bio-control of pathogens in fresh and ready-to-eat food products (Hagens and Loessner, 2010) but the cost associated with their use is very high. Other methods include bacteriocin-activated films high-hydrostatic pressure, high-pressure homogenization, in-package pasteurization, food irradiation, pulsed electric fields, or pulsed light and electrolysed water (Gálvez *et al.*, 2010).

Sanitizers such as carvacrol, vanillin, peroxyacetic acid, hydrogen peroxide, N-acetyl-l-cysteine and citrox among others have also been tested (Abadias *et al.*, 2011). Sanitizers affect microbial cell components for example proteins, DNA, RNA, cell wall constituents through physicochemical interactions or chemical reactions. They cause irreversible damage to these cell structures and loss of cell contents thereby rendering the bacteria inactive or dead (Cerf *et al.*, 2010).

Governing the action of sanitizers is contact time (exposure time), pH and temperature among other factors. Other researchers concluded that sanitizers are not effective in eradicating food borne pathogens when used individually, however, a combination of these agents increases the sanitizer ability (Sagong *et al.*, 2011, Zhao *et al.*, 2009). Recent studies have also shown that if not used properly, sanitizers can affect the quality of fresh produce (Salgado *et al.*, 2013, Guan *et al.*, 2010). With regard to tomatoes, pH and acidity are the most important determinants of tomato quality (Anthon *et al.*, 2011), hence the interaction of tomatoes with sanitizers during washing should be monitored. Levelunic acid and SDS are among the new sanitizers being tested in food industry in order to create an alternative to routine sodium hypochlorite washing that has been proven through research as not effective. The study was performed to evaluate the effectiveness of SDS, sodium hypochlorite solution, levulinic acid in reducing the viability of *L. monocytogenes* on tomatoes and the effect these sanitizers on pH, titratable acidity and total soluble solids.

3.3 Materials and methods

3.3.1 Materials

3.3.1.1 Fresh produce

Tomatoes were purchased from a local supermarket in Durban, South Africa on three separate occasions. On the day of purchase the tomatoes were washed in running water. The tomatoes were then washed in 70% alcohol (Ijabadeniyi *et al.*, 2011a). Prior to subjection to different sanitizer treatments the tomatoes were tested for the presence of *L. monocytogenes.*

3.3.1.2 Bacterial strains

Listeria monocytogenes ATCC 7644 (Merck, South Africa) was used for this study. The strain was cultured in Fraser broth for 24 hours at 37 ºC and stored at 4 ºC (Ijabadeniyi *et al.*, 2011a). Prior to each experiment, a fresh culture was prepared from the stock culture by sub culturing

in Fraser broth for 24 hours at 37 ºC. An 8 log CFU/ml culture of *L. monocytogenes* prepared using McFarland Standards and was used for inoculation (Ji *et al.*, 2010).

3.3.1.3 Chemicals and chemical treatments

Sodium dodecyl sulphate (SDS), levulinic acid, sodium hypochlorite solution, all purchased from Merck, South Africa were tested, individually or combined with contact times (1, 3, 5 minutes); for their effect on *L. monocytogenes* ATCC 7644 in tomatoes. The chemicals were used as follows;

1% SDS individually
0.5% levulinic acid individually
200 ppm sodium hypochlorite solution individually
0.5% levulinic acid/0.05% SDS mixture.

3.3.2 Methods

3.3.2.1 Inoculation of bacterial strains into tomatoes

The method of Zhao *et al.* (2009) was followed. A 25 g sample of tomatoes was cut into approximately 5 cm long pieces in the lamina flow hood. The samples were submerged into bacterial suspension (10^8 CFU/ml, 50 ml of bacterial solution into 950 ml of distilled water) for 60 seconds and then air dried for 20 minutes in the lamina flow hood. The samples were then suspended into 500 ml test solutions and agitated by a magnetic stirrer at 100 rpm for 1, 3, 5 minutes. Following treatment, the individual samples were placed in double zipper bags containing 25 ml of phosphate buffered saline and pummelled for one minute. The suspension was serially diluted (1:10) in 0.1% buffered peptone water and enumerated for *L. monocytogenes* ATCC 7644.

3.3.2.2 Enumeration of *L. monocytogenes*

A method by Taormina and Beuchat (2001) was followed. Populations of *L. monocytogenes* ATCC 7644 were determined by surface plating serially diluted samples; 0.1 ml in duplicates on *Listeria* Selective Agar (Oxoid Ltd, Wade Road, Basingstoke, Hants UK). Plates were incubated for 24 hours at 37 ºC after which colonies were counted using a colony counter.

3.3.2.3 Preparation of samples for scanning electron microscopy (SEM)

For SEM viewing, untreated samples and samples subjected to sodium hypochlorite solution, levulinic and SDS/levelunic acid mixture were used. A method used by Ijabadeniyi *et al.* (2011b) was followed with a few modifications. Pieces of tomatoes inoculated with *L. monocytogenes* ATCC 7644 and subjected to different treatments were cut in small pieces 2 x 2 mm using a sterile blade. Primary fixation was carried out in 2.5% glutaraldehyde for 12 hours, and rinsed three times in phosphate buffer (0.1 M, pH 7.0). Post fixation was done using 0.5% osmium tetroxide for one hour. Fixed samples were dehydrated in graded ethanol (30, 50, 75 and 100%) each for 5 minutes. The samples were then dried in a critical point dryer with carbon dioxide as a transition gas. The samples were mounted on specimen stubs and coated with gold palladium. The samples were then analysed using Desmond Clarence scanning electron microscopy.

3.3.3 Analysis of tomato physicochemical properties

3.3.3.1 Preparation of samples

The method of Zhao *et al.* (2009) was followed for sample preparation, except that tomato was further homogenised into slurry. A 25 g sample of tomatoes was cut into approximately 5 cm long pieces. The samples were then suspended into 500 ml test solutions as follows;

25 grams of tomatoes + 500 ml de-ionised water (control)
25 grams of tomatoes + 500 ml 1% SDS
25 grams of tomatoes + 500 ml of 0.5% levulinic acid
25 grams of tomatoes + 500 ml of 200 ppm sodium hypochlorite solution
25 grams of tomatoes + 500 ml of 0.5% levulinic acid/0.05% SDS (mixture)

The samples were agitated by a magnetic stirrer at 100 rpm for 1, 3 and 5 minutes (contact time). After each contact time was achieved, samples were immediately drained and tomatoes homogenised to form a slurry using Waring Commercial Laboratory blender (Thermo Fisher Scientific). The slurry was used to test for pH, titratable acidity and total soluble solids.

3.3.3.2 Determination pH

Determination of pH was done on freshly made tomato slurry using Themo Scientific Orion 2star pH meter. The electrodes were rinsed with distilled water in between samples.

3.3.3.3 Determination of titratable acidity

For estimating titratable acidity, the slurry was filtered using Whatman syringe filters. A 100 ml of the filtrate was titrated by adding 0.1 N NaOH until a pH of 8.1 was attained. The volume of the NaOH added to the solution was multiplied by a correction factor of 0.064 to estimate titratable acidity as percentage of citric acid (Cheema *et al.*, 2014, Turhan and Seniz, 2009).

3.3.3.4 Determination of soluble solids content

Total soluble solids is an index of soluble solids concentration in fruit. For estimation of soluble solids content, 1.5 mL of tomato slurry was centrifuged at 10,000 rpm (15 min, 25 °C), and the supernatant was filtered through Whatman nonsterile syringe filters (0.45 μm). The filtered tomato serum (40 μL) was measured using a digital refractometer ATAGO (ATAGO, Inc. Kirkland,WA,USA). Measurements were taken once for each sample, and 70% ethanol was used to clean in between samples. The refraction index was expressed as percent soluble solids in °Brix (Wilkerson *et al.*, 2013, Javanmardi and Kubota, 2006).

3.4 Data analysis

Three trials were conducted for each experiment. Analysis of the data was performed using SPSS version 21 (IBM Statistics). Analysis of variance was conducted with repeated measures and Greenhouse Geisser correction to study the effect of contact time on the survival of *L. monocytogenes*, ATCC 7644 and the effect of each sanitizer on the survival of *L. monocytogenes* ATCC 7644 at varied time intervals (0, 24, 48, 72 hours). The number of surviving LM was plotted against contact time (1, 3, 5 minutes) and also against time interval (0, 24, 48, 72 hours). Log reduction for each contact time and sanitizer were also calculated and presented in a table. Pairwise comparison with Bonferroni adjustment was used to determine any significance difference between treatments. To analyse results for physicochemical attributes, ANOVA was used to assess if there was a significant difference in pH, total soluble solids and titratable acidity of treated and untreated tomato samples.

3.5 Results

3.5.1 Effect of storage time, sanitizer treatments and contact time on survival of *L. monocytogenes* ATCC 7644

Treatment of *L. monocytogenes* with sanitizers resulted in a decrease in the populations of bacteria. All the sanitizers tested had the ability to reduce the surviving LM, with varying degree of effectiveness. Among the sanitizers tested, sodium hypochlorite solution was the least effective, with highest counts of surviving LM. Following in that list, is levulinic acid, a mixture of SDS and levulinic (termed mixture) and SDS being the most effective completely destroyed the bacteria even at 1 minute. The results of repeated measures ANOVA, with Greenhouse-Geisser correction showed that there was a significant difference ($P \leq 0.05$) between the sanitizers used. The surviving LM were reduced progressively as storage time increased from 0 hours to 72 hours. The means of surviving bacteria are shown in Table 3.1.

Table 3.1 Mean [1] counts of *L. monocytogenes* ATCC 7644 after treatment with different sanitizers at different contact times and storage times

Sanitizer	Contact time (minutes)	Time intervals			
		0 Hours	**24 Hours**	**48 Hours**	**72 Hours**
NaClO	1	5.36 ± 0.02^{a}	5.14 ± 0.03^{a}	5.02 ± 0.03^{a}	4.75 ± 0.04^{a}
	3	5.06 ± 0.03^{a}	5.06 ± 0.03^{a}	4.78 ± 0.05^{a}	4.45 ± 0.04^{a}
	5	4.17 ± 0.09^{b}	3.77 ± 0.09^{b}	3.33 ± 0.10^{b}	2.60 ± 0.09^{b}
SDS/lev	1	4.60 ± 0.01^{c}	4.59 ± 0.02^{c}	4.27 ± 0.08^{c}	4.01 ± 0.06^{c}
	3	4.35 ± 0.05^{c}	4.24 ± 0.06^{c}	3.39 ± 0.36^{c}	2.53 ± 0.08^{c}
	5	1.33 ± 0.15^{c}	1.40 ± 0.03^{c}	0.56 ± 0.09^{c}	0.00^{c}
Levulinic	1	4.68 ± 0.03^{e}	4.60 ± 0.02^{e}	4.15 ± 0.14^{e}	4.06 ± 0.11^{e}
	3	4.68 ± 0.03^{e}	4.34 ± 0.09^{e}	4.12 ± 0.10^{e}	2.60 ± 0.30^{e}
	5	3.17 ± 0.07^{e}	2.06 ± 0.04^{e}	1.50 ± 0.10^{e}	0.43 ± 0.20^{e}
SDS	1	0.00^{g}	0.00^{g}	0.00^{g}	0.00^{g}
	3	0.00^{g}	0.00^{g}	0.00^{g}	0.00^{g}
	5	0.00^{g}	0.00^{g}	0.00^{g}	0.00^{g}

Mean counts ±Standard Deviation (Log_{10} CFU/ml) [1] Means followed by different letters in the same column are significantly different, ($P \leq 0.05$).

Marginal means for each sanitizer contact time were also plotted in Figure 3.1 for 1, 3, 5 minutes. As shown, sodium hypochlorite solution has the highest mean values, meaning, a highest number of surviving LM was observed after exposure to this sanitizer compared to

other solutions. Sodium hypochlorite solution was thus not very efficient in reducing survival of the pathogen in this particular study.

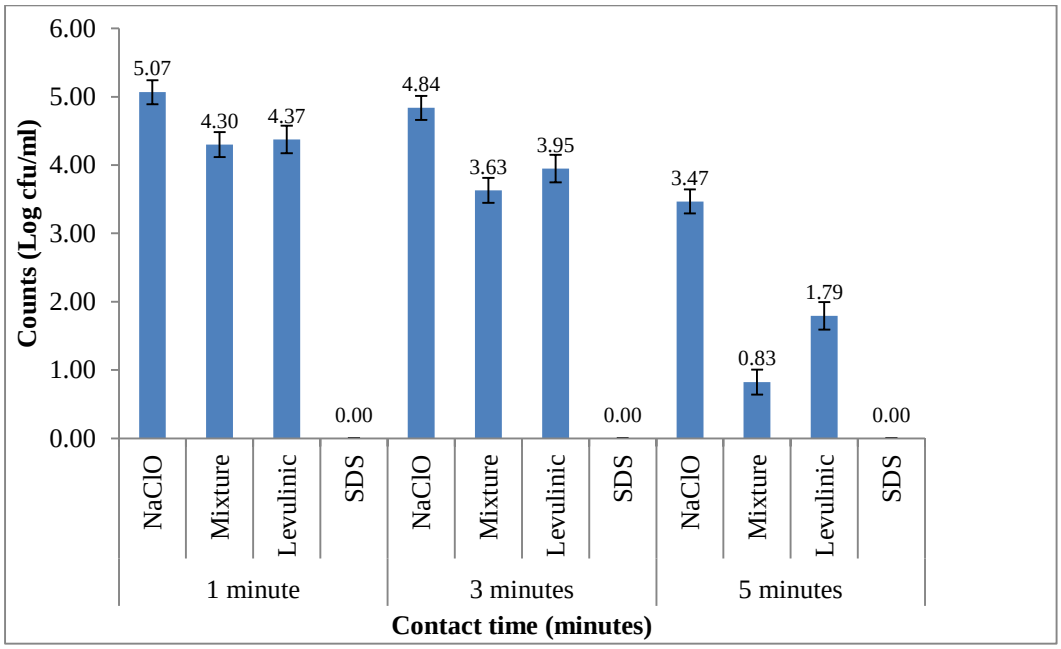


Fig 3.1 Means of surviving LM; based on marginal means. The highest mean values associated with sodium hypochlorite show that it was least effective

Increasing the contact time (1, 3 and 5 minutes) significantly reduced ($P \leq 0.05$) the surviving LM for all sanitizers tested. However, the results of ANOVA with Green House Geisser correction showed that the reduction for 1 and 3 minutes treatment were not significantly different ($P \leq 0.05$). This shows that increasing contact time of each of the sanitizer to 3 minutes did not make much difference.

3.5.2 Overall log reductions

When exposed for 1 minute to 200 ppm sodium hypochlorite solution, *L. monocytogenes* were inactivated by 2.93 log CFU/ml. A log reduction of 3.16 and 4.53 log CFU/ml was achieved after increasing contact time to 3 and 5 minutes, respectively. A mixture of 0.5% levulinic acid and 0.05% SDS (mixture) reduced the surviving LM to 3.69, 4.4 l and log 7.97 CFU/ml after exposure for 1, 3 and 5 minutes, respectively. Using 0.5% levulinic acid resulted in log reduction of 3.63, 4.05 and 6.71 CFU/ml after exposure for 1, 3 and 5 minutes. The overall log reduction is presented in Table 3.2.

Table 3.2 Log reduction of *L. monocytogenes* (CFU/ml) for sodium hypochlorite solution, SDS, levulinic acid and mixture at 1, 3 and 5 minutes

	Overall log reduction		
Sanitizer	1 minute	3 minutes	5 minutes
NaClO	2.93	3.16	4.53
Mixture	3.69	4.40	7.17
Levulinic	3.63	4.05	6.71
SDS	8.00	8.00	8.00

3.5.3 Scanning electron microscopy study of *L. monocytogenes* ATCC 7644

Scanning electron microscopy was used to study the existence of LM even after exposure to sanitizers. The results in this current work showed that there were surviving LM after exposure to sodium hypochlorite solution, levulinic acid and a mixture. However, viewing of samples treated according to above sanitizers did not clearly show the existence of surviving bacteria. Figure 3.2 (a) shows the picture before treatment with any of the sanitizer (untreated sample). Figure 3.2 b, c and d shows pictures after treatment with levulinic, sodium hypochlorite solution and SDS/ Levulinic mixture, respectively.

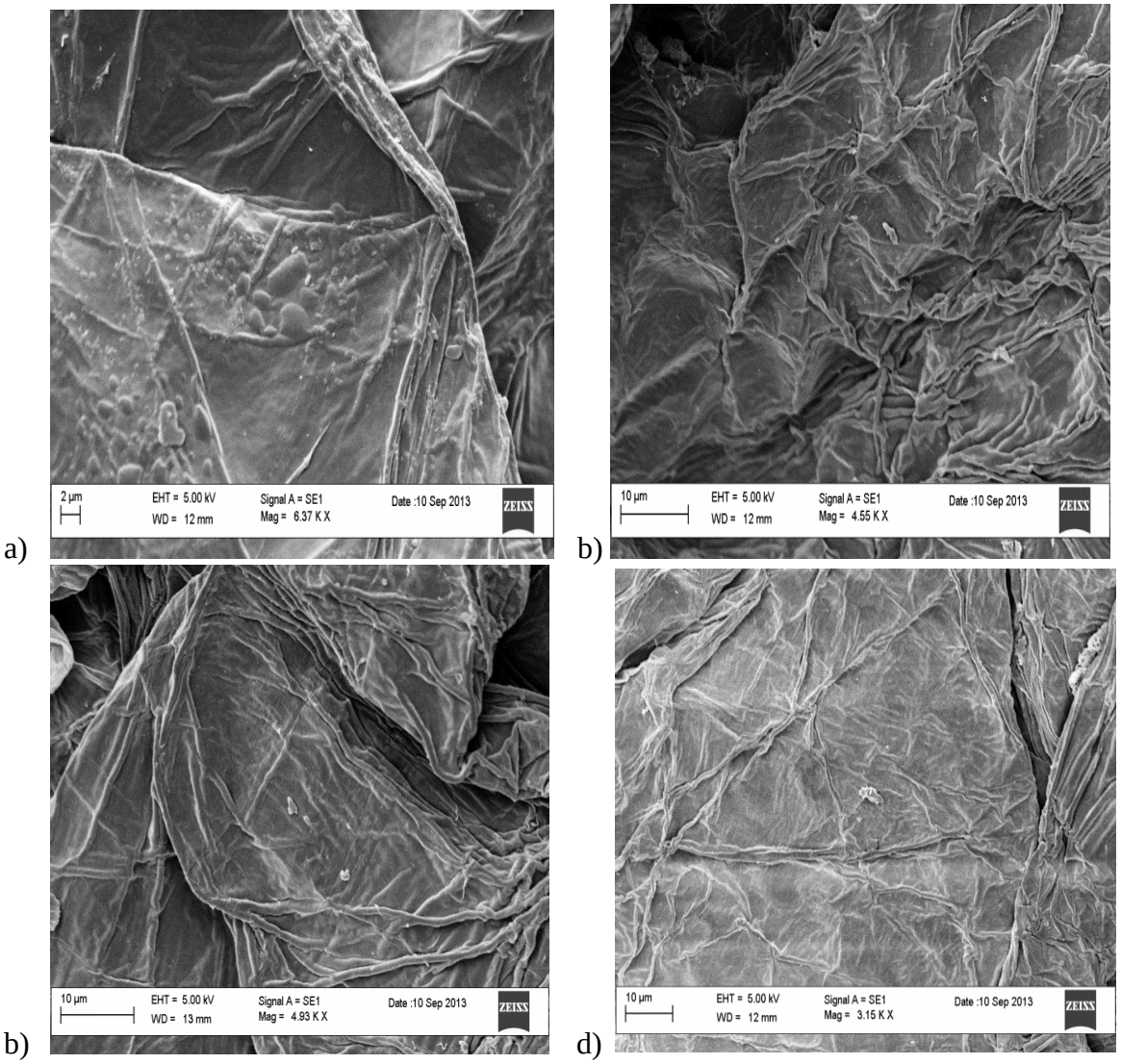


Fig 3.2 SEM of *L. monocytogenes* ATCC 7644 viewed under SEM. (a) before treatment with sanitizers, (b), (c) and (d) after treatment with levulinic acid, sodium hypochlorite solution and SDS/ Levulinic mixture, respectively.

The SEM images above do not show the presence of an abundance of *Listeria* on the surfaces. It is possible that the *Listeria* that were inoculated on the surfaces could have been washed out during sample preparation procedure. *Listeria* might also have migrated into other hidden sections of the pictures due to irregularities of the topography.

3.5.4 Titratable acidity, pH and total soluble solids

Table 3.3 presents the results of TA, pH and TSS. The TA of samples treated with levulinic acid and mixture was significantly different from the control ($P \leq 0.05$). The TA for levulinic acid was 2.78, 2.81 and 2.81%; while TA for mixture was 3.81, 3.73 and 3.74% for 1, 3 and 5 minutes, respectively. The pH for levulinic acid and mixture was relatively lower than the pH of the control sample as shown in Table 3.3. There was no significant difference between the

TA and pH of mixture and levulinic acid. The TA for samples treated with SDS was 0.16 and for samples treated with sodium hypochlorite solution were 0.15, 0.14 and 0.14% when exposed for 1, 3 and 5 minutes, respectively. These results did not vary significantly from those of the control. The pH for SDS and sodium hypochlorite solution treated samples were also slightly different from the control sample as shown in Table 3.3. TSS for levulinic acid were significantly ($P \leq 0.05$) reduced to 3.20 % brix for 1, 3, 5 minutes, while the TSS for mixture was reduced to 3.24, 3.26, 3.24 % brix, respectively. Though TSS for SDS treated and sodium hypochlorite solution treated samples were reduced, the effect was not that significant. Contact time was varied from 1 to 5 minutes and there were no significant changes in theses parameters from 1 to 3 minutes contact times.

Table 3.3 Effects of levulinic acid, sodium hypochlorite solution, mixture and SDS on the physicochemical properties of tomatoes

Tomato treatment	Contact time	pH of sample	Titratable acidity (% citric acid)	Total soluble solids (%Brix)
Distilled water	1	4.77 ± 0.02 [a]	0.16 ± 0.01 [a]	4.90 ± 0.02 [a]
	3	4.78 ± 0.01 [a]	0.14 ± 0.03 [a]	4.90 ± 0.01 [a]
	5	4.78 ± 0.12 [a]	0.16 ± 0.13 [a]	4.90 ± 0.10 [a]
Levulinic acid	1	3.61 ± 0.09 [b]	2.78 ± 0.05 [b]	3.20 ± 0.03 [b]
	3	3.67 ± 0.01 [b]	2.81 ± 0.01 [b]	3.20 ± 0.01 [b]
	5	3.69 ± 0.04 [b]	2.81 ± 0.03 [b]	3.20 ± 0.04 [b]
Mixture (SDS/lev)	1	3.81 ± 0.03 [b]	2.76 ± 0.02 [b]	3.24 ± 0.01 [b]
	3	3.73 ± 0.01 [b]	2.78 ± 0.02 [b]	3.26 ± 0.03 [b]
	5	3.74 ± 0.03 [b]	2.78 ± 0.01 [b]	3.24 ± 0.01 [b]
NaClO	1	5.09 ± 0.10 [a]	0.15 ± 0.01 [a]	4.60 ± 0.02 [a]
	3	5.17 ± 0.04 [a]	0.14 ± 0.05 [a]	4.63 ± 0.03 [a]
	5	5.20 ± 0.02 [a]	0.14 ± 0.03 [a]	4.61 ± 0.02 [a]
SDS	1	4.68 ± 0.01 [a]	0.16 ± 0.01 [a]	4.65 ± 0.02 [a]
	3	4.88 ± 0.03 [a]	0.16 ± 0.03 [a]	4.61 ± 0.01 [a]
	5	4.87 ± 0.02 [a]	0.16 ± 0.02 [a]	4.63 ± 0.01 [a]

Each value represents the mean + SD of three trials. For each parameter, the values significantly different at $P \leq 0.05$ are indicated by different letters. Samples treated with distilled water were used as control.

3.6 Discussion

The food manufacturing industry has depended on the use of sanitizers for reducing the health risk associated with food borne pathogens. Many sanitizers have been tested but to date, food borne pathogens are still a problem in the food and fresh produce industry. Some researchers have suggested that this is due to the development of resistance by the bacteria due to repeated exposure to sanitizers (Mani-López *et al.*, 2012, Riazi and Matthews, 2011).

Most fruit and vegetable processing facilities resort to chlorine based sanitizers because they are cheaper and have long standing credibility of reducing surviving bacteria which has however, been proved otherwise in this current research as well as other previous researches. Findings from this study show that though chlorine based sanitizer has been widely used for washing produce and sanitising food surfaces, it is not capable of killing all food borne pathogens. This is shown by high mean counts of surviving bacteria associated with sodium hypochlorite solution as presented in results above. Observations on the ineffectiveness of sodium hypochlorite solution to remove *L. monocytogenes* were also reported in a similar work by Keskinen *et al.* (2009) against *Escherichia coli* O157:H7 and *Salmonella.* Other researchers also agree that sodium hypochlorite solution cannot effectively eradicate food borne pathogens from fresh produce or work surfaces (Ijabadeniyi *et al.*, 2011b, Allende *et al.*, 2009, Mahmoud *et al.*, 2007).

Several researches are ongoing to try and find other alternative sanitizers due to challenges that are associated with sodium hypochlorite solution (Keskinen *et al.*, 2009). Some researchers have cited that its pH sensitivity affects its effectiveness (Zhao *et al.*, 2009). Another challenge is that it diminishes quickly upon contact with organic matter and hence leads to reduced effectiveness (Neal *et al.*, 2012). Other researchers raised concerns over environmental and health risks associated with the formation of carcinogenic halogenated disinfection by-products such as trihalomethanes (Gil *et al.*, 2009, Kim *et al.*, 2009). For these reasons chlorine based sanitizer has not been gainfully useful in the fresh produce industry for the past years. Though it has been a long standing sanitizer in food industry, other sanitizers that have been shown to be more effective than sodium hypochlorite solution; through this research and previous research can be employed for the betterment of microbiological quality of fresh produce.

Levulinic acid has been applied in the food manufacturing industry as a food additive. It has been designated as a generally safe additive to food by the Food and Drug Administration

(FDA)(Zhao *et al.*, 2009). Levulinic acid disrupts the membrane structure of bacteria due to its polarity thereby exposing cell constituencies and lethality (Thompson *et al.*, 2008). It can be used over a wide pH and temperature range (Sagong *et al.*, 2011). In this study, levulinic acid had mean counts of surviving bacteria that were much lower than those of sodium hypochlorite solution. With these findings, it can be concluded that levulinic acid at 0.5% can perform better than 200 ppm sodium hypochlorite solution against *L. monocytogenes* ATCC 7644. Other researchers also tested levulinic acid in their work and obtained related findings. Thompson *et al.* (2008) concluded that it was effective in inhibiting growth of *L. monocytogenes* in ready to eat meat products. Other studies using lactic acid, acetic acid and levulinic acid on meat revealed that though levulinic acid is effective, it does not provide as much effective decontamination as lactic acid nor residual protection as much as acetic acid (Carpenter *et al.*, 2011). Levulinic acid shows potential in the fresh produce industry, therefore further research can be pursued on the most usable concentrations.

Sodium dodecyl sulphate is generally regarded as safe (GRAS) food additive (Lu and Wu, 2012). In this study, using 1% SDS alone resulted in 8 log CFU/ml reduction of *L. monocytogenes.* SDS has amphilic properties (12 carbon chain attached to sulphate group) and its anti-microbial effectiveness increases when pH is decreased. It has the ability to denature cell proteins and damage cell membranes irreversibly (Zhao *et al.*, 2009). The action of SDS was much better than that of levulinic acid in this study when they were used individually. This is because levulinic acid has a shorter carbon chain (5 carbons and a hydroxyl group) which makes it a weak acid therefore its effectiveness is lower than SDS. Extra care must be taken if SDS is to be used in fresh produce as it was established through this study that very low concentrations of 1% can have a very huge impact on survival of pathogens. Its possibility of causing detrimental effects on quality can be put into consideration as well.

A combination of 0.05% SDS and 0.5% levulinic acid was also used in this study. Findings show that this mixture achieved better results as compared to levulinic acid alone. Many researchers have reported on the advantages of mixing SDS and levulinic acid. Zhao *et al.* (2009)'s findings showed an increased antimicrobial activity of the combination of SDS and levulinic acid against *Salmonella* and *E. coli* O157: H7. Gurtler and Jin (2012) found that a combination of 2% acetic acid, lactic acid and levulinic acid reduced *Salmonella* on tomatoes. Ortega *et al.* (2011) reported that a combination of levulinic acid and SDS was highly effective against *E. coli* when exposure times were increased to 30 and 60 minutes. On the contrary, Guan *et al.* (2010) reported that a combination of these had no commercial value as they have

detrimental effects to the quality of fresh produce. Combining sanitizers has shown to have a positive contribution in the food market. This has potential for implementation in fresh produce industry. Implementation of a combination of sanitizers can be tested alongside an assessment on their effects on sensory qualities.

Increasing exposure time decreased significantly the surviving *L. monocytogenes.* In this study, a greater drop in surviving LM was achieved at 5 minutes exposure time. This showed that, the longer the bacteria are exposed to chemicals, the greater the chances of reducing their survival. Park *et al.* (2011) also reported that log reduction increased with increasing contact time. Other writers have indicated that an exposure time of 3 minutes is effective against food pathogens (Mattson *et al.*, 2011). Ding *et al.* (2011) and Møretrø *et al.* (2012) also reported that effectiveness of sanitizer depended on treatment time. Other researchers also noted significant decrease in bacterial counts occurs in the first minute and that subsequent decrease after one minute is not significant (Stebbins *et al.*, 2011, Tirpanalan *et al.*, 2011). In view of this, it can be concluded that contact time is one of the factors that should be monitored when using sanitizers. Insufficient contact time will lead to high survival rate after treatment while extended, contact time may also lead to damage in sensory qualities of fresh produce.

Listeria monocytogenes was further stored for a period of 72 hours at 4 °C. During this storage period bacterium survived up to 72 hours after being treated with sanitizers except in the case SDS. Survival of pathogens after storage period of 72 hours was also reported by Ijabadeniyi *et al.* (2011b). Elif *et al* (2006) cited by Ijabadeniyi *et al.* (2011b) had similar findings with *Salmonella* and concluded that it can survive up to 220 hours after exposure to sanitizers. Sufficient exposure of pathogens to sanitizers is paramount to reduce surviving bacteria, as some have the ability to recover even after being treated with sanitizer.

Two important quality attributes of processed tomatoes are pH and titratable acidity (Anthon *et al.*, 2011). For sanitizers to be effectively used on tomatoes, they should cause negligible changes to pH and TA of the tomatoes. In this research it was revealed that sanitizers can alter the quality attributes of fresh produce if they come into contact with the sub-surfaces. Other recent studies also point out that to some extent, sanitizers can affect sensory qualities of fresh produce (Pérez-Gregorio *et al.*, 2011). On the other hand, some researchers used the mentioned sanitizers as well as other sanitizers on tomatoes, other fresh fruits and vegetables and reported varying results (Jemni *et al.*, 2014, Pérez-Gregorio *et al.*, 2011, Silveira *et al.*, 2008, Martínez-Sánchez *et al.*, 2006).

In previous studies, SDS was tested together with organic acids and hydrogen peroxide on blue berries, but no significant difference was found on pH and total anthocyanin value between untreated and treated blueberries (Li and Wu, 2013). Studies using sodium lactate and levulinate on turkey meat and pork sausages did not have any effects on sensory properties of these products (Vasavada *et al.*, 2003). SDS and chlorine based sanitizer were also tested on Iceberg lettuce and the conclusion was that SDS used alone or in combination with other sanitizers such as levulinic acid are of low commercial value compared to chlorine washing, since they cause detrimental effects to sensory attributes (Guan *et al.*, 2010). In another study using Iceberg and Romane lettuce sodium hypochlorite solution had high quality scores for Romane lettuce but caused quality deterioration on Iceberg lettuce. A combination of SDS and Tsunami also did not show any effect on the sensory attributes of Iceberg lettuce (Salgado *et al.*, 2013).

In this study, TSS was reduced for all treatments with levulunic acid having the lowest reductions followed by the mixture. This could have been attributed to leaching of contents into treatment solutions as a larger surface area of the sub-surface area of tomatoes was exposed. Leaching of materials was also reported by Alegria *et al.* (2009). Though previous studies have also reported that longer contact times result in deterioration of sensory characteristics (Rico *et al.*, 2007), there was no significant difference for all attributes in relation to contact time as found in this study.

3.7 Conclusion

This work confirms that the use of sanitizers in food processing at shorter contact time of 1 minute may not eradicate food borne pathogens. SDS alone is capable of destroying *L. monocytogenes* causing no detrimental effect on sensory attributes of tomatoes. It is also important to consider exposure time to increase the effectiveness of sanitizers. Sanitizers can have detrimental effect on sensory attributes of fresh produce hence careful consideration is required when selecting sanitizers for particular produce. Further studies are required to validate the application of levulinic acid and SDS as sanitizers in food processing as well as their efficacy.

CHAPTER FOUR: INACTIVATION OF HEAT ADAPTED AND CHLORINE ADAPTED *Listeria monocytogenes* ATCC 7644 ON TOMATOES USING SODIUM DODECYL SULPHATE, LEVULINIC ACID AND SODIUM HYPOCHLORITE SOLUTION

4.1 Abstract

A study was done to evaluate the effectiveness of sodium dodecyl sulphate (SDS), sodium hypochlorite solution and levulinic acid in reducing the survival of heat adapted and chlorine adapted *Listeria monocytogenes* ATCC7644. The results against heat adapted *L. monocytognes* revealed that sodium hypochlorite solution was the least effective, achieving log reduction of 2.75, 2.94 and 3.97 log CFU/ml for 1, 3 and 5 minutes, respectively. SDS was able to achieve 8 log reduction for both heat adapted and chlorine adapted bacteria. When used against chlorine adapted *L. monocytogenes* sodium hypochlorite solution achieved log reduction of 2.76, 2.93 and 3.65 log CFU/ml for 1, 3 and 5 minutes, respectively. Using levulinic acid on heat adapted bacteria achieved log reduction of 3.07, 2.78 and 4.97 log CFU/ml for 1, 3, 5 minutes, respectively. On chlorine adapted bacteria levulinic acid achieved log reduction of 2.77, 3.07 and 5.21 log CFU/ml for 1, 3 and 5 minutes, respectively. Using a mixture of 0.05% SDS and 0.5% levulinic acid on heat adapted bacteria achieved log reduction of 3.13, 3.32 and 4.79 log CFU/ml for 1, 3 and 5 minutes while on chlorine adapted bacteria it achieved 3.20, 3.33 and 5.66 log CFU/ml, respectively. Increasing contact time also increased log reduction for both test pathogens. A storage period of up to 72 hours resulted in progressive log reduction for both test pathogens. Results also revealed that there was a significant difference ($P \leq 0.05$) between contact times, storage times and sanitizers. Findings from this study can be used to select suitable sanitizers and contact times for heat adapted and chlorine adapted *L. monocytogenes* in the fresh produce industry.

Keywords: Heat adapted *L. monocytogenes,* chlorine adapted *L. monocytogenes*, adapted food borne pathogens, food borne illnesses.

4.2 Introduction

Food borne pathogens encounter various stress factors in food processing. These may result in pathogens developing resistance towards stress inducers over time (Battesti *et al.*, 2011). Pathogens are able to adapt to environmental stress factors such as cold, acid, heat, starvation and osmotic stress (Soni *et al.*, 2011). These environments are inherent in food manufacturing units. During food production, pathogens adapt to repeated use of sanitising chemicals, heat, temperature changes and substrate changes (Bridier *et al.*, 2011).

Adaptation may also be due to intrinsic factors (Moorman *et al.*, 2008). When bacteria adapt to a particular environment, they further develop cross protection for other stress factors, and this is when a pathogen develops further extended protection towards multiple stressors (Ágoston, 2009). Cross protection is a defence mechanism employed by bacteria to several other stresses including various food preservation techniques. Previous studies by Taormina and Beuchat (2001) explain the existence of cross protection on *Salmonella* and *Enterococcus faecalis* which were resistant to heat after alkaline stress as well as *L. monocytogenes* which were resistant to heat following starvation conditions, use of ethanol, acid and hydrogen peroxide. Bacon *et al.* (2003) also found that *Salmonella* spp. showed a cross protection against heat treatments after exposure to acid treatment. It was also suggested that induction of cross protection increases with increasing levels of stress (Ágoston, 2009).

Exposure of bacteria to sub-lethal doses of the same stressor also results in increased resistance to subsequent lethal treatment of the same stressor (Bridier *et al.*, 2011). De Angelis and Gobbetti (2004) termed this as 'limited' response. Ágoston (2009) found that *L. monocytogenes* exhibits unique physiological, genomic, and proteomic responses when exposed to sub-lethal temperatures and developed resistance to subsequent lethal heat treatment. Arku *et al.* (2011) found that *Cronobacter* spp. survived better a lethal temperature of 52 °C after adaptation at 46 ˚C for 30 minutes. Bacterial cells may also develop general stress resistance. The general stress response is regulated by sigma factors. During nutrient deprivation and stress cells increase the accumulation of sigma factor RpoS. RpoS-dependent gene expression leads to general stress resistance of cells (Battesti *et al.*, 2011).

Heat resistance in *L. monocytogenes* is influenced by factors such as strain variation, previous growth conditions, prior exposure to heat shock, acid stressor or other stressors (Bridier *et al.*, 2011, Ágoston, 2009, Moorman *et al.*, 2008). Heat resistance can occur during food processing

especially in foods that require prolonged heating at low temperatures. Sergelidis and Abrahim (2009) reported on bacteria that increased thermo-tolerance after exposure to moderately elevated temperatures for a prolonged period. Heating processes induces the production of heat shock proteins (HSPs). When temperatures are elevated, genes for hsp70 and hsp90 encode proteins which increase heat resistance. Incubation temperature also determines the extent of heat shock tolerance (Hu *et al.*, 2007).

Pathogens that have been repeatedly exposed to sanitizers also develop resistance to subsequent treatment with the same sanitizer or different sanitizers especially when used below recommended concentrations. Resistance to sanitizers such as quaternary ammonium compounds is associated with *mdrL* gene which encodes efflux pumps responsible for sanitizer resistance (Gandhi and Chikindas, 2007). Studies revealed that *Staphylococcus aureus* has an effective efflux system that confers resistance to QAC sanitizers (Smith *et al.*, 2008). The same results were observed with trichlosan and chlorhexidine (Villagra *et al.*, 2008).

The objective of this study was to determine the effectiveness of SDS, levulinic acid and sodium hypochlorite solution in reducing the presence of heat adapted and sodium hypochlorite solution adapted *L. monocytogenes* on tomatoes. Tomatoes are widely consumed worldwide and can be consumed raw, partially cooked or as an ingredient in other food products due to their high carotenoid and Vitamin C content. Since they can be consumed raw and/or partially cooked, chances of growth of *L. monocytogenes* are high. As a result studies are necessary to come up with best treatment methods that destroy the food borne pathogens that would possibly contaminate tomatoes.

4.3 Materials and methods

4.3.1 Materials

4.3.1.1 Fresh produce

Tomatoes were purchased from a local supermarket on separate three occasions in Durban South Africa. On the day of purchase the tomatoes were washed in running water. The tomatoes were again washed in 70% ethanol (Ijabadeniyi *et al.*, 2011a). Prior to subjection to different sanitizer treatments, the tomatoes were tested for the presence of *L. monocytogenes*.

4.3.1.2 Bacterial strains

Listeria monocytogenes ATCC 7644 (Merck, South Africa) was used for this study. The strain was cultured in Fraser broth for 24 hours at 37 ºC and stored at 4 ºC (Ijabadeniyi *et al.*, 2011a). Prior to each experiment, a fresh culture of 8 log CFU/ml of *L. monocytogenes* was prepared using McFarland Standards from the stock culture by sub culturing in Fraser broth for 24 hours at 37 ºC (Ji *et al.*, 2010).

4.3.1.3 Sanitizers

Sodium dodecyl sulphate (SDS), levulinic acid, sodium hypochlorite solution, all purchased from Merck, South Africa were tested, individually or combined with contact times (1, 3, 5 minutes); for their killing effect on *L. monocytogenes* in tomatoes. The sanitizers were used as follows;

1% SDS individually
0.5% Levulinic acid individually
200 ppm Sodium hypochlorite solution individually
0.5% levulinic acid/0.05% SDS mixture.

4.3.2 Methods

Apart from the strains prepared below, non-adapted *L. monocytogenes* strains were used as control and the results are reported in Chapter 3.

4.3.2.1 Preparation of heat adapted *L. monocytogenes*

The method of Ágoston (2009) was followed except that Fraser Broth was used in place Brain Heart Infusion. A fresh culture of *L. monocytogenes* ATCC 7644 was grown in Fraser broth for 24 hours at 37 °C. The cultures were harvested by centrifugation at 4000 rpm for 5 minutes at 4 °C. The pellets were washed twice in phosphate buffer (pH 6.8) to remove unspent media and inoculated gradually into Tryptone Soy Broth (pH 7.3) to yield a population of 10^8 CFU/ml using the McFarlan standard (Ji *et al.*, 2010). Proportions of 1 ml were transferred to 1.5 ml Epperndorf tubes. The samples were submerged in thermostatically controlled water bath at 60 °C for 15 minutes. Immediately after heat treatment, samples were transferred to an ice bath to cool them and then sanitised using 70% alcohol. The suspensions were used to inoculate

tomatoes as detailed below. Three replicate experiments were done for each trial and a fresh suspension was prepared for each trial.

4.3.2.2 Preparation of chlorine adapted *L. monocytogenes*

The method of Taormina and Beuchat (2001) was followed. A fresh culture of *L. monocytogenes* grown overnight in Tryptose phosphate broth. A 25 ml culture was dispensed into a 50 ml centrifuge tube and centrifuged at 5000 rpm for 10 minutes at 4 $^{\circ}$C. Pellets were then washed three times in pre-cooled potassium phosphate buffer (pH 7.1) and re-suspended in 25 ml of phosphate buffer. Cells of *L. monocytogenes* (10 ml) were added to 50 ml of 6 ppm of 12.5% w/ v sodium hypochlorite. After 5 minutes 10 ml were drawn and neutralised by adding into 30 ml of 0.01 N sodium thiosulphate solution and vortexing for 10 seconds for inoculation on tomatoes.

4.3.2.3 Inoculation of bacterial strains into tomatoes

As implemented by Zhao *et al.* (2009), A 25 g sample of tomatoes was cut into approximately 5 cm long pieces in the lamina flow hood. The samples were submerged into bacterial suspension (either heat adapted or chlorine adapted) (10^8 CFU/ml, 50 ml of bacterial solution into 950 ml of distilled water) for 60 seconds and then air dried for 20 minutes in the lamina flow hood. The samples were then suspended into 500 ml test solutions and agitated by a magnetic stirrer at 100 rpm for 1, 3, 5 minutes. Following treatment, the samples were placed in double zipper bags containing 25 ml of phosphate buffered saline and pummelled for one minute. The suspension was serially diluted (1:10) in 0.1% buffered peptone water and enumerated for *L. monocytogenes* ATCC 7644.

4.3.2.4 Enumeration of *L. monocytogenes*

In line with Taormina and Beuchat (2001) experimental methodology, populations of *L. monocytogenes* ATCC 7644 were determined by surface plating serially diluted samples; 0.1 ml in duplicates on *Listeria* Selective Agar (Oxoid Ltd, Wade Road, Basingstoke, Hants UK). The treated samples were kept at 4 °C and analysed at 0, 24, 48 and 72 hours for assessment. Plates were incubated for 24 hours at 37 ºC after which colonies were counted using a colony counter.

4.4 Data analysis

Three trials were conducted for each experiment for the purposes of reducing the margin of error, thereby improving the quality of the results. Data was analysed using SPSS version 21 (IBM Statistics). Analysis of variance was conducted with repeated measures and Greenhouse Geisser correction to study the effect of contact time on the survival of adapted *L. monocytogenes*, ATCC 7644 and the effect of each sanitizer on the survival of adapted *L. monocytogenes* ATCC 7644 at varied time intervals (0, 24, 48 and 72 hours). The number of surviving LM was plotted against contact time (1, 3 and 5 minutes) and also against time interval (0, 24, 48 and 72 hours). Log reduction for each contact time and sanitizer was also calculated and presented in a table. Pairwise comparison with Bonferroni adjustment was used to determine any significance difference between treatments.

4.5 Results

4.5.1 Effect of sanitizer treatments and storage time intervals on the survival of heat adapted and chlorine adapted *L. monocytogenes*

Three trials were conducted for this study and means determined as shown in Table 4.1. The mean values show the surviving heat adapted *Listeria* (a) and chlorine adapted *Listeria* (b). The heat adapted bacteria was more resistant to the sanitizer compared to the chlorine adapted bacteria. However; the difference between the mean of surviving bacteria for heat adapted and chlorine adapted bacteria was not significant.

Table 4.1 Mean [1] bacterial count of (a) heat adapted and (b) chlorine adapted *L. monocytogenes* ATCC 7644 after treatment with different sanitizers at different contact times.

(a)Sanitizer	Contact time (minutes)	0 Hours	24 Hours	48 Hours	72 hours
NaClO	1	5.42 ± 0.01	5.37 ± 0.01	5.27 ± 0.04	4.94 ± 0.04
	3	5.25 ± 0.01	5.10 ± 0.02	5.04 ± 0.03	4.85 ± 0.03
	5	5.17 ± 0.06	4.17 ± 0.45	4.10 ± 0.45	2.67 ± 0.06
Mixture	1	5.03 ± 0.01	4.93 ± 0.03	4.81 ± 0.06	4.72 ± 0.08
	3	4.77 ± 0.04	4.73 ± 0.04	4.61 ± 0.02	4.59 ± 0.07
	5	3.87 ± 0.12	3.47 ± 0.59	2.80 ± 0.70	2.70 ± 0.10
Levulinic	1	5.28 ± 0.02	5.22 ± 0.02	5.21 ± 0.01	5.18 ± 0.01
	3	5.23 ± 0.01	4.99 ± 0.09	4.86 ± 0.09	4.65 ± 0.08
	5	4.20 ± 0.00	3.77 ± 0.15	2.67 ± 0.06	1.50 ± 0.10
SDS	1	0.00	0.00	0.00	0.00
	3	0.00	0.00	0.00	0.00
	5	0.00	0.00	0.00	0.00

(b)Sanitizer	Contact time (minutes)	0 Hours	24 Hours	48 Hours	72 hours
NaClO	1	5.42 ± 0.01	5.37 ± 0.01	5.21 ± 0.02	4.96 ± 0.06
	3	5.22 ± 0.02	5.09 ± 0.03	5.02 ± 0.02	4.96 ± 0.03
	5	5.23 ± 0.06	4.77 ± 0.49	4.13 ± 0.8	3.27 ± 0.66
Mixture	1	5.00 ± 0.03	4.86 ± 0.03	4.76 ± 0.04	4.59 ± 0.05
	3	4.76 ± 0.04	4.73 ± 0.05	4.60 ± 0.04	4.60 ± 0.02
	5	2.90 ±0.10	2.77 ± 0.06	2.17 ± 0.37	1.53 ± 0.06
Levulinic	1	5.28 ± 0.01	5.24 ± 0.01	5.21 ± 0.01	5.18 ± 0.01
	3	5.24 ± 0.02	4.96 ± 0.03	4.9 ± 0.04	4.63 ± 0.08
	5	4.20 ± 0.53	3.37 ± 0.49	2.00 ± 0.50	1.60 ± 0.10
SDS	1	0.00	0.00	0.00	0.00
	3	0.00	0.00	0.00	0.00
	5	0.00	0.00	0.00	0.00

Mean counts ±Standard Deviation (Log $_{10}$ CFU /ml)
[1] Means were not significantly different ($P \leq 0.05$).

The surviving bacteria were also stored for 0, 24, 48 and 72 hour series so as to assess the effect of storage time on surviving LM. Varying storage time intervals reduced both heat adapted bacteria ($P \leq 0.05$). A progressive reduction in surviving bacteria was observed for both heat adapted and chlorine adapted bacteria.

4.5.2 Effect of sanitizer contact time on heat adapted and chlorine adapted *L. monocytogenes* ATCC 7644

Increasing sanitizer contact time reduced the survival of both heat adapted and chlorine adapted *L. monocytogenes.* Among the tested sanitizers, SDS destroyed all the bacteria. Marginal

means were plotted in Figure 4.1 to show the means of surviving bacteria for each sanitizer and contact time.

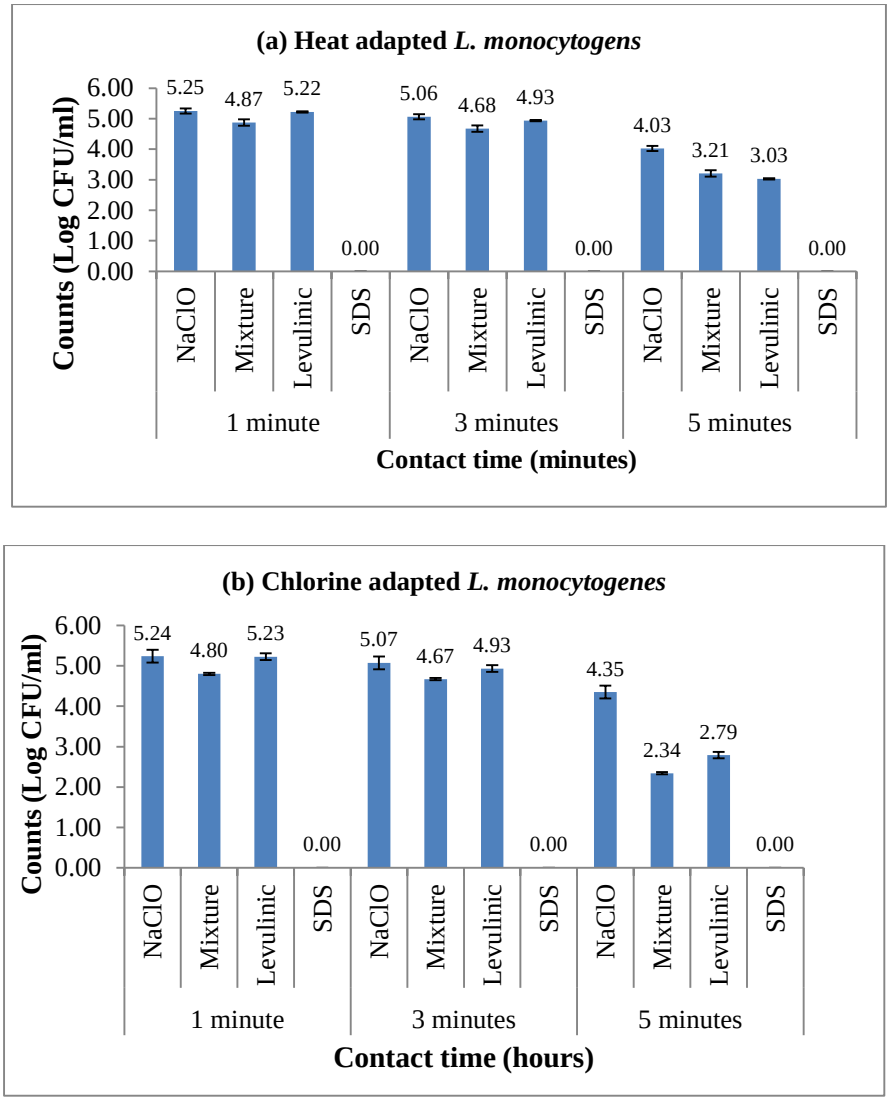


Figure 4.1 Means of surviving heat adapted (a) and chlorine adapted *L. monocytogenes* (b)

4.5.3 Overall log reductions

Overall log reduction of surviving bacteria for the entire storage period were also calculated (Table 4.2). Heat adapted bacteria were reduced by approximately 2.75, 3.13 and 2.78 log CFU/ml when exposed to sodium hypochlorite solution, mixture and levulinic acid, respectively. When the contact time was increased to 3 minutes, the log reduction also increased to 2.94, 3.32 and 3.07 log CFU/ml, respectively for sodium hypochlorite solution, mixture and levulinic acid. A further reduction in bacteria was achieved with a contact time of 5 minutes for all sanitizers.

Log reduction of 2.76, 3.20 and 2.77 log CFU/ml was achieved by exposing chlorine adapted *L. monocytogenes* to sodium hypochlorite solution, mixture and levulinic acid for 1 minute. Increasing contact time to 3 minutes increased the log reductions by 2.93, 3.33 and 3.07 log CFU/ml, respectively, while a contact time of 5 minutes achieved a log reduction of 3.65, 5.66 and 3.21 log CFU/ml. The log reduction for heat adapted bacteria were lower than those of chlorine adapted bacteria. This is evident that the chlorine adapted *L. monocytogenes* was more responsive to sanitizer stress compared to heat adapted bacteria. In all cases SDS achieved an 8 log CFU/ml reduction.

Table 4.2 Log reduction (CFU/ml) for all sanitizers at 1, 3 and 5 minutes: heat adapted (a), chlorine adapted *L. monocytogenes* (b)

(a) Overall log reduction of heat adapted *L.monocytogenes*

Sanitizer	1 minute	3 minutes	5 minutes
NaClO	2.75	2.94	3.97
Mixture	3.13	3.32	4.79
Levulinic	2.78	3.07	4.97
SDS	8.00	8.00	8.00

(b) Overall log reduction of chlorine adapted *L. monocytogenes*

Sanitizer	1 minute	3 minutes	5 minutes
NaClO	2.76	2.93	3.65
Mixture	3.20	3.33	5.66
Levulinic	2.77	3.07	5.21
SDS	8.00	8.00	8.00

4.6 Discussion

In this study, sodium hypochlorite solution, levulinic acid, a mixture of SDS and levulinic, and SDS were able to reduce the surviving bacteria of both heat adapted and chlorine adapted *L. monocytogenes* ATCC 7644. Their action on the same pathogens treated differently was significantly different. Sodium hypochlorite solution was the least effective followed by levulinic acid and then SDS/Lev mixture. SDS was able to destroy the pathogen totally for both sanitizers. It has been reported that effectiveness of sanitizer depends on the pathogen being subjected to that particular sanitizer (Beltrame *et al.*, 2012, Møretrø *et al.*, 2012, Ding *et al.*, 2011, Stebbins *et al.*, 2011, Tornuk *et al.*, 2011). The reports from previous researchers suggest the importance of taking extra care when selecting sanitizers to use against a particular pathogen.

Heat adapted pathogens were more resistant to these sanitizers compared to the chlorine adapted pathogens. It has been reported that previously adapted pathogens are more resistant to subsequent stress (Ágoston, 2009) and that non-adapted pathogens are more susceptible to sanitizer stress compared to adapted pathogens (Gandhi and Chikindas, 2007). This was also confirmed in our earlier studies reported in Chapter 3 using non-adapted *L. momocytogenes*. Effects of heat adaptation, acid adaptation and sanitizer adaptation have also been widely reported. Studies have also shown that particularly in *L. monocytogenes*, resistance to sanitizer is caused by the presence of sigma B factor, a protein required for RNA synthesis (Ryan *et al.*, 2008, Gandhi and Chikindas, 2007).

Other studies indicated that *L. monocytogenes* are resistant to alkaline stress at high temperatures (Taormina and Beuchat, 2001). Due to cross protection adapted cells were more stable to sanitizer treatment. Acid adaptation was also reported to increase the viability of *L. monocytogenes* and *Salmonella* spp. to other sanitizers (Lin *et al.*, 2011). Another study showed that heat adapted *L. innocua* could not survive the action of cetrimide (Moorman *et al.*, 2008). Neo *et al.* (2013) also reported similar results using peroxyacetic acid and sodium hypochlorite solution on bean sprouts. Lin *et al.* (2013) found that *Vibrio parahaemolyticus* was more resistant to chlorine-containing disinfectant (Clidox-S) and a quaternary ammonium compound (Quatricide) at 25 and 40 °C after pre exposure to heat shock, cold shock and acid adaptation.

Kim *et al.* (2012) also found that acid adaptation of *C. sakazakii* by pre-exposure to acidic pH can enhance the resistance of cells against subsequent environmental stresses such as acidic

pH, heat, and organic acids. Another study by Mavri and Smole Možina (2012) using *Campylobacter jejuni* and *Campylobacter coli* also show an increased tolerance to sanitizers. Ethanol and isopropanol concentration of 70% reduced the infectivity of murine norovirus by 2.6 log units, whereas 50 and 70% ethanol reduced the infectivity of feline calicivirus by 2.2 log units after exposure for 5 min (Park *et al.*, 2010). On the contrary Riazi and Matthews (2011) found that previously adapted pathogens were still susceptible to sanitizers.

Observations of this study are not different from previous studies that showed that the effect of sanitizers can be changed based on contact time with increasing contact time resulting in a decrease in viability of pathogens (Beltrame *et al.*, 2012, Møretrø *et al.*, 2012, Ding *et al.*, 2011, Mattson *et al.*, 2011, Park *et al.*, 2011, Tornuk *et al.*, 2011). In this study increasing contact time significantly reduced surviving bacteria of either heat adapted or chlorine adapted *L. monocytogenes.* Through this study it was also established that adaptive treatments using sodium hypochlorite solution and heat separately can impose resistance on *L. monocytogenes*.

Some studies have shown that a 3 minute contact time is enough to destroy pathogens (Ding *et al.*, 2011, Mattson *et al.*, 2011, Stebbins *et al.*, 2011), while other studies showed that a contact time of up to 10-15 minutes is required if pathogens are to be significantly reduced (Beltrame *et al.*, 2012). Research using peracetic acid to remove *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus mutans*, *Candida albicans* and *Bacillus subtilus* revealed that a contact time of 2 minutes can achieve satisfactory results (Salvia *et al.*, 2011). In other studies using ozone, prolonging contact time to 5 minutes could not reduce *E. coli* effectively (Ölmez, 2010). Some studies have also indicated that contact time varies and depends on the pathogen under study (Park and Sobsey, 2011). It is important to consider carefully the contact time suitable for better results when using antimicrobials.

Varying the storage time period up to 72 hours resulted in progressive reduction in surviving LM of both heat adapted and chlorine adapted *L. monocytogenes*. In other studies, growth was observed only for the samples stored at 22 °C for 18 hours, whereas in the rest of the incubation conditions no significant change in the *E. coli* count was observed (Ölmez, 2010). Although this study did not determine the effect of sanitizers on organoleptic properties or antioxidant capability, a study by Ruiz-Cruz *et al.* (2010) revealed that sanitizers were capable of controlling microbial growth without inducing major loss of antioxidant capacity and photochemical characteristics after a storage period of 27 days. Similar results were reported

by Tomás-Callejas *et al.* (2011) using 40, 70 or 100 mg L^{-1} free chlorine, neutral and acidic electrolyzed water on fresh-cut mizuna bay leaves for 11 days at 5 ˚C.

4.7 Conclusion

Exposure to adaptation conditions cause resistance of *L. monocytogenes* to sanitizer. Sodium hypochlorite solution, levulinic acid and SDS were able to reduce the microbial populations of heat adapted and chlorine adapted *L. monocytogenes* in tomatoes. Levulinic acid and SDS achieve greater log reduction as compared to sodium hypochlorite solution. A mixture of levulinic acid and SDS achieves best results compared to results when levulinic acid was used alone. Contact time can be increased to increase the effectiveness of sanitizers however extra care should be taken so as not to cause negative impact on the sensory properties of fresh produce.

CHAPTER FIVE: INACTIVATION OF *Listeria monocytogenes* ATCC 7644 BIOFILMS USING SODIUM DODECYL SULPHATE, LEVULINIC ACID AND SODIUM HYPOCHLORITE SOLUTION

5.1 Abstract

A study was done to assess the effectiveness of 200 ppm sodium hypochlorite (NaClO), 1% sodium dodecyl sulphate (SDS) and 0.5% levulinic acid in reducing *L. monocytogenes* ATCC7644 biofilms. 0.05% SDS and 0.5% levulinic acid were also used combined (mixture). After treatment with sanitizers, the biofilms were stored at 4 °C and samples were tested at 0, 24, 48 and 72 hours. The contact time was varied to 1, 3 and 5 minutes. Results revealed that biofilms were still viable after treatment with these sanitizers with no significance difference observed between storage times. Varying contact times from 1 to 3 minutes did not show any significant difference, however, there was a significant difference ($P \leq 0.05$) when the contact time was increased to 5 minutes. Non-adapted biofilms had highest log reduction when compared to chlorine adapted and heat adapted biofilms. Treatment with sodium hypochlorite solution was the least effective in reducing viability of biofilms, followed by levulinic acid, then SDS/Lev mixture. SDS when used alone had the highest log reduction. Application of sanitizers at different contact times individually or combined may be successful in reducing biofilms in food manufacturing units. However, a careful selection of sanitizer for each specific pathogen may be required if sanitizers are to work effectively against biofilms.

Keywords: Biofilms, adapted biofilms, food borne pathogen, fresh produce

5.2 Introduction

Biofilms are a community of microorganisms attached to a surface (Abee *et al.,* 2011). They are formed when a group of single celled organisms come together and then encased in an exo-polysaccharide matrix (Niemira, 2010). The resultant body exhibit different characteristics to a singular bacterium from which they were made (Bridier *et al.*, 2011) and can either be mixed species or one type of bacteria. Biofilms are a concern in food manufacturing as their presence may lead to post processing contamination (Kim and Wei, 2007) since they attach to food preparation surfaces as well as equipment. It has been established that biofilms are resistant to chemical decontamination and sanitizers (Bridier *et al.,* 2011). The exopolysaccharide matrix formed by biofilms and cross linking of flagella and fimbriae create a body that is difficult to penetrate and hence sanitizers cannot reach the internal layers (Bridier *et al.*, 2011). Mixed

biofilms have also been implicated as being more resistant to sanitizers than their single specie biofilm (Abee *et al.*, 2011). Previous studies by Van der Veen and Abee (2011) found that a mixed biofilm of *Lactobacillus planturum* and *Listeria monocytogenes* was resistant to benzalkonium chloride and peracetic acid.

Resistance to sanitizers is also enhanced by stress-associated genes formed during biofilm formation (Abee *et al.*, 2011, Wood *et al.*, 2011). Studies have also shown that biofilms are capable of dispersing and their dispersal may be triggered by environmental or nutritional changes in their community (Wood *et al.*, 2011). Biofilms may also develop an adaptation characteristic due to repeated exposure to the biocides (Bridier *et al.*, 2011). These and other factors have caused the control of biofilms to remain a challenge in food processing plants.

Among other pathogenic bacteria, *L. monocytogenes* has been known to form biofilms that are resistant to biocides (Bae *et al.*, 2012). *L. monocytogenes* can form single species biofilms, however in food manufacturing environments they are likely to form a mixed biofilm with other species either Gram positive or Gram negative bacteria (Van der Veen and Abee, 2011). *L. monocytogenes* poses serious threat to human health (Pan *et al.*, 2006). The elimination of *L. monocytogenes* biofilms in processing plants is critical for improving food safety. The objective of this study was to identify a sanitizer that can best reduce or eliminate *L. monocytogenes* ATCC7644 biofilms on tomatoes. The findings of this study will inform the food industry on the potential sanitizers that could be used to combat the long standing problem of biofilms thereby improving food safety.

5.3 Materials and methods

5.3.1 Materials

5.3.1.1 Bacterial strains

Listeria monocytogenes ATCC 7644 (Merck, South Africa) was used for this study. The strain was cultured in Fraser broth for 24 hours at 37 ºC and stored at 4 ºC (Ijabadeniyi *et al.*, 2011a). Prior to each experiment, a fresh culture was prepared from the stock culture by sub culturing in Fraser broth for 24 hours at 37 ºC. An 8 log CFU/ml culture of *L. monocytogenes* was prepared using McFarland Standards (Ji *et al.*, 2010).

5.3.1.2 Preparation of biofilms

Preparation of biofilms was carried out according to the method of Niemira (2010). Pre-cleaned glass microscope slides were wrapped in aluminium foil paper and sterilised by autoclaving for 15 minutes at 121 °C. After autoclaving, a single slide was aseptically placed into clearly labelled 50 ml centrifuge tubes using a sterile forceps. A 25 ml of tryptose soy broth (TSB-Oxoid Ltd, Wade Road, Basingstoke, Hants UK) was added to these centrifuge tubes. The solutions were inoculated with 200 µl of approximately 10^8 CFU/ml of non-adapted bacterial solutions and incubated at 37 °C for 48 hours under static conditions to form a biofilm which was used as a test pathogen. The same procedure was followed for heat adapted biofilms and chlorine adapted biofilms using heat adapted and chlorine adapted *L. monocytogenes* ATCC 7644 prepared following the methods reported in Chapter 4.

5.3.1.3 Chemicals and chemical treatments

Sodium dodecyl sulphate (SDS), levulinic acid, sodium hypochlorite solution, all purchased from Merck, South Africa were tested, individually or combined. Contact times 1, 3 and 5 minutes were investigated. The chemicals were used as follows;

1% SDS individually
0.5% Levulinic acid individually
200 ppm sodium hypochlorite solution individually
0.5% levulinic acid/0.05% SDS mixture.

5.3.2 Methods

5.3.2.1 Biofilm treatment with different sanitizer solutions

Following biofilm formation, the slides were carefully removed from TSB solution using sterile a forceps gripping the clean, dry upper portion of the slide. They were rinsed for 10 seconds under a stream of sterile distilled water to remove unattached cells. Each slide was transferred into different treatment solutions in another centrifuge tube containing 25 ml of test solutions (sodium hypochlorite solution, levulinic acid, SDS or SDS/lev mixture). Exposure times were varied on 1, 3, 5 minutes intervals. After treatment, the slides were then vigorously shaken in 25 ml of phosphate buffer (PB) in a clean, sterile 50 ml centrifuge tube. The suspension was serially diluted (1:10) in 0.1% buffered peptone water and enumerated for *L. monocytogenes* ATCC 7644. Samples of suspensions were also kept for 24, 48 and 72 hours for assessment.

5.3.2.2 Enumeration of *L. monocytogenes* biofilms

A method by Taormina and Beuchat (2001) was followed. Populations of *L. monocytogenes* ATCC 7644 biofilms were determined by surface plating serially diluted samples; 0.1 ml in duplicates was plated on *Listeria* Selective Agar (Oxoid Ltd, Wade Road, Basingstoke, Hants UK). Plates were incubated for 24 hours at 37 ºC after which colonies were counted using a colony counter.

5.4 Data analysis

Three trials were conducted for each experiment. Analysis of the data was performed using SPSS version 21 (IBM Statistics). Analysis of variance was conducted with repeated measures and Greenhouse Geisser correction to study the effect of contact time on the survival of *L. monocytogenes* ATCC 7644 biofilms and the effect of each sanitizer on the survival of *L. monocytogenes* ATCC 7644 at varied time intervals (0, 24, 48 and 72 hours). The number of surviving LM was plotted against contact time (1, 3 and 5 minutes) and also against time interval (0, 24, 48 and 72 hours). Log reductions for each contact time and sanitizer were also calculated and presented in a table. Pairwise comparison with Bonferroni adjustment was used to determine any significance difference between treatments.

5.5 Results

5.5.1 Effect of sanitizer and contact time on the survival of non-adapted, chlorine adapted and heat adapted biofilms

The sanitizers tested were able to reduce the surviving LM for non-adapted, heat adapted and chlorine adapted biofilms as seen by overall log reductions achieved as well as plotted marginal means. Increasing contact time reduced significantly the surviving bacteria for non-adapted biofilms. Though increasing contact times from 1, 3 and 5 minutes resulted in a significant fall in surviving bacteria for non-adapted biofilms, results showed that increasing contact time from 1 minute to 3 minute did not cause any significant reduction in bacteria, while a significant ($P \leq 0.05$) drop was achieved at 5 minutes. The drop in bacteria was insignificant for heat adapted and chlorine adapted biofilms.

5.5.2 Overall log reductions

When exposed for 1 minute to 200 ppm sodium hypochlorite solution, non-adapted biofilms were reduced by 1.73 log CFU/ml. A log reduction of 1.77 and 1.80 log CFU/ml was noted after increasing contact time to 3 and 5 minutes, respectively. A mixture of 0.5% levulinic acid and 0.05% SDS (mixture) reduced bacteria by a log reduction of 1.78, 1.80 and log 2.86 CFU/ml after exposure for 1, 3 and 5 minutes, respectively. Using 0.5% levulinic acid resulted in log reduction of 1.74, 1.75 and 2.0 CFU/ml after exposure for 1, 3 and 5 minutes, respectively. SDS achieved a reduction of by 1.79, 1.86 and 3.54 log CFU/ml for 1, 3, 5 minutes, respectively (Table 5.1[a]). The chlorine adapted and heat adapted biofilms had lower log reduction compared to non-adapted biofilms. This showed that the adapted biofilms had developed a resistance to the treatments and hence they survived more due to adaptive response.

Chlorine adapted biofilms were reduced by 1.69, 1.70 and 1.77 log CFU/ ml when exposed to 200 ppm sodium hypochlorite solution for 1, 3, 5 minutes, respectively. Log reduction for levulinic acid was relatively lower than sodium hypochlorite solution with 1.73 log CFU/ ml, 175 log CFU/ ml and 1.80 log CFU/ ml, respectively. A mixture of 0.5% levulinic acid and 0.05% SDS performed better than levulinic acid when used alone with log reduction of 1.74, 1.77 and 2.09 log CFU/ml for 1, 3, 5 minutes, respectively. SDS had highest log reduction of 1.78, 1.88 and 3.17 log CFU/ml for the tested contact time (Table 5.1[b]).

The log reduction for heat adapted biofilms were more or less similar to those of chlorine adapted biofilms. Log reductions of 1.69, 1.70 and 1.70 log CFU/ml were achieved when heat adapted biofilms were subjected to 200 ppm sodium hypochlorite solution for 1, 3 and 5 minutes, respectively. A mixture of 0.05% SDS and 0.5% levulinic acid led to a log reduction of 1.74, 1.77 and 2.33 log CFU/ml while exposure to 0.5% levulinic acid when used alone achieved 1.73, 1.75 and 1.80 CFU/ml for 1, 3, 5 minutes, respectively. High log reduction were achieved by use of 1% SDS (Table 5.1[c]).

Table 5.1 Log reduction (CFU/ ml) for all sanitizers at 1, 3, 5 minutes; [a] non-adapted biofilms [b] chlorine adapted biofilms [c] heat adapted biofilms

Overall log reduction non adapted biofilms [a]

Sanitizer	1 minute	3 minutes	5 minutes
NaClO	1.73	1.77	1.80
Mixture	1.78	1.80	2.86
Levulinic	1.74	1.75	2.00
SDS	1.79	1.86	3.54

Overall log reduction chlorine adapted biofilms [b]

Sanitizer	1 minute	3 minutes	5 minutes
NaClO	1.69	1.70	1.77
Mixture	1.74	1.77	2.09
Levulinic	1.73	175	1.80
SDS	1.78	1.83	3.17

Overall log reduction heat adapted biofilms [c]

Sanitizer	1 minute	3 minutes	5 minutes
NaClO	1.69	1.70	1.70
Mixture	1.74	1.77	2.33
Levulinic	1.73	1.75	1.80
SDS	1.78	1.80	3.32

The marginal means of surviving LM were plotted against sanitizers for each contact time. The results presented in Figure 5.1 show that among the sanitizers tested sodium hypochlorite solution was least effective as it had highest mean values of surviving bacteria regardless of whether the biofilms were adapted or not.

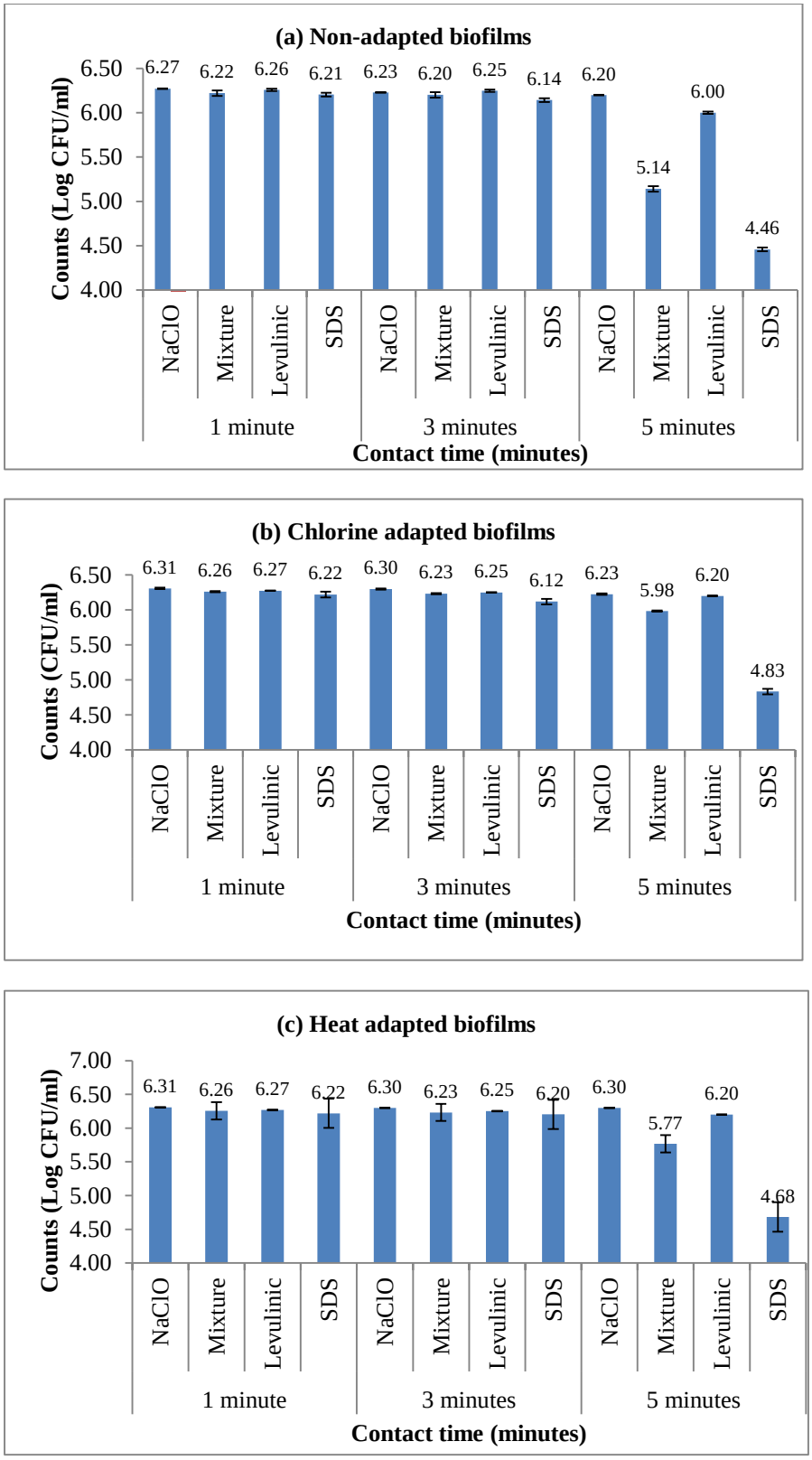
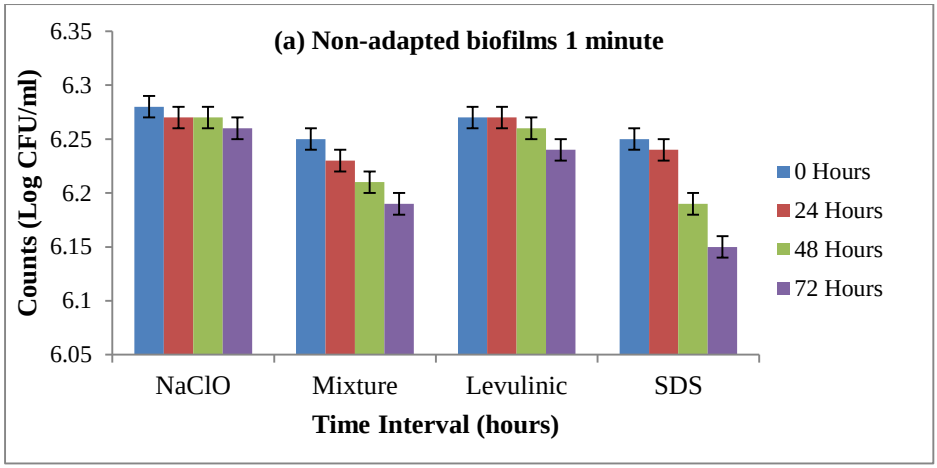
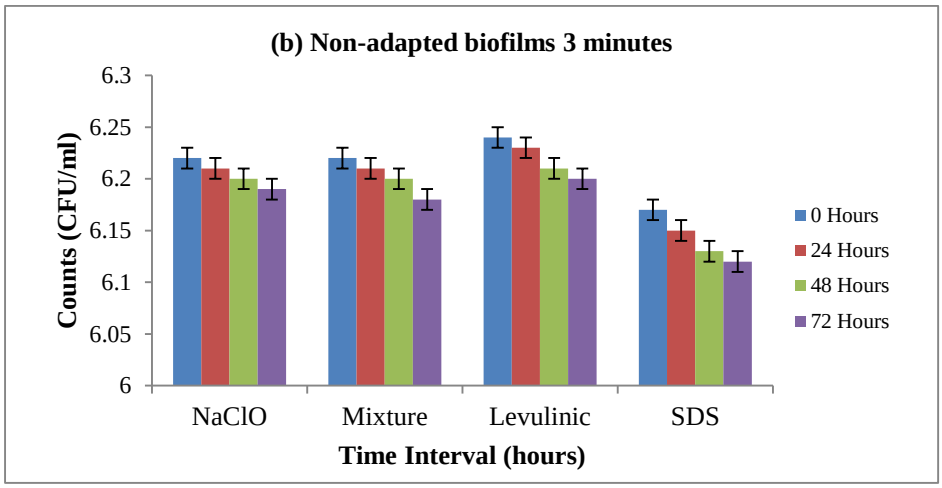


Figure 5.1 Marginal means of surviving non-adapted (a), chlorine adapted (b) and heat adapted *L. monocytogenes* biofilms (c). The low mean counts associated with sodium hypochlorite solution show that it was least effective among other sanitizers.

5.5.3 Effect of sanitizer treatments and storage time on the survival of non-adapted, chlorine adapted and heat adapted biofilms

The action of sodium hypochlorite solution, SDS, levulinic acid and mixture was significantly different for all tested bacteria. As reported earlier in this article, sodium hypochlorite solution was least effective. Increasing storage time (time intervals) from 0 to 72 hours did not have a significant decrease in surviving bacteria, as observed in this research. Though there was a reduction in bacteria for up to a storage time of 72 hours, the reduction was not significantly different. Figure 5.2, 5.3 and 5.4 shows the trends.





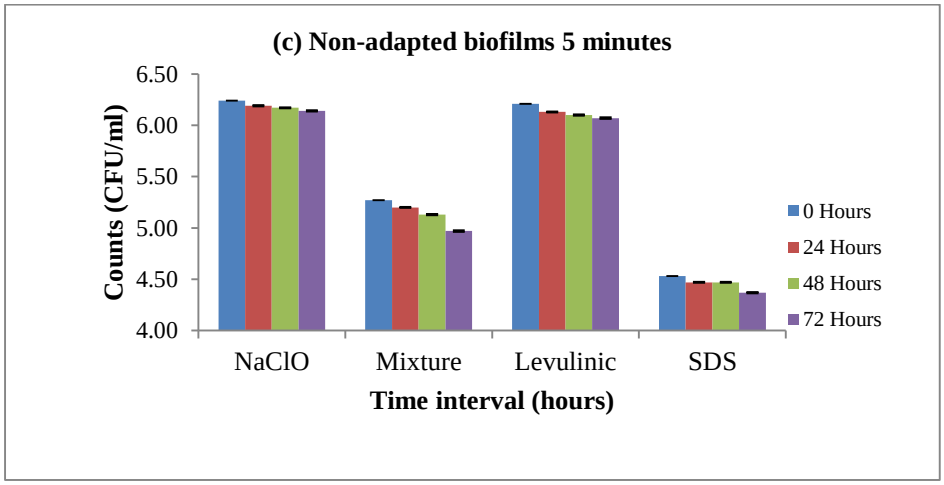
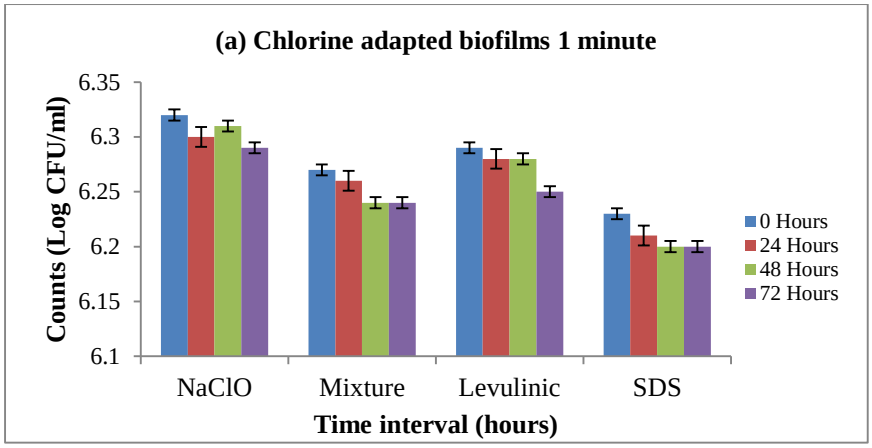
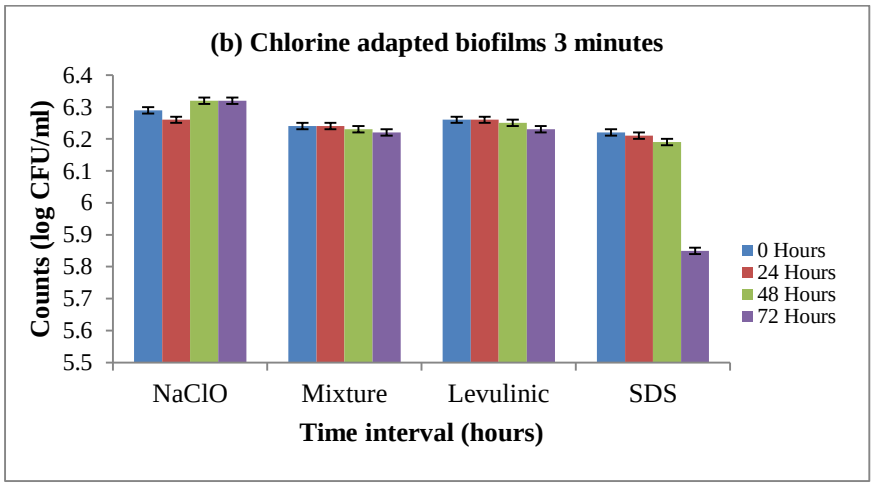


Figure 5.2 Survival of non-adapted *L. monocytogenes* ATCC 7644 biofilms following sanitiser treatment for 1 minute (a), 3 minutes (b) and 5 minutes (c)





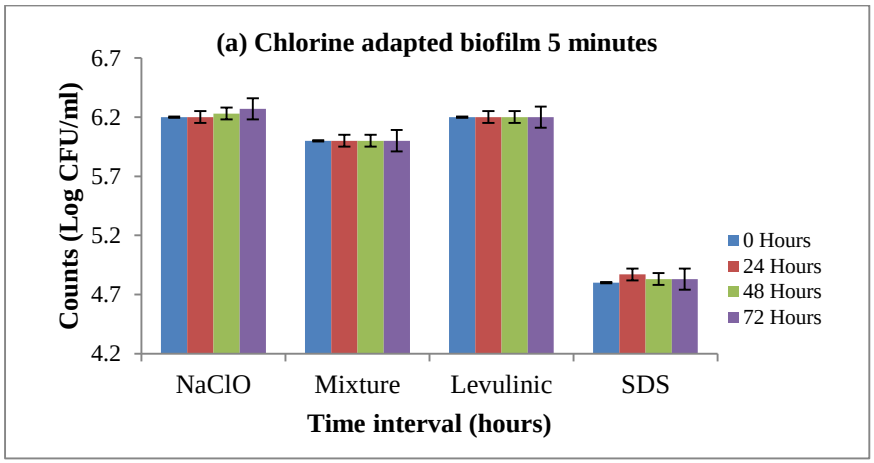
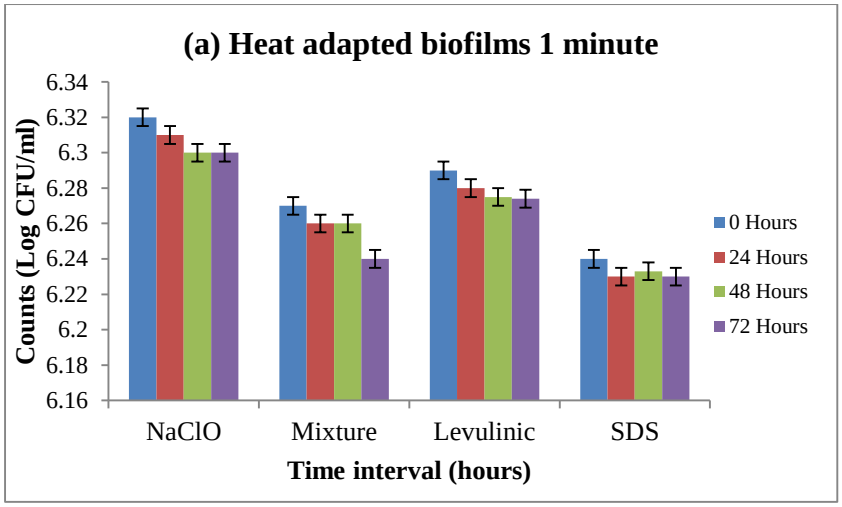
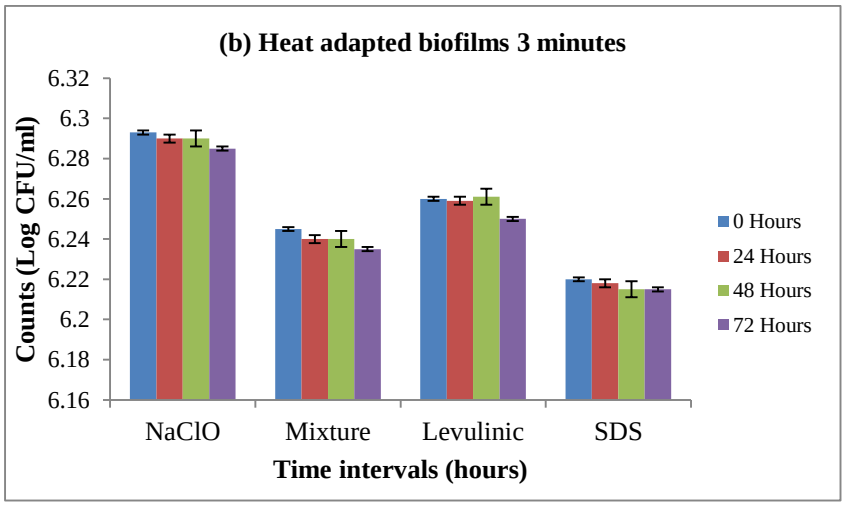


Figure 5.3 Survival of chlorine adapted *L. monocytogenes* ATCC 7644 biofilms following sanitiser treatment for 1 minute (a), 3 minutes (b) and 5 minutes (c)





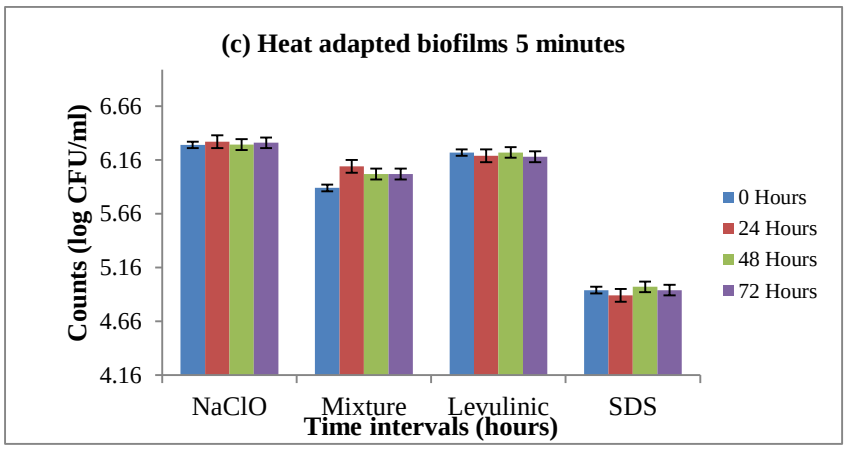


Figure 5.4 Survival of heat adapted *L. monocytogenes* ATCC 7644 biofilms following sanitiser treatment for 1 minute (a), 3 minutes (b) and 5 minutes (c)

5.6 Discussion

Sanitizers are chemical agents used to inactivate bacteria in food processing units. Previous studies showed that biofilms are resistant to sanitizers (Abee *et al.*, 2011, Renier *et al.*, 2011, Wood *et al.*, 2011). In this study, there were surviving bacteria after treatment with sanitizers. In order of performance, sodium hypochlorite solution had the lowest log reduction followed by levulinic acid and mixture, meanwhile SDS had the highest log reduction.

Despite its wide use as a sanitizer, the ineffectiveness of sodium hypochlorite solution in eradicating pathogens has been previously reported (Allende *et al.*, 2009, Gil *et al.*, 2009, Kim *et al.*, 2009, Mahmoud *et al.*, 2007). Previous studies reported that SDS or levulinic acid when used in isolation does not achieve significant results (Cannon *et al.*, 2012, Zhao *et al.*, 2009). In this study, a concentration of 0.05% SDS mixed with 0.5% levulinic acid could not achieve total reduction of biofilms during the entire storage period. Also SDS alone could not totally eradicate *Listeria monocytogenes* biofilms though it acted most effectively against non-adapted and stress adapted bacteria. Other studies using higher concentrations 3% levulinic acid and 2% SDS achieved a reduction of *Salmonella* from 19% before treatment to 1% after treatment; coliform counts were reduced from between 6-8 to 2-4 log CFU/cm^2, and aerobic plate counts were reduced from 7-9 to 4-6 log CFU/cm^2 (Zhao *et al.*, 2011). A 5% levulinic acid and 2% SDS also showed to be effective on influenza virus (Aydin *et al.*, 2013). These findings suggest that higher concentrations of sanitizers may be required to effectively reduce biofilms. However, higher concentrations may interfere with sensory properties of food items and could be hazardous.

From the results, it can be concluded that non-adapted biofilms tested were more responsive to sanitizer treatment compared to chlorine adapted and heat adapted biofilms. The bacterial counts for chlorine adapted biofilms were more or less similar to those of heat adapted biofilms, with heat adapted counts slightly higher than those of chlorine adapted. From these findings it can be concluded that sodium hypochlorite solution, levulinic acid and SDS are not able to completely eradicate biofilms. Other researchers have also reported on biofilm resistance to sanitizer. Machado *et al.* (2012) using benzalkonium chloride found that adapted biofilms of *Pseudomonas aeruginosa* and *Escherichia coli* maintained their mass and activity after treatment, while Stopforth *et al.* (2002) found no differences between previously acid-adapted and non-adapted *L. monocytogenes* with regard to sensitivity to sanitizers using sodium hypochlorite solution and quaternary ammonium compound.

Apart from these sanitizers, biofilms have also been reported to be resistant to benzalkonium chloride, peracetic acid and nisin (Ibusquiza *et al.*, 2011). Cruz and Fletcher (2012) found that out of the twenty one sanitizers tested against *L. monocytogenes* in biofilm, only peroxyacetic acid, chlorine dioxide and acidified sodium chlorite-based products gave a 5 log CFU/ml reduction. It is well known that biofilms are more resistant than their planktonic counterparts (Abee *et al.*, 2011, Bridier *et al.*, 2011, Van der Veen and Abee, 2011, Gandhi and Chikindas, 2007, Kim and Wei, 2007, Joshua *et al.*, 2006, Pan *et al.*, 2006) and that mixed biofilms are more resistant that single species biofilms (Van der Veen and Abee, 2011). A use of combined methods could help in reducing the viability of biofilms. Other researchers suggested mechanical abrasion with subsequent spray applications of sanitizer to reduce biofilm and non-biofilm populations of *L. monocytogenes* from stainless steel surface (Chambliss-Bush, 2012).

Resistance of biofilms to sanitizers is attributed to many factors. It can be intrinsic, genetically acquired or phenotypically induced. According to Lambert and Johnston (2001), sanitizer effectiveness can be impaired by the presence of organic molecules such as proteins, nucleic acid and carbohydrates. This is because sanitizers are highly chemically reactive and may thus interact with these organic molecules. Ganeshnarayan *et al.* (2009) mentioned the presence of electrostatic forces as having a negative effect on the movement of cationic surfactants across negatively charged biofilms thereby reducing biocide effectiveness. Hydrophobic interactions due to long carbon-chains have also been implicated in reducing sanitizer effectiveness (Habimana *et al.*, 2010, Sandt *et al.*, 2007). Leriche *et al.* (2003) reported that *Staphylococcus sciuri* was protected from chlorine treatment due to a mixed biofilm it formed with a more resistant strain of *Kocuria* spp.

Increasing contact time decreased significantly the surviving LM of *L. monocytogenes*. This is evident that, the longer the bacterium is exposed to chemicals, the greater the chances of reducing their survival. Several researchers agree that increasing contact time increases effectiveness of sanitizers (Møretrø *et al.*, 2012, Ding *et al.*, 2011, Mattson *et al.*, 2011, Park *et al.*, 2011) as more time is allowed to penetrate the three dimensional aggregate of biofilms. When biofilms were stored up to 72 hours, their numbers did not decrease significantly. These results suggest that biofilms were either able to recover during the storage period or continued to multiply since sanitizers had not completely inactivated them.

5.7 Conclusion

Sanitizers cannot completely eradicate *L. monocytogenes* biofilms. Adapted biofilms are more resistant to sanitizers compared to non-adapted biofilms. A contact time of 5 minutes is not enough to eradicate biofilms and hence a higher contact time coupled with increase in concentration of sanitizers may achieve better results.

CHAPTER SIX: GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary of research approach

Surface decontamination of fresh fruits and vegetables is paramount to improve their microbiological quality and reduce the occurrence of food borne illness (WHO., 2011). In view of the global trade, increasing productivity of fresh produce coupled with the shift in consumer interest; more technologies are required for minimising safety issues relating to fruits and vegetables. Novel methods like irradiation and high pressure pasteurisation have been approved for use in other products, however, these may not work well with fresh produce. This is because, fresh produce require gentle processing to maintain the sensory qualities. Researchers have continued to use different sanitizers on fresh produce and ready-to-eat foods. Chlorine based sanitizers have been widely used for sanitizing, however, reduced microbiological efficiency coupled with sensorial changes and the eventual formation of carcinogenic chlorinated compounds have led to further research on the application of alternative decontamination technologies (Stopforth *et al.*, 2008, Hellstrom *et al.*, 2006, Gonçalves *et al.*, 2005).

Tomatoes are amongst the most widely consumed fresh produce as they are highly recognised not only for their nutritional value and ease to prepare but they are used as a food condiment (Rao and Rao, 2007). Tomatoes can be widely used either raw or processed in many dishes and products. They are also recognised for their high oxidant value. Being a fresh produce, it is prone to any sort of contamination that can be found in fresh produce. Decontamination before and after processing is necessary to achieve microbiological safety of tomato and its products. Tomatoes contain fragile nutrients that are water soluble such as Vitamin B and Vitamin C (Frusciante *et al.*, 2007). The nutrients are also sensitive to heat and prolonged processing methods that have previously been used on tomatoes and other foods to destroy food borne pathogens. Less extensive sterilisation methods are suitable for tomatoes to maintain sensory quality.

This study set out to investigate the effect of sodium dodecyl sulphate, levulinic acid and sodium hypochlorite solution on the survival of *L. monocytogenes* ATCC 7644 on tomatoes. Specifically, non-adapted, heat adapted and chlorine adapted *L. monocytogenes* species were tested. The study also investigated the effect of these sanitizers on biofilms grown on glass slides; which were either non-adapted, heat adapted or chlorine adapted. The study further

investigated the effect of these sanitizers on the physicochemical characteristics of tomatoes; specifically pH, TSS and TA. The attachment of *L. monocytogenes* on tomatoes after treatment with these sanitizers was also assessed.

An experimental approach was used to achieve the set objectives. The methods included treatment experiments with the above-mentioned sanitizers, storage of treated samples over 72 hours at 4 $^{\circ}$C to assess any changes that may arise due to storage. Study of treated samples using SEM was also done to observe how the pathogen attaches itself to tomatoes after treatment.

The study is innovative in that many tests of the above mentioned sanitizers have been done on Gram negative bacteria, but this study focused on Gram positive bacteria. This study pioneered an investigation on tomatoes as well as the effects thereof due to these sanitizers on the physicochemical properties of tomatoes. This study contributes to the knowledge of effects of sanitizers on tomato physicochemical properties, as a function of sensory quality. It paves a way for further studies on the use of these particular sanitizers on other Gram positive food pathogens. Through the investigation of effects of sanitizers on physicochemical properties, the study sets to show the importance of selecting a suitable sanitizer for particular fresh produce. This study will also contribute to knowledge to the growing fruit and vegetable industry in South Africa.

6.2 Findings and conclusions

The study revealed that 200 ppm sodium hypochlorite solution is not the best sanitizer for decontamination of fresh produce. According to this study, SDS used alone best destroys *L. monocytogenes* on tomatoes, followed by a mixture of SDS and levulinic acid, then levulinic acid alone. Investigations done with non-adapted, heat adapted and chlorine adapted *L. monocytogenes* revealed that non-adapted *L. monocytogenes* are more responsive to the tested sanitizers. Highest log reduction was achieved on non-adapted *L. monocytogenes*. The log reduction achieved for heat and chlorine adapted *L. monocytogenes* were lower than those of non-adapted *L. monocytogenes*. From these findings, the researcher concluded that stress adapted pathogens are more resistant to the action of sanitizers. The resistance of stress adapted pathogens has also been reported by several researchers. This is because, the stress adapted pathogens acquire cross protection against other treatments when they have previously subjected to one form of stress.

A good sanitizer is capable of maintaining produce quality while enhancing product safety. Further investigations done on physicochemical properties revealed that SDS and sodium hypochlorite solution have minimal effects on the physicochemical properties of tomatoes. The use of levulinic acid alone and a mixture of SDS/ Lev resulted in a significant drop in pH and increase in TA. pH and TA contributes very much to the sensory properties of tomatoes, hence use of levulinic acid and mixture produced unfavourable results in this study and would not be recommended for use as decontaminants should these sanitizers get into contact with sub-surfaces. Total solids and titratable acidity have direct implications in the tomato processing industry, therefore sanitizers that cause minimal or no damage to these parameters are recommended. The researcher concluded that since SDS can achieve better log reduction and minimal damage to physicochemical properties, it can be effectively used to replace the routine chlorine washing. Sanitizers are best used on surface decontamination; contact with sub-surfaces can affect sensory qualities.

With regards to biofilms, the sanitizers did not achieve significant log reduction. Highest log reduction was achieved when using SDS on non-adapted, heat adapted and chlorine adapted biofilms. Sodium hypochlorite solution achieved the lowest log reduction in all biofilms. Though there was reduction in surviving LM for all biofilms; the non-adapted biofilms were more responsive to the sanitizers. For effective applications of sanitizers against biofilms, higher sanitizer concentrations or longer contact time may be required.

Storage of tomato samples treated with sanitizers for 72 hours resulted in significant decrease ($P \leq 0.05$) in surviving LM, both for adapted and non-adapted *L. monocytogenes*. This showed that the pathogens failed to recover after treatment with these sanitizers. This could be explained that there were no sufficient nutrients and growth conditions to promote recovery of injured pathogens. For biofilms, recovery was noted but it was not significant enough to restore the levels to match surviving LM at Day 0. It is known that biofilms are resistant to sanitizers due to their 3 dimensional structures, hence more vigorous treatment may be required to achieve better results.

This study also revealed that varying contact times also has an effect on surviving LM. Findings indicated that varying contact time from 1 to 3 minutes did not result in any significant changes, however increasing contact time to 5 minutes caused a significant decrease ($P \leq 0.05$) in surviving LM. This was the case with non-adapted pathogens, chlorine adapted and heat adapted bacteria. With regards to biofilms, varying contact time from 1 to 5 minutes did not

cause a significant drop in surviving LM. Previous research agrees that increasing contact time increases the effectiveness of sanitizers. However, high contact time may have detrimental effect on the sensory quality of the final product.

Though results from these experiments indicate that there were residual pathogens after sanitizer treatment, observation of these samples under SEM did not show any pathogen attached to the surfaces. This could be because, the method used for preparation of samples for SEM study was not relevant in this particular case. Pathogens may also have migrated to other areas which were not viewable under SEM. Other methods can be implemented for viewing of samples under SEM.

6.3 Recommendations

Interventions to reduce pathogenic microorganisms on fresh produce are important elements in decreasing the risk of foodborne illnesses. In light of the findings from this study, it is recommended that further studies may be done on tomatoes, using the same sanitizers but varying their concentrations. Studies can further be carried investigate other physicochemical properties that were not investigated in this study. The effect of these sanitizers on the surface and sub-surface of tomatoes could also be compared. Despite the fact that SDS proved to be a better sanitizer than other studies tested herein, further studies are recommended on the allowable levels and effects on other quality parameters not addressed herein. Studies examining synergistic interactions between sanitizers can also address issues around the use of single sanitizers. Further studies can also focus on evaluating different concentrations of SDS that could effectively reduce biofilm survival as they are still a concern in food manufacturing settings. The findings on levulinic acid and mixture can be used effectively to research on other fresh produce that could make use of these sanitizers. Understanding the ecology of pathogens on fresh produce is essential for the development of methods to eliminate them from these products with further research targeting factors affecting survival, attachment and internalization of food borne pathogens in fresh produce.

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