Effect of bacteriophage control and artificial neural networks prediction in the inactivation of *Listeria monocytogenes* on fresh produce

By

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DECLARATION

This is to declare that this work was done by Adebola Olubukola Oladunjoye, student number 21357016, Department of Biotechnology and Food Technology, Durban University of Technology, South Africa.

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DEDICATION

This thesis is dedicated to the Almighty God for his support throughout my study. I would also like to especially dedicate this thesis to my lovely wife- Oladunjoye Bolade Victoria, who has demonstrated immense courage, integrity and support all through my absence from home. I love you all the way my love.

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ABSTRACT

There has been a global increase in fresh produce consumption, due to its attendant nutritional and health benefits. On the other hand, increase in the outbreak of diseases, accompanied with health and economic implications, have been traced to this development. A good number of pathogenic contaminants along the food chain have been identified as causative agents with *Listeria monocytogenes* identified as one of such. Among other control strategies, the use of bacteriophage, was recommended as a palliative measure. Furthermore, the application of artificial neural networks (ANN) in food safety remains an emerging concept in risk assessment study. Therefore, the aim of this research is to investigate the effect of bacteriophage or phage control and artificial neural network prediction in the inactivation of *L. monocytogenes* ATCC 7644 on fresh produce. Fresh-cut tomato and carrot were artificially inoculated with *L. monocytogenes* (10^8 CFU/ml) and subjected to antimicrobial treatment of Listex P100 bacteriophage (10^8 PFU/ml), sucrose monolaurate (SML at 100, 250 and 400 ppm), with chlorine (sodium hypochlorite at 200 ppm) used as control. Also, application of ANN to predict the risk effect of antimicrobial treatments of bacteriophage, sucrose monolaurate and chlorine was evaluated on the fresh-cut produce. Mathematical models were developed using a linear regression and sigmoid (hyperbolic and logistic) activation function-(120). Data sets were trained using Back propagation ANN, containing one hidden layer with four hidden neurons. Furthermore, carbon utilization profile of phage-treated *L. monocytogenes* using phenotypic micro array method was evaluated. In the first phase, susceptibility of *L. monocytogenes* subjected to certain stress-adapted conditions (acid,-adapted AA, chlorine-adapted CA, heat-adapted HA) and non-adapted-NA to phage treatment inoculated on the fresh-cut produce stored for 10 days at 4, 10 and 25^{o}C was evaluated. The second phase investigated the combination of bacteriophage and sucrose monolaurate (using chlorine at 200 ppm as control) to inhibit the *L. monocytogenes* growth on the fresh-cut produce stored for 6 days at 4, 10 and 25^{o}C. Physicochemical properties (pH, titratable acid-TTA, total soluble solids-TSS, and colour values-CIE L* a* b*) of the fresh produce after treatment were evaluated. In the third phase, ANN as a predictive tool was used to evaluate the risk involved in the relationship among the initial bacterial load, fresh-produce type, antimicrobial concentration and residual bacteria. In the final phase, 100 µL of phage-treated *L. monocytogenes* was introduced into a 96-micro well plate impregnated with a tetrazolium dye. The Carbon utilization profile was evaluated at intervals of 4 hours for 48 hours using a biolog micro station. Generally, *L.*

monocytogenes grew on both fresh-cut produce and the storage temperature did not adversely affect the lytic ability of the phage treatment. Antimicrobial treatment of phage and sucrose monolaurate had minimal variations on the physicochemical properties of both fresh-cut samples. All stress-adapted and non-adapted *L. monocytogenes* were ($p \leq 0.05$) susceptible to bacteriophage control. Phage treatment reduced non-adapted, acid adapted, chlorine-adapted, and heat-adapted *L. monocytogenes* population by 0.57, 0.81, 0.86 and 0.95 log CFU/ml in fresh-cut tomato, and 2.26, 2.41, 2.49 and 2.54 log CFU/ml in fresh cut carrot respectively. Furthermore, the additive effect of SML at 100 and 250 ppm had no significant effect on phage lysis. However, combination of phage with SML at 400 ppm significantly ($p \leq 0.05$) resulted in 1 and 3 fold reductions in tomato and carrot respectively. Control treatment with chlorine resulted in 1-2 log reductions on both fresh produce. Algorithm data set trained using ANN gave 100% accuracy. Prediction with logistic activation function showed the highest positive correlation relationship between predicted and observed values with ~ 0.99 R^2-value and MSE of 0.0831. Carbon utilization profile showed hexose and pentose sugars-ribose, glucose, fructose and sugars were maximally utilized while oligosaccharide sugars of sucrose, cellobiose and gentiobiose were similarly observed to be utilized. Notably, utilization of glucose-6-phosphate which determines *L. monocytogenes* pathogenicity was not very pronounced in the carbon profile. Bacteriophage application in the inactivation of *L. monocytogenes* contamination of fresh produce provides a safe means of control. Its perceived limitation however, can be overcome by combining with other antimicrobials. Similarly, the use of artificial neural networks prediction, remains an improved approach to harness the potential risk that could occur through this method.

CHAPTER ONE

1.0 Introduction.

Fresh or slightly processed produce constitutes an integral component of human diet. They represent excellent micronutrient sources of vitamins, minerals and some phytochemicals which offers nutritional and health benefits (Abadias *et al.* 2008; Warriner *et al.* 2009). Also, development in agro-processing, preservation techniques and trade globalization has enhanced the availability of exotic fresh produce throughout the year (Sela *et al.* 2009).

Studies have shown that consumption of ≥400 g per day of fresh produce, which corresponds to "five-serving per day", offers a prophylactic means of reducing certain diseases such as cancer, diabetes, neurological and certain cardiovascular disorders (Allende *et al.* 2006; CAC 2010). This guideline has been strongly advocated by relevant global organizations such as World Health Organization (WHO), Food and Agricultural Organization (FAO), Center for Disease Control and Prevention (CDC) and European Food Safety Authority (EFSA), as part of contributing to a balanced and healthy society (WHO 2003; FSA 2006).

However, outbreak of food borne illnesses, together with concomitant clinical cases and deaths linked to fresh produce consumption, have also been reported (Warriner *et al.* 2009; Scallan *et al.* 2011). It is therefore not surprising that numerous safety challenges are associated with fresh produce since they are often eaten raw or minimally processed. This makes fresh produce highly tolerable to several pathogenic contaminations along the food chain (Beuchat 2002). Furthermore, non-lethal treatments prior to consumption have been found to facilitate the proliferation of these pathogenic contaminants (Scolari and Vescovo 2004; Tournas 2005; Allende *et al.* 2006; Froder *et al.* 2007; Sánchez, Elizaquível and Aznar 2012).

The commonly identified pathogenic contaminants of safety and public health importance in fresh produce includes certain species of bacteria such as *Salmonella, Shigella, Escherichia coli, Listeria*, viruses (hepatitis A virus and norovirus) and protozoans such as *Cyclospora cayetanensis* and *Cryptosporidium parvum* (Rangel *et al.* 2005; Aruscavage *et al.* 2006). Meanwhile, the major contributing pathogens of these outbreaks have been reported to be mostly from bacterial origin (Sivapalasingam *et al.* 2004).

Amongst these bacterial pathogens which constitute the main focus of this research, the most worthy is *Listeria monocytogenes*. It is a gram-positive, facultative bacterium which can be found across many locations in the environment. It has a low prevalence level, but a high

lethality rate of (20-30%) compared to other bacterial pathogens of health importance. Furthermore, it has the ability to survive a number of environmental and stress conditions (Farber and Peterkin 1991; Todd and Notermans 2011).

Foodborne listeriosis resulting from *L. monocytogenes* contamination has been identified as the third-leading cause of death reported every year in the USA (Hoffmann, Maculloch and Batz 2015). Incidentally, the disease can affect people of all ages and the immuno-deficient people in a given society (Leverentz *et al.* 2003; Bae *et al.* 2013). Strategies to eradicate *L. monocytogenes* contamination in foods have resulted in legislation of "zero tolerance" approach by the United States Department of Food and Agriculture (Klima and Montville 1995).

Consequent to this, a number of strategies have been recommended against the proliferation of this bacterial pathogen in fresh produce. As a result of toxicity and health concerns associated with certain chemical-based sanitizers, naturally sourced antimicrobial has become increasingly in demand (Raybaudi-Massilia *et al.* 2009; Siroli *et al.* 2015). Preliminary work to investigate the use of nisin and its combinations with organic acids and salts against *L. monocytogenes* on fresh-cut tomato has been published in the course of this research (See Appendix).

However, the use of bacteriophage was recommended as one of the methods (FDA 1998b). Bacteriophages or phages are viruses which invade and kill bacteria through a lytic process. They are natural commensals of bacteria and are very abundant in the environment (Kutateladze and Adamia 2010; Ackermann and Węgrzyn 2014; Dalmasso, Hill and Ross 2014). They have a specific target host they lyse without causing secondary infection (Spricigo *et al.* 2013). They are abundantly available and reproduce quickly within their host system in a small amount making them an economic means of biocontrol (Hughes *et al.* 1998). They also adapt to phage-resistant bacteria mutants, and do not constitute any toxicity risk to mammalian cells (Guenther and Loessner 2011; Sillankorva, Oliveira and Azeredo 2012).

Predictive microbiology describes the influence of environmental factors on the growth and survival of microorganisms via a mathematical model (Pérez-Rodríguez and Valero 2013; Huang 2014). Evolutionary models are used in food-based products as risk assessment tools to determine the response of microorganisms in an altered environment. However, due to the large set of data associated with predictive microbiology, models have been limited in combining both the kinetic growth in a wide range of experimental conditions and probability growth under a specified condition (McKellar and Lu 2001).

The use of Artificial neural networks (ANNs) as a data-driven model, have been found to offer better prediction on the growth and survival of microorganisms (Yu, Davidson and Yang 2006; Yolmeh, Najafi and Salehi 2016). Artificial neural network basically operates by simulating activity of the human brain. It has gained popularity in the field of medicine, meteorology and financial management. However, its utilization in the field of food safety is new and recent (Huang, Kangas and Rasco 2007).

1.1 Problem statement

Global increase in production and consumption of fresh or slightly processed produce in recent times has been well documented (Abadias *et al.* 2008; FAO/WHO 2008b; Olaimat and Holley 2012). Similarly, outbreak of diseases resulting from pathogenic contamination of fresh produce along the food chain have also been on the rise, with reported cases of hospitalization and death (Lynch, Tauxe and Hedberg 2009; Warriner *et al.* 2009).

Food borne listeriosis occasioned by certain strain of *L. monocytogenes* bacteria has been implicated in wide varieties of fresh produce as one of the notable disease of public health importance (Beuchat 2002; Scallan *et al.* 2011; Botticella *et al.* 2013). In the USA, it has been estimated that about 1600 illnesses and 260 deaths occur every year due to *Listeria* contamination in Ready-to-eat (RTE) foods including fresh produce (Scallan *et al.* 2011). In Canada, about 178 cases of food illnesses caused by *Listeria* contamination have been reported (Thomas *et al.* 2013). In Europe, over 2100 cases of *Listeria* contamination are reported every year in an increasing trend since 2009 (Iannetti *et al.* 2016).

In South Africa, where the domestic consumption of this fresh commodity either as a whole, or minimally processed (desserts, food adjuncts or fruits and vegetable salad) has been reported to have increased from 1.8 to 2.1 million tons between 1988 and 1998 (Rathogwa *et al.* 2000), information on listeria contamination and its control on fresh produce has been poorly investigated. Recently, three different isolates of *L. monocytogenes* contaminations have been identified in some patients in the Western Cape province (Smith *et al.* 2016).

This recalcitrant pathogen has been found to evolve adaptive mechanism to circumvent certain stress (post-harvest) conditions such as acid shock (fermentation), heat shock (during pasteurization) and resistance to common sanitizers (such as chlorine) in fresh produce processing (Yousef and Juneja 2002).

Based on the above assertions, there is a need to adopt a more virile measure against the conventional use of chemical-based sanitizers (such as chlorine), to the use of natural, safer

and eco-friendly control measures. The use of bacteriophage or phage either singly or in combination with other antimicrobials as intervention measure has received creditable attention in recent times. This is due to the advantages it provides over other antimicrobial agents. Bacteriophage control of *Listeria monocytogenes* in fresh produce has been reported in most industrialized countries of the world, but very little or no information of its application against stress-adapted *L. monocytogenes* and in combination with sugar esters such as sucrose monolaurate is available. Also, information on its utilization in South Africa as the highest producer of fresh produce in Sub-Saharan Africa has been very little.

However, as the need for phage control of *L. monocytogenes* in fresh produce becomes imminent, it is also important to assess the risk associated with the residual bacteria levels as total elimination may seem practically impossible. Artificial Neural Networks (ANNs) as a risk assessment tool have proven to offer ideal and better risk predictions especially in addressing uncertainties and variations often linked with microbial growth (Jeyamkondan, Jayas and Holley 2001). Furthermore, application of ANN risk prediction on some fresh produce when treated with certain sanitizers has been reported (Keeratipibul, Phewpan and Lursinsap 2011; Ozturk *et al.* 2012), but no investigation on its use in phage biocontrol of fresh produce.

Meanwhile, from genomic studies, *L. monocytogenes* has been found to resist certain macrophages (such as bacteriophage therapy) by utilizing certain pentose and hexose carbon sugar sources. These are usually cellular metabolites from its glycolytic pathway and are used as principal nutrient for growth in intracellular environment (Glaser *et al.* 2001; Hain, Steinweg and Chakraborty 2006). Further studies on this showed that the pathogen mainly utilize a carbon-6-phosphorylated as sugar metabolite (Grubmüller *et al.* 2015).

However, a simplified growth assessment of bacteria using phenotype microarrays has been reported to quantify this (Bochner 2009). Following its application in profiling antibacterial activity of red cabbage (Bakar, Hafidh and Abdulamir 2012), evaluating the carbon utilization of phage-treated *L. monocytogenes* grown on fresh produce using phenotype micro array could provide more insight into application of phage as a veritable tool of controlling Listeriotic disease in an implicated patient resulting from fresh produce contaminations.

CHAPTER TWO

2.0 Literature review

2.1 Global production and economic importance of fresh produce

Global production of exotic fresh produce has increased in recent times due to a continuous demand and consciousness for a healthy life style (Williams 1995; Lampe 1999; Randhawa *et al.* 2015). Fruits and vegetables remain one of the world's fastest growing agricultural commodities due to its contributive nutritional, health and economic benefits (Ramos *et al.* 2013; Panda *et al.* 2016). According to a report by FAO, production of over 2.4 billion tons (Figure 2.1) of fresh produce were reported in the year 2008 with China and India as leading producing countries (FAO/WHO 2008b; Negi and Anand 2015).

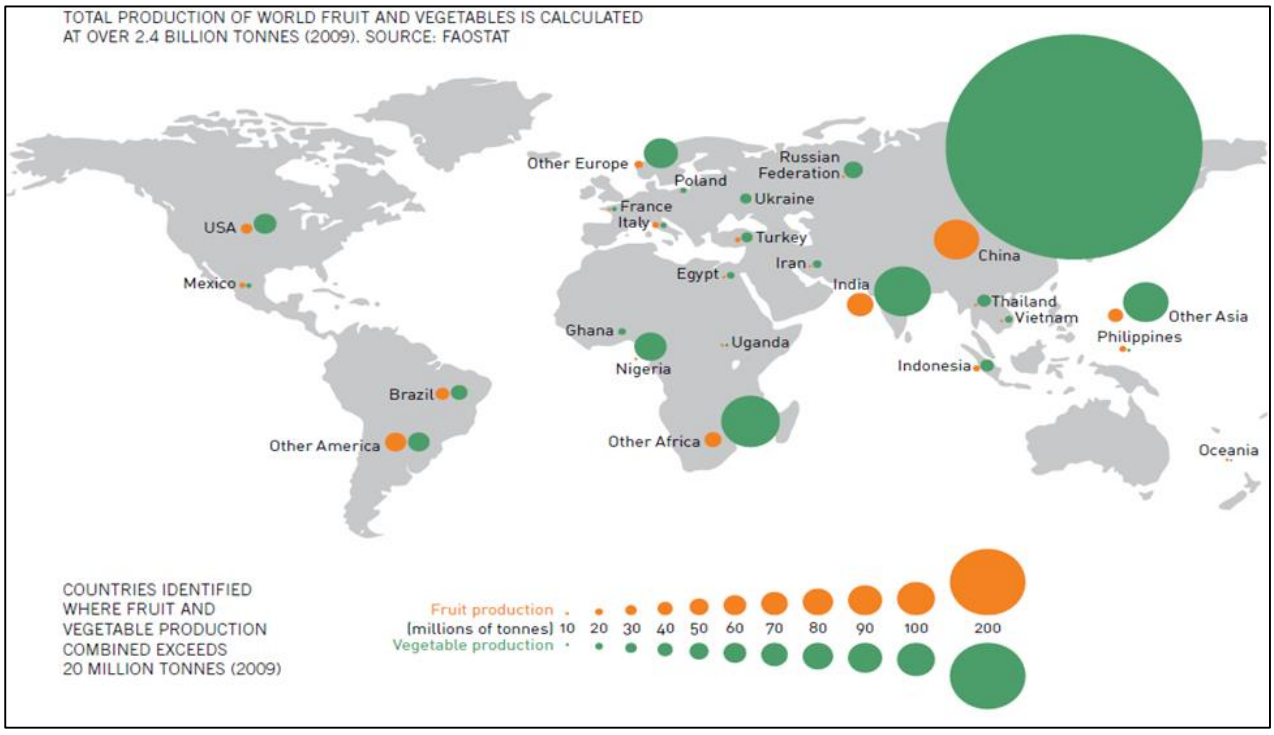


Figure 2.1 Global production of fruits and vegetables

(FAO 2009)

Fresh produce recorded an increased global production by 94% between 1980-2004 and 52% (Figure 2.2) between 1992-2004 (EU 2007). Fruit production alone in 2000-2011 rose by 38% while global fruit production in the year 2004 was estimated at 500 million tons (FAOSTAT

2013). This increasing trend yielded 9.4% production in the year 2013 above the preceding year. India, being the second largest producer of fruits and vegetables, shared 10 and 14% of this output respectively (Panda *et al.* 2016). China currently produces and exports one third of fruits and half of vegetables consumed all over the world, while Europe and North America takes the next five position (IBIS 2016).

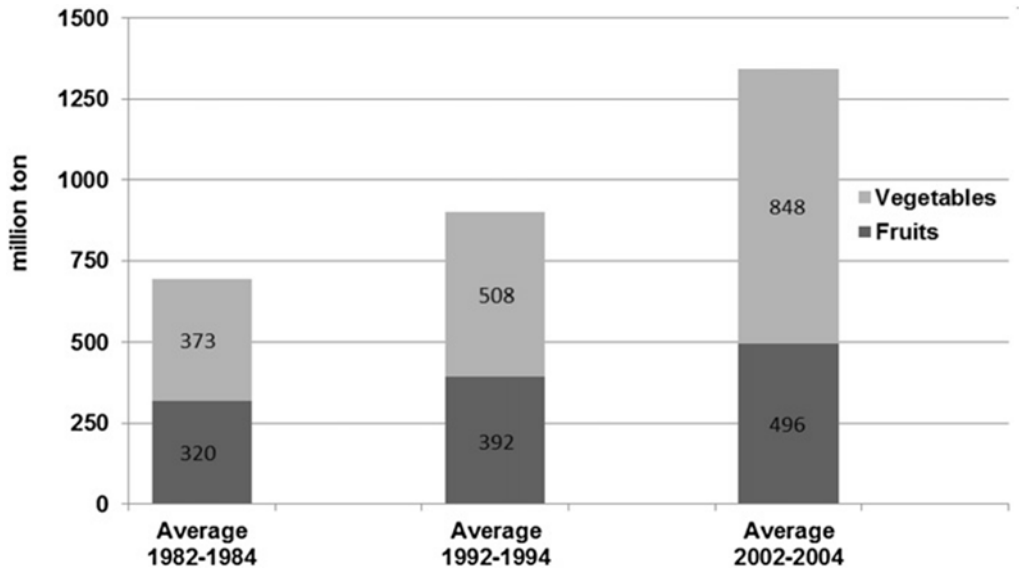


Figure 2.2 Global fruit and vegetable production from 1982-2004

(EU, 2007)

The production and consumption of fresh produce continues to contribute favorably to the global economy. United States of America (USA) ranks the first in terms of importation and exportation of produce commodities, whose level of exportation has doubled since the mid 90's (Bruinsma 2003). Import value on fresh produce in 2011 was reported to be more than $7 billion while export value rates over $12 billion (Johnson 2013). In Canada, consumption of fruits have been reported to have increased by 56% and vegetables by 26% yearly from 1963-2010 (Olaimat and Holley 2012). In 2010, reported values of various imported vegetables have been estimated at $2.8 billion, while in 2011, about 1.2 million tons of vegetables valued at $614 million were produced (Allen *et al.* 2013).

South Africa's fresh produce industry has been growing in the last decade, thus contributing to her agro-economy. Between 1980 and 2007, an increase of 18-26% was reported in fresh produce output (Barrientos and Visser 2013). As a leading nation of fresh produce export in Africa especially to the EU, over 50% of citrus weighing about 1.3 million tons had been

reported to have been exported between 2001-2011, and also the net worth of this production valued at $302 million for fruits and $291 million for vegetables (Barrientos and Visser 2013; Meitz-Hopkins *et al.* 2013).

2.2 Fresh-cut product and economic trend

Fresh-cut products basically comprises fruits or vegetables that have undergone minimal pre-processing operations such as trimming, peeling and/or cutting into a final convenient product and packaged to offer healthy and nutritious ready-to-eat diet while maintaining its wholesomeness (IFPA 2000). Consumers demand for fresh-cut produce as convenience food has increased in recent dispensation in response to the need for a healthier and natural product. Fresh produce industry has however responded to this through innovative and cutting edge techniques to enhance its value.

In the USA, fresh-cut products continue to contribute increasingly to the food-based sector as convenient food. In the year 2006, 27% of this food commodity was sold to this sector, while 73% was sold in the retail market. The total estimated value on fresh-cut produce increased from US$3.3 billion in 1999 to US$15.5 billion in 2007(Cook 2009). This development has been occasioned by the synergy of the fresh-cut industry across all levels in the food chain; including the food service and retail sector to form a conglomerate.

In Europe, similar growth in the fresh-cut industry has been recorded due to consumer emphasis for a healthier living with the retail market providing major distributive channels (Rabobank 2009). These retail markets have already been strongly trending in countries such as Netherlands, Switzerland, Italy and Spain.

The fresh-cut industry in the developing countries especially in African countries has not been growing unlike the developed countries due to post-harvest losses occasioned by poor preservation of product quality via the food value and market chain, poor cold chain distribution and logistics, inadequate processing and storage facilities, lack of updated research on tropical fresh-cut produce. This has equally affected the export value of this commodity where it can be presented as novel-based product to the developed countries (James, Ngarmsak and Rolle 2011).

2.3 Food safety in developing countries

Developing countries have been greatly challenged with food borne diseases. This has been linked to certain phenomenon such as increased consumption of contaminated foods and lack of adequate attention to food safety management practices (Grace *et al.* 2015). This challenge is mostly noticeable in countries in South East Asia, South Asia and Africa (Hotez *et al.* 2014). According to a UN report, the full extent of food borne diseases in developing countries is not completely known (UN 2013). However, previous reports suggest that foodborne diseases may be higher in developing countries than developed countries (Hop *et al.* 2007; Greenwood *et al.* 2008; FAO 2014; WHO 2014). Major factors of these food borne diseases includes physical agents; like iron shavings, nails, broken glass, and heavy metals, chemical agent; such as pesticides and biological agents such as rodents and pathogenic microorganisms. Increase in fresh food consumption in East and South Africa have been projected to increase four times by year 2040 where the market size will also increase eight times (Tshchirley 2014). In Nigeria, an estimate of about 3 $3 billion was expended on food borne diseases in 2010 (Grace *et al.* 2015). In the US and Europe, food safety concerns are on the increase as a result of urbanization and industrial development, and it is now becoming noticeable in fast developing countries (ECDC 2012; EFSA 2013).

2.4 Pathogens associated with fresh produce

Fresh produce has been widely reported to provide a good ecological niche for the proliferation of many species of pathogens linked to its consumption along the food chain (Beuchat 2002; Buck, Walcott and Beuchat 2003; Sivapalasingam *et al.* 2004; Aruscavage *et al.* 2006). These pathogens are mostly enteric in nature as they are often found in the human intestines and animal dungs. They display structural similarities, but physiological variations in growth and survival mechanism are noted (Beuchat 2002). They consist of some species of virus, protozoa and bacteria (Table 2.1). Common ones, of public health importance include hepatitis 'A' Virus and norovirus, protozoans such as *Cyclospora cayetanensis* and *Cryptosporidium parvum*. Pathogens of bacterial origin are more dominant and they include *Aeromonas hydrophila, Bacillus cereus, Clostridium spp., E. coli* O157:H7, *Listeria monocytogenes, Salmonella spp., Shigella spp., Vibrio cholera, Campylobacter spp.,* and *Yersinia enterolitica* (Olaimat and Holley 2012). Outbreak of food borne diseases linked to viral origin accounts for about 20%

with norovirus widely reported, while parasites account for about 16% of reported outbreak cases in which *Cyclospora* parasite was mostly implicated (Sivapalasingam *et al.* 2004).

Pathogens of bacterial origin have continued to play a prominent role in fresh produce contamination with *E.coli* O157:H7 and *Salmonella spp* mostly reported (Buck, Walcott and Beuchat 2003; Warriner *et al.* 2009; Nithya, Gothandam and Babu 2014). *L. monocytogenes* have also been reported as pathogen of public health concern. Even though its prevalence level is low compared to *Salmonella* and *E. coli* O157: H7, its mortality rate is very high due to its ability to survive a broad range of environmental and stress conditions, also its ability to form biofilms makes its inactivation by common sanitizers difficult (Liu 2006; Gandhi and Chikindas 2007; Milillo *et al.* 2012).

Table 2.1 Major fruits and vegetable pathogens associated with disease outbreaks

Pathogens		**Product**
Bacteria	*Clostridium botulinum*	Cabbage, pepper, garlic, potato and carrots
	E.coli O157:H7	Alfalfa sprouts, cabbage, celery, coriander, watercress, lettuce, berries, melons, and apple juice
	L. monocytogenes	Bean sprouts, cabbage, chicory, cantaloupe, eggplants, lettuce, potatoes, radish and lettuce
	Salmonella spp.	Alfalfa sprouts, artichokes, beet leaves, celery, cabbage, cantaloupe, cauliflowers, eggplants, endive, fennel, green onions, lettuce, mung bean sprouts, mustard cress, pepper, salad greens, spinach, unpasteurized fruit juice, tomatoes, watermelon, mamey and mango
	Shigella spp	Celery, lettuce, green onions, salad vegetables and parsley
	Staphylococcus spp	Lettuce, parsley, radish, salad vegetables, and seed sprout
	Vibrio cholera	Cabbage and coconut milk
	Yersinia enterolitica	Carrots, cucumbers, lettuce and tomatoes
Viruses	*Norovirus*	Lettuce, green onions, watercress, sliced melon, salads, diced tomatoes and fresh cut fruits
	Hepatitis A	Lettuce, green onions, watercress, raspberries, frozen strawberries, and berries
Protozoans	*Cryptosporidium spp*	Lettuce, onions, and green onions
	Cyclospora spp	Lettuce, onions, green onions, raspberries and blackberries

(Señorans, Ibáñez and Cifuentes 2003; Warriner *et al.* 2009; Abadias *et al.* 2012; Olaimat and Holley 2012; Seow *et al.* 2012; Van Boxstael *et al.* 2013).

2.5 Listeria monocytogenes

Listeria monocytogenes is a gram positive, facultative, anaerobic bacterium which belongs to one of the species *L. marthii, L, innocua, L. seeligeri, L. welshimeri, L.ivanovii, L. gray* and *L. rocourtiae* (Graves *et al.* 2010; Leclercq *et al.* 2010). Two new strains -*L. fleischmannii* and *L. weihenstephanensis* were later reported (Bertsch *et al.* 2013; Halter, Neuhaus and Scherer 2013). Also, seven new species with limited occurrence have recently been reported (Orsi and Wiedmann 2016). However, *L. monocytogenes*, *L. ivanovii* and *L. seeligeri* have been found to be pathogenic (Gouin, Mengaud and Cossart 1994). *L. monocytogenes* typically comprises 13 different serotypes (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4ab, 4c, 4e and 7). However, most human listeriosis invasive types are associated with 1/2a, 1/2b and 4b (Kathariou 2002).

L. monocytogenes is widely distributed in the environment such as soil, water, animal dungs and more importantly on a variety of foods including dairy, meat, sea and fresh produce (Colburn *et al.* 1990; Bell and Kyriakides 2012; Strawn *et al.* 2013). One reason for the wide distribution of *L. monocytogenes* is its ability to resist several adverse environmental conditions. This bacterial pathogen can survive temperature range of 1.7 to 45^{o}C and a pH range from 4.7 to 9.2 (Junttila, Niemelä and Hirn 1988; Petran and Zottola 1989). It tolerates high salinity condition up to 10% sodium chloride (McClure, Kelly and Roberts 1991). This resistance spectrum of the bacteria to evade well-established food conservation interventions such as acidification, lowering of water activity or cooling and this forms the basis for its relevance as a foodborne pathogen. Recent reviews by some authors reported its occurrence in harborage sites of processing equipment. This was linked to its saprophytic attribute and poor sanitary practices (Carpentier and Cerf 2011; Ferreira *et al.* 2014). Though its prevalence level is low, with a record of 0.1 – 10 per million human reported cases, however, its death rate is comparatively high (20 – 40%) in terms of impact (Farber and Peterkin 1991; Travier and Lecuit 2014).

Infection of the human system with this pathogen causes listeriosis disease. It is characterized by septicemia, encephalitis, and meningitis as well as neurodegenerative disorder. Susceptible people to this disease includes neonates, young elderly, immunosuppressed, and pregnant women (Vázquez-Boland *et al.* 2001; Carpentier and Cerf 2011).

There has been about 1600 cases of food borne listeriosis that are reported in the USA, thus making approximately 19% of the total death that is reported yearly (Scallan *et al.* 2011; Hoffmann, Maculloch and Batz 2015). In Europe, the prevalence level of 12.1% of *L. monocytogenes* contamination of food has been reported. However, a general increase in

reported cases of food borne listeriosis within the European sub regions have been observed in the last five years (EFSA 2014, 2015). Investigation into occurrence of this pathogen in the African context has been poorly reported (Niehaus *et al.* 2011).

In South Africa, a recent case of increased human listeriosis disease from Western Cape province has been reported (Smith *et al.* 2016). Ajayeoba *et al.* (2015) reported isolation of 244 species of *L. monocytogenes* from 555 composite samples of five different ready-to-eat vegetables in Nigeria.

2.5.1 Prevalence of *Listeria monocytogenes* in food and food industry

Prevalence describes the occurrence, persistence and survival of a particular pathogen over a set of substrates either natural or artificial for a particular period of time (Berger *et al.* 2010; Ferreira *et al.* 2014). However, statistical facts to authenticate the prevalence of *L. monocytogenes* in food and non-food matrix has been affected by study size, variations in food sources, and processing platforms (Strydom and Witthuhn 2015). Furthermore, RTE foods have been found to be more susceptible to *L. monocytogenes* contamination due to the absence of non-thermal treatments to kill the pathogen (Beuchat 1996a; Luber *et al.* 2011). Various subtyping approaches employed reported conflicting results possibly because of limitations in methods used and various media upon which they are identified (Leong, Alvarez-Ordóñez and Jordan 2015).

The occurrence and prevalence of *L. monocytogenes* in various food commodities have been reported (Heisick *et al.* 1989; MacGowan *et al.* 1994; Sant'Ana *et al.* 2012). About 67 different isolates of *L. monocytogenes* from 698 samples were identified in raw meat with one third of these isolates having virulent genes that were pathogenic in nature (Shen *et al.* 2013). Furthermore, Jamali, Radmehr and Thong (2013) reported 21.7% *L. monocytogenes* contamination from 446 raw milk samples examined, showing the potential risk of the pathogen on human health. *L. monocytogenes* contamination in a number of fresh-cut produce has been reported. Heisick *et al.* (1989), and MacGowan *et al.* (1994) reported the presence of this pathogen in some vegetables such as potatoes, cabbage, cucumber and tomato radish. Other authors reported similar contamination in some ready-to-eat vegetables stored under modified atmosphere (Berrang, Brackett and Beuchat 1989; Kallander *et al.* 1991).

In packaged ready-to-eat vegetables, Sant'Ana *et al.* (2012) reported 3.1% contamination of *L. monocytogenes* from 512 samples with some countable level above 4 log fold cycle. A metagenomics assay on some fresh and minimally processed vegetables reported about 2.63 CFU/g level of *L. monocytogenes* contamination (FAO/WHO 2008a).

The food processing plants have also been reported to harbor *L. monocytogenes* contamination. This observation was reported in a dairy and non-dairy food products processing plant (Harvey and Gilmour 2001). Moreover, Miettinen and Wirtanen (2006) reported occurrence of *L. monocytogenes* in a fish processing plant. Also, Strydom *et al.* (2013) reported its occurrence in an avocado processing plant in South Africa. Different authors reported varied opinions as to the cause of this prevalent contamination within the food processing plants. For example, cross-contamination from resident strains in harborage sites of processing equipment was reported by Carpentier and Cerf (2011), While Ojeniyi, Christensen and Bisgaard (2000) reported certain environmental factors such as floor drains, and personnel more as medium of contamination than the raw material. However, safety measures which includes microbial testing of the processing environment and equipment has been commonly recommended to address this prevalent syndrome (Ferreira *et al.* 2014).

2.5.2 Survival mechanism of *Listeria monocytogenes*

L. monocytogenes has been regarded as a recalcitrant bacterial pathogen because it has the ability to survive a wide range of stress and environment factors (Farber and Peterkin 1991). This unique attribute of *L. monocytogenes* raises safety concerns in the food safety industry as reflected in continuous food product recalls during listeriosis outbreaks. Previous authors have linked this behavior to certain phenomenon such as attachment, internalization, energy utilization, stress adaptation, biofilm formation and adherence to food contact surfaces (Lou and Yousef 1997; Lundén *et al.* 2000; Holah *et al.* 2002; Wulff *et al.* 2006; Bochner 2009).

2.5.2.1 Attachment

Attachment of pathogens to fresh produce is dependent on certain external and internal factors including the pathogen's motility, adaptations with other organisms and availability of nutrients from the fresh produce (Frank 2001; Aruscavage *et al.* 2006). Pathogens sometimes persist in fissures of produce outer layer which eventually contaminate edible parts during pre-processing operations such as cutting, slicing and peeling, or they may rather penetrate and proliferate inside the produce via the stem scar (FDA 2009). Motility plays an important role in this approach as it promotes entry into cuts, stomata, and other entry points via the mechanism of chemotaxis, motility and quorum sensing (Kroupitski *et al.* 2009). *L. monocytogenes* is no exception under this concept. The pathogen attaches itself to host tissues by inducing their own endocytosis with subsequent transport across host protective barriers

(Farber and Peterkin 1991). However, it has been found that attachment of this pathogen to fresh produce is dependent on its flagellar motility and temperature (Gorski, Palumbo and Mandrell 2003). Also, this flagellar motility facilitates its attachment to food contact surfaces such as stainless steel (Vatanyoopaisarn *et al.* 2000). Furthermore, from genomic studies, the PrfA protein has been identified to be expressed during the attachment (Bruno Jr and Freitag 2010).

2.5.2.2 Internalization

Pathogens possess the ability to internalize themselves in the tissues of plants. Previous authors have reported that pathogens can enter into the vascular tissues of plants through the cuts, stomata to a depth of 20-100µm (Sharma, Ryu and Beuchat 2005; Gomes *et al.* 2009). Internalization of *L. monocytogenes* has been observed within the stomata of lettuce. Several studies have identified internalization of pathogens in fresh produce could be rare but it is generally dependent on the type of fresh produce, climatic conditions and contamination level and strain types (Jablasone, Warriner and Griffiths 2005). However, Jablasone et al. (2005) reported that internalization process of three pathogens- *E. coli* O157:H7, *S. Typhimurium* and *L. monocytogenes* was determined by type of plant, maturity index of harvest and the type of pathogen. Microbes were inoculated on cress, lettuce, radish and spinach seeds. After 9 days of germination, *E. coli* O157:H7 was internalized in all plants while lettuce and radish had S. Typhimurium attached. However, no internalization by *L. monocytogenes* was observed. It was significant that the pathogens were not recovered from mature plants (49 days post-germination). Similarly, investigations reported lack of internalization by some food borne pathogens in plant tissues, certain other bacteria does better by colonizing outer surfaces than others (Olaimat and Holley 2012). Internalization mechanism of *L. monocytogenes* in most host cells is often facilitated by internalin, listeriolysin, and phospholipases.

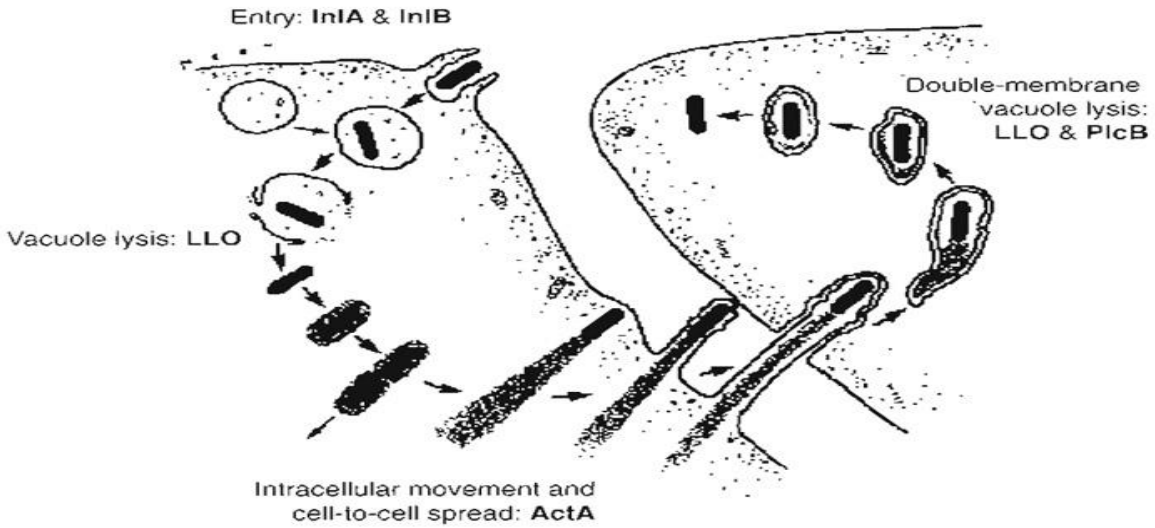


Figure 2.3 Intracellular cycle of *L. monocytogenes*

(Dussurget, Pizarro-Cerda and Cossart 2004).

2.5.2.3 Energy utilization

Intracellular bacteria can survive and multiply inside a host cells vacuole or cytosols. However, presence of membranous coverings in the vacuole often limits access to nutrient availability by the bacteria for energy utilization as compared to the cytosol where more access to nutrient avails.

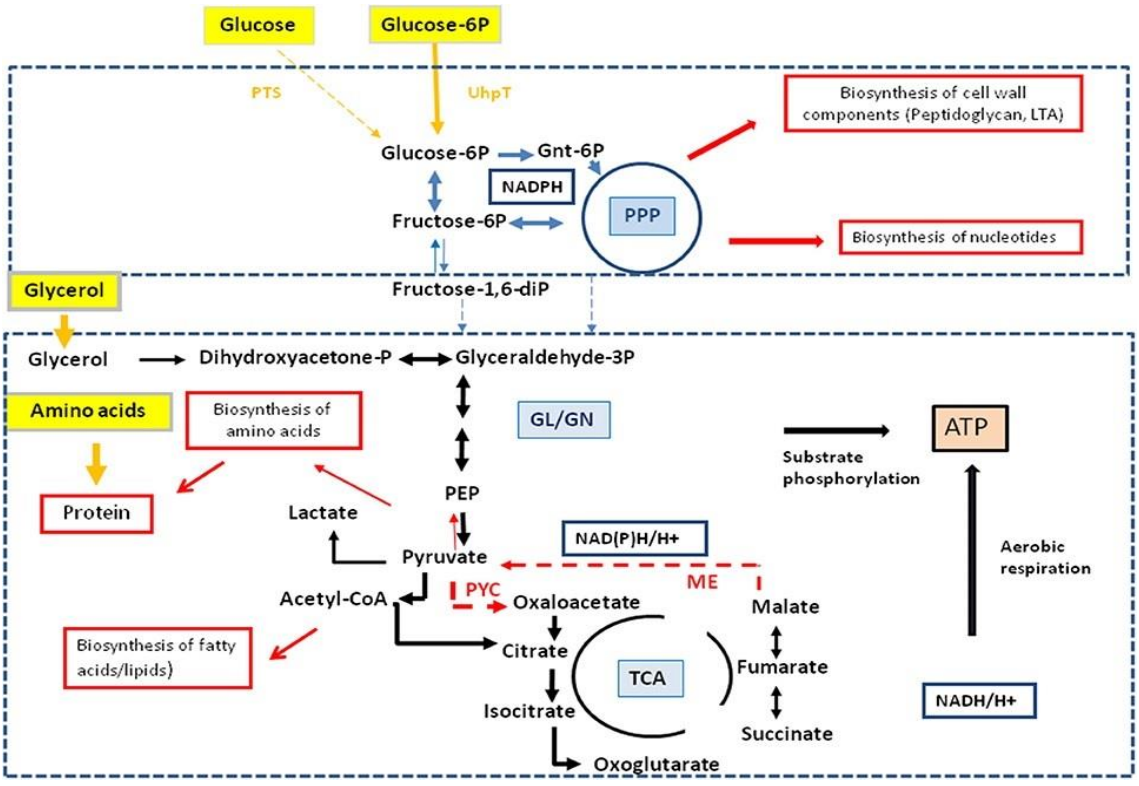
Phenotypic study has shown that bacteria utilizes basic elemental nutrients in their environment such as carbon, nitrogen, phosphorus, sulphur, oxygen and hydrogen to survive harsh and lethal conditions such as chemicals, irradiations, temperature (Bochner 2009). This author reported that *Listeria* utilizes hexose-phosphate carbon as an energy source for survival in intracellular medium (Fig 2.4).

Energy utilization of intracellular bacterial pathogens such as *L. monocytogenes* depends on active utilization of the basic elemental nutrients from the host cells. Within the cytosols of the host cells, *L. monocytogenes* effectively replicate with a generation time of 1 h which thereby spreading to nearby cells (Hamon *et al.* 2012). From genomic analysis, it has been found that *L. monocytogenes* has a complete glycolytic and pentose phosphate pathway where carbon-based metabolites of glucose and glucose-6-phosphate can be readily broken down by either of the pathways (Glaser *et al.* 2001).

However, due to lack of oxoglutarate and malate dehydrogenase enzyme reported in the citrate cycle, the formation of oxaloacetate molecules by the intracellular *L. monocytogenes* will rely absolutely on the pyruvate carboxylation which is broken down by pyruvate carboxylase

enzyme (PycA). These cellular metabolites usually represent energy source for the *L. monocytogenes* survival (Schär *et al.* 2010). Uptake of this energy source by the pathogen has been reported to be facilitated by a hpt *gene* encoded from PrfA protein (Chico-Calero *et al.* 2002).

The expression of this energy uptake could occur at transcriptional, translational and post-translational level (Eylert *et al.* 2008). The principle of post-translational modification of the PrfA protein has not been fully ascertained, but could be linked to certain phosphorylation process of the permeases of specific PEP: phosphotransferase systems (PTS) (Stoll *et al.* 2008). Active transport of PTS carbohydrates, such as glucose or cellobiose, results in unphosphorylated PTS permeases which could inhibit PrfA activity. Conversely, in the presence of non-PTS carbon sources, such as glycerol or glucose-6-phosphate, these PTS permeases stays phosphorylated and high PrfA activity takes place (Mertins *et al.* 2007; Joseph *et al.* 2008).



(Hamon *et al.* 2012)

Figure 2.4 Carbon utilization mechanism of *L. monocytogenes*

(Grubmüller *et al.* 2015)

2.5.2.4 *Listeria monocytogenes* Biofilms

One major attribute of *L. monocytogenes* is biofilm formation. Biofilms are microbial aggregates enclosed in an exo-polysaccharide matrix (Lappin-Scott and Costerton 2003). Formation of biofilm (Fig 2.3) involves attachment of cells to a surface, followed by a permanent binding to the surface, which further forms small colonies that grows into a large matrix (Marshall 1992). Biofilm formation has been found to enhance the proliferation of bacteria under unfavorable environment via phenotypic display of new genetic traits which would adapt more to antimicrobial factor such as common sanitizers than parent traits (Davey and O'toole 2000).

L. monocytogenes biofilms represents a serious safety concern in the fresh produce industry due to their long persistence in processing environment thus serving as a potential source of continuous contamination (Møretrø and Langsrud 2004; Ferreira *et al.* 2014). Some authors have reported varied opinions on biofilm formation of *L. monocytogenes*. While Norwood and Gilmour (2000) reported a better biofilm adherence of adapted strains from a food processing plant, and farm environment on stainless steel than non-adapted strains when observed after 24 h at 25ºC. Djordjevic, Wiedmann and McLandsborough (2002) however reported no difference in biofilms formation in both strains from a fish processing plant after 40 h of incubation.

This variation in biofilm formation could be linked to method of isolation and strain differentials and medium of proliferation. Molecular determinants of biofilm formation in *L. monocytogenes* have been linked to motility of its flagella, *Prfa* virulence factor and its quorum sensing mechanism (Botticella *et al.* 2013).

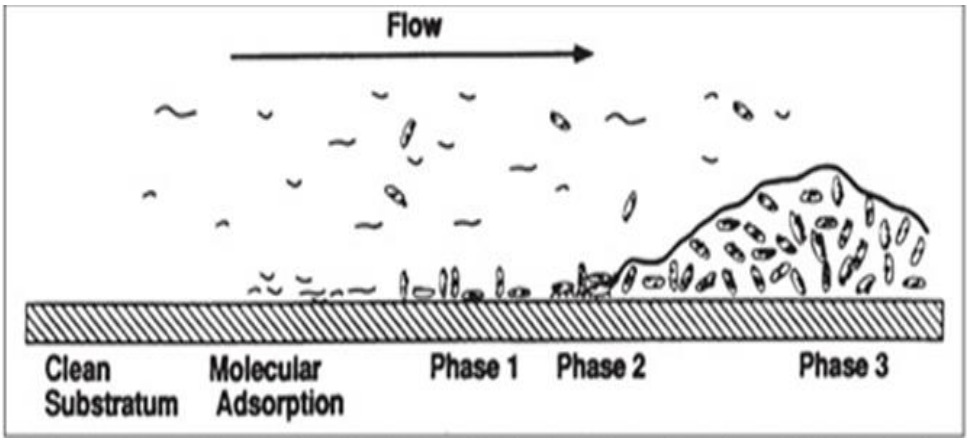


Figure 2.5 Process of microbial biofilm formation

(Deibel and Schoeni 2002)

2.5.2.5 Stress Adaptation

Stress has been defined as any unfavorable condition which negatively affects the growth and survival of microorganisms (Lou and Yousef 1997). Adaptation to stress factors such as depletion in availability of nutrients, low pH, altered temperature conditions and high osmotic have been reported to alter the cellular constituent and responses of bacteria (Lou and Yousef 1997; Storz and Hengge 2010). These stress factors can be lethal to cause permanent damage or death to the bacterial cells or it can be sub lethal to give room for survival. Mild or sub-lethal stress survival of bacteria increases their chances of surviving further exposure to antimicrobial compounds. For example, *L. monocytogenes* subjected to osmotic stress have been reported to enhance adaptation to peroxide stress (Hill *et al.* 2002). Also, treatment of acid-adapted *L. monocytogenes* with lauric arginate on carrot juice showed increased survival by approximately 2 log CFU/ml than its non-adapted cells (Shen, Soni and Nannapaneni 2015).

This phenomenon plays a significant role in the food safety industry especially when adopting control measures. Studies have shown that response of *L. monocytogenes* to various stress conditions is motivated by gene expression of certain stress protein which is induced in the cellular structure. This response is principally mediated by an alternative sigma factor σ^B (Ferreira, O'Byrne and Boor 2001; Kallipolitis and Ingmer 2001; Chaturongakul and Boor 2004).

Proteomic study as reported by Soni, Nannapaneni and Tasara (2011) identified certain number of stress-induced proteins in *L. monocytogenes.* These include cold shock proteins (GroEL, Dnak, Csp1-Csp4, CspA and CspD), heat shock proteins (Hsps, Dnak, and GroES), osmotic stress proteins (Ctc, Dnak, GbuA, GroEL and AppA), acid shock proteins (such as ASP, GbuA, GroEL and ClpP), and alkaline stress protein (such as DdlA, GroEL, and Dnak).

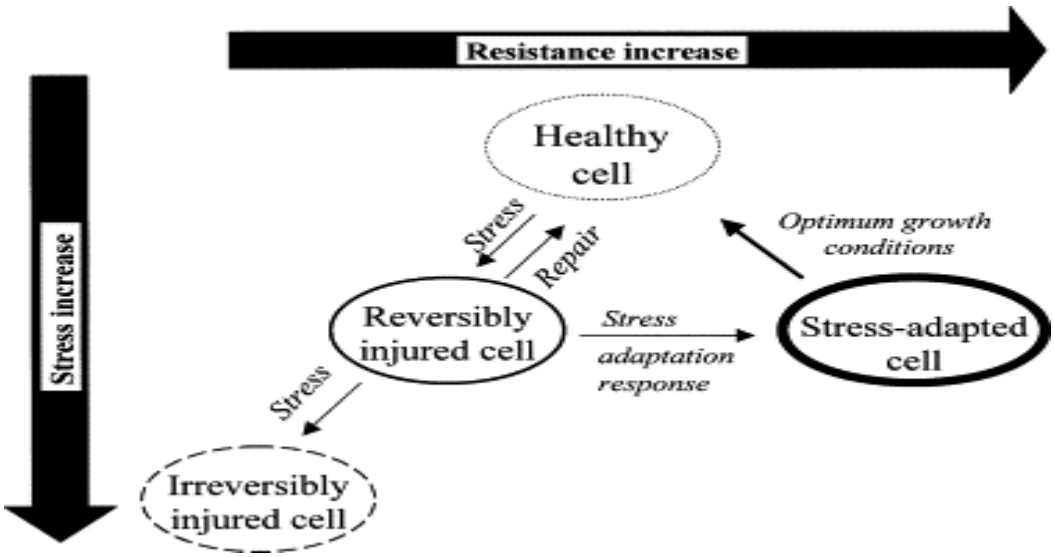


Figure 2.6 Bacteria stress adaptive networks

(Yousef and Juneja 2002).

2.6 Control of *L. monocytogenes* in fresh produce

As the demand for fresh produce consumption increases globally, the need for measures to control microbial contamination becomes more important. Due to the high mortality rate and recalcitrant nature of *L. monocytogenes,* the United States Department of Food and Agricultural enacted a "zero tolerance" approach to checkmate this pathogen in RTE foods (Klima and Montville 1995). In the year 1998, WHO and FAO endorsed a good number of intervention measures to curtail challenges of fresh produce safety from "farm to fork" (FDA 1998b). Sequel to this, fundamental quality management principles of Good Agricultural practices (GAP), Good hygiene practices (GHP) and Good manufacturing practices (GMP) have been encouraged among all stakeholders in the food value chain to ensure safety of fresh produce (FDA 2004).

These measures consist of chemical, physical and biological methods, with emerging techniques which incorporates the use of thermal, non-thermal and a combination of approaches (Olaimat and Holley 2012; Ramos *et al.* 2013; Meireles, Giaouris and Simões 2016).

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2.6.1 Chemical methods

2.6.1.1 Chlorine

Traditionally, the use of chlorine (sodium hypochlorite) at 50-200 ppm for a contact time of 1-2 mins has been established (Beuchat *et al.* 1998; Rico *et al.* 2007). Furthermore, the inhibition of *L. monocytogenes* growth in some fresh produce has been reported (Parish *et al.* 2003). A good number of fresh produce washed with this sanitizer reported varying degrees of *L. monocytogenes* reduction (Hoelzer *et al.* 2014). Results of analysis suggested that the comparative efficacy of the chlorine is dependent to some extent on the nature of the fresh produce, method of application and concentration (Baur *et al.* 2005; Casteel, Schmidt and Sobsey 2008).

The advantage of chlorine use lies in its ease of availability and low cost (Ramos *et al.* 2013). However, continuous application of this sanitizer in fresh produce has been discouraged. Despite the reported 1-3 fold reduction commonly achieved, development of toxic residues, which are carcinogenic in nature, has been reported (Baur *et al.* 2005; Pao *et al.* 2012). Furthermore, this pathogen forms biofilm which resists the efficacy of common sanitizers such as chlorine (Pan, Breidt and Kathariou 2006). Alternative chlorine-based sanitizers have been developed and applied in the fresh produce industry.

2.6.1.2 Peroxyacetic acid

The use of peroxyacetic acid at not more than 80 ppm in wash water has been recommended by the FDA (USDA 2000). Its inactivation mechanism involves oxidation of cell wall membrane via electron transfer. This application has been reported to be effective in the inhibition of pathogens on various types of fresh produce (Rodgers *et al.* 2004). Furthermore, Hellström *et al.* (2006) reported log reduction of 1.7 fold on *L. monocytogenes* population in lettuce. Further studies by other authors reported that this concentration is not sufficient enough for significant reduction of pathogens in fresh produce (Rico *et al.* 2007; Artés *et al.* 2009).The development may be due to variations in application conditions as the efficacy of this antimicrobial compound has been reported to be influenced by pH and temperature (Joshi *et al.* 2013).

2.1.6.3 Acidified sodium chlorite

The use of acidified sodium chlorite (ASC) dip at 500-1200 ppm has been reported to exhibit bactericidal effects on fresh produce pathogens (Allende et al. 2009). Similarly, (Ruiz-Cruz et

al. 2007) reported 2.5 log CFU/g reduction of L. monocytogenes population on fresh-cut carrots. Several authors equally reported 0-6 log fold reduction on several fresh produce when acidified sodium chlorite was applied against L. monocytogenes (Han et al. 2001; Rodgers et al. 2004; Wu and Kim 2007; Kim, Kim and Song 2009).

Similarly, Stopforth *et al.* (2008) reported 3.0-3.8 log CFU/g reduction of *L. monocytogenes* population on green leafy vegetables when treated with ASC. However, more information on its utilization in fresh produce is needed. This sanitizer has earned the approval of the U.S. Food and Drug Administration as secondary food additive particularly as an antimicrobial intervention for raw agricultural commodities (USDA 2000).

2.6.1.4 Chlorine dioxide

The use of chlorine dioxide at about 3 ppm has been reported to exhibit better bactericidal effect than sodium hypochlorite with no toxic by-products after treatment. It has also been found to retard enzymatic browning (Rico *et al.* 2007; Chen *et al.* 2010). Furthermore, Hoelzer *et al.* (2014) reported an average reduction of 2.5-5 log CFU/g of *L. monocytogenes* population on a good number of fresh produce. This sanitizer has been used to remove *L. monocytogenes* biofilms (Robbins *et al.* 2005). However, its application has been found to be limited by flammability, photolytic nature and pH dependence (Hirneisen *et al.* 2010).

2.6.1.5 Organic acids

The use of weak organic acids and their salt equivalence remains a common method of inhibiting microbial growth in foods (Hirshfield, Terzulli and O'Byrne 2003; Lianou *et al.* 2012). Most fresh produce are inherently composed of organic acids and this has enhanced its GRAS (Generally Regarded as Safe) status and commercial application in the food industry. Principally, they exhibit antimicrobial effect by cytoplasmic acidification, alteration of proton motive force across cell membrane and high osmotic stress of the cellular environment (Hirshfield, Terzulli and O'Byrne 2003; Carpenter and Broadbent 2009).

Organic acid such as citric acid, acetic acid applied on artificially inoculated *L. monocytogenes* has been reported to reduce the pathogen population by 1 and 1.3 log CFU/cm^2 on fresh lettuce stored at 20^{o}C for 1 to 5 min respectively (Akbas and Ölmez 2007; Samara and Koutsoumanis 2009). Other chemical-based sanitizers with significant reduction of *L. monocytogenes* population in fresh produce includes ozone, hydrogen peroxide and electrolyzed oxidizing water (Joshi *et al.* 2013; Hoelzer *et al.* 2014).

2.6.1.6 Ozone

Ozone has been regarded as a strong antimicrobial agent due to the strong oxidizing power of its radicals against genetic composition of microbial cells (Joshi *et al.* 2013). It is normally produced in gaseous form, and can be used in small concentrations of 1-5 ppm when dissolved in water. However, a higher concentration is required in its gaseous form to effect its antimicrobial capacity (Horvitz and Cantalejo 2014). It has been found to be effective against a wide spectrum of microorganisms (bacteria, fungi, virus and protozoan) associated with fresh produce contamination (Khadre, Yousef and Kim 2001). Treatment of lettuce with 5 ppm of ozone for a contact time of 10 mins at 4 and 22^{o}C has been reported to reduce *L. monocytogenes* population by 1.1 and 0.8 respectively (Zhang and Farber 1996).

Different opinions on the use of ozone in fresh produce have been reported. Some authors reported its antimicrobial efficacy against fresh produce such as shredded lettuce, blackberries, grapes, black pepper, broccoli, carrots and tomatoes (Kim, Yousef and Dave 1999; Tiwari and Muthukumarappan 2012).

However, other authors reported that it is not suitable for use on fresh produce due to impact on the physicochemical properties (Foong-Cunningham *et al.* 2012). Efficacy has therefore been linked to the type of microorganism, fresh produce type, and storage condition, residual concentration of ozone in the medium, and organic matter present around the cell (Pascual, Llorca and Canut 2007). Meanwhile, its limitations include instability and rapid decomposition, health risk via inflammation of eyes and throat, and a strong corrosive effect on equipment (Artés *et al.* 2009; Chawla *et al.* 2012).

2.6.1.7 Hydrogen peroxide

Hydrogen peroxide acts on microorganism via the production of cytotoxic radicals which exerts both bacteriostatic and bactericidal effect (Rico *et al.* 2007; Ölmez and Kretzschmar 2009). Lower concentration of about 2-4% hydrogen peroxide has been reported to be optimum for reducing bacterial load in contaminated fresh produce (Beltrán *et al.* 2005). A reduction of 2.0-3.5 log CFU/cm^2 of *L. monocytogenes* population on melon surface when treated with higher concentration of hydrogen peroxide has been reported (Ukuku and Fett 2002). Despite being granted a GRAS status, its use in fresh produce against microorganism has not been approved (Ölmez and Kretzschmar 2009). This is due to its slow pace of penetration where the

possibility of cross contamination with other pathogens is high (Van Haute *et al.* 2015). Furthermore, the impact on organoleptic qualities due to development of browning on vegetables with the use of hydrogen has been reported (Beuchat *et al.* 1998; Rico *et al.* 2007).

2.6.1.8 Quaternary Ammonium Compounds

Quaternary Ammonium Compounds (QAC) are biocidal surface active compounds with positively charged molecules which are used as decontaminants on food-contact surfaces (Ramos *et al.* 2013). Their antimicrobial mechanism is basically facilitated via interaction with lipid layers of cell membrane due to their lipophilic property (del Carmen Velázquez *et al.* 2009). QAC at concentration of 200-400 ppm has been reported to be optimum in the inactivation of food contact surfaces to exhibit antimicrobial activity (Chauret 2014).

Gram positive bacteria such as *L. monocytogenes* have been found to be highly susceptible to QAC (Chaidez, Lopez and Castro-del Campo 2007; Ohta *et al.* 2008). Advantageously, it is stable, eco-friendly, non-corrosive, and adaptable to long range of pH and temperature conditions (Bari 2014; Holah 2014). Examples of QAC commonly used as decontaminants include Benzalkonium chloride, benzethonium chloride and cetylpyridinium (Izumi 2014). However, Wang, Li and Slavik (2001) reported reduction of 3.70 log CFU/g of *L. monocytogenes* population in cauliflower when treated with 5 x 10^3 ppm cetylpyridinium chloride. Meanwhile, it is not approved for direct contact with food due to interaction with organic matter (Ramos *et al.* 2013).

2.6.1.9 Electrolyzed oxidizing water (EOW)

Electrolytic combination of sodium chloride solution (12%) and electrolyzed water through a charged membrane either to form electrolyzed acidic solution (AcEOW) and Electrolyzed alkaline solution (AIEOW) has been found to possess antimicrobial properties (Issa-Zacharia *et al.* 2011; Cheng *et al.* 2012).

The mode of action of Electrolyzed oxidizing water (EOW) is based on redox potential, splitting of water molecules and pH changes (Huang *et al.* 2008). The electrolyzed acidic solution with lower pH (2.5-3.5) has been found to be very effective as it possesses higher redox potentials (1000-1200 mV) which could cause denaturation of cell proteins and enzymes. However, Deza, Araujo and Garrido (2003) reported 6 log CFU/cm reduction on *L. monocytogenes* population in tomatoes when treated with EOW with no deleterious impact on the organoleptic qualities. A similar result was reported on lettuce contaminated with *L.*

monocytogenes. However, further studies have been recommended on the residual chemical composition of EOW especially in response to interaction with organic component of the food

2.6.1.10 Sugar fatty acid esters

Esterified product of carbohydrates and fatty acids (CFA) has been found to exert antimicrobial ability on a number of microorganisms (Lee, Sandhu and Walsh 2015; Zhao *et al.* 2015). They are basically non-toxic, biodegradable, non-ionic surfactants with application in food, cosmetic and pharmaceutical industry (Hill and Rhode 1999; Piccicuto *et al.* 2001). Their inhibitory ability has been reported on both gram positive and gram negative bacteria (Monk, Beuchat and Hathcox 1996; Watanabe *et al.* 2000; Devulapalle *et al.* 2004; Xiao *et al.* 2011).
This can either be bacteriostatic or bactericidal depending on certain factors such as nature of carbohydrate, extent of esterification, type and chain length of fatty acid (Wagh *et al.* 2012; Chen, Nummer and Walsh 2014; Zhao *et al.* 2015). Examples of these esters include sucrose monolaurate, lactose monolaurate, sucrose palmitate, fructose laurate (Monk, Beuchat and Hathcox 1996; Watanabe *et al.* 2000). Sucrose monolaurate has been reported to be lethal to L. monocytogenes (Monk, Beuchat and Hathcox 1996; Nobmann *et al.* 2009).

2.6.2 Physical methods of control

Physical method of control involves the use of irradiation, packaging techniques and certain technologies using physical properties of air and water as antimicrobial media.

2.6.2.1 Irradiation

The use of irradiation as a decontaminating medium of pathogens in fresh produce at 1.0 KGy has been reported (Gomes *et al.* 2009). Mahmoud (2010), reported 4.1 log CFU/5 cm^2 reduction of *L. monocytogenes* population on shredded iceberg lettuce when exposed to ionizing radiation of 1.0 KGy. However, the efficacy of this method is dependent on the state of produce type, nature of pathogen, and packaging condition (Niemira, Fan and Sokorai 2005). Furthermore, post-treatment impact on fresh produce and the psychological aversion of consumers to an irradiated food has been found to be one of the limiting factors in the use of irradiation (Sulakvelidze 2013). Effort therefore has been made to reduce the dosage treatment and to also combine with other antimicrobial treatments. Similarly, Gomes, Moreira and Castell-Perez (2011) reported 5 log CFU/g reduction of *L. monocytogenes* population on baby spinach leaves when exposed to 0.7 KGy in combination with 100% oxygen.

2.6.2.2 Ultrasound

The use of ultrasonic waves at high amplitudes which generates certain bubbles to form free radicals has been reported to possess antimicrobial properties (Otto *et al.* 2011; Sagong *et al.* 2011). This method has been applied in various foods and in water sanitation. Birmpa, Sfika and Vantarakis (2013) reported 1.88 log reduction of *Listeria* population on lettuce at 37 kHz for 30 min. High intensity needed to effect inactivation has been reported as the major demerit of this method due to deleterious impact on organoleptic qualities of the food (Seymour *et al.* 2002).

2.6.2.3 Ultraviolet light (UV)

Ultraviolet (UV) light which is categorized into classes of wavelength: UV-A (400-320 nm), UV-B (320-280 nm), UV-C (190-280 nm) and vacuum UV (Artés *et al.* 2009; Gray 2014) has been found to generate antimicrobial properties via denaturation of DNA molecule of cells (Birmpa, Sfika and Vantarakis 2013). Its application especially at UV-C wavelength has been reported to produce no toxic residue, but lengthy time of application could affect organoleptic qualities of the food (Demirci and Krishnamurthy 2011). However, log reduction of 1.27 CFU/g of *Listeria* population was reported on lettuce via this method.

2.6.2.4 Pulsed Light (PL)

This technique, also referred to as pulsed light is an upcoming method of inhibiting pathogens in the food industry (Goodburn and Wallace 2013). Its application involves the use of wide-range light spectrum at 100-110 nm to induce morphological changes in DNA of bacterial cells. It's decontaminating effect on food contact against *L. monocytogenes* in a meat industry has been reported (Rajkovic *et al.* 2010). Furthermore, a reduction of 2.79 log CFU/g *Listeria spp* population was reported on fresh water melon. The efficacy of this method depends on absorption ratio of the microorganism and food component.

2.6.2.6 Pulsed Electric Field (PEF)

Pulsed electric field (PEF) is a non-thermal technique of inactivating microorganism especially in liquid foods (Min *et al.* 2003). It involves introducing the food into a compartment having two electrodes. These electrodes generate high-pulsed voltage (20-80 Kv/cm) which passes

through the food material for a very short time (Knorr *et al.* 1994). This process results in loss of cellular structure such as cell membrane leading to death. Due to the short period of electric passage, minimal loss of nutrients and organoleptic qualities of the food is observed. Cserhalmi *et al.* (2006), reported 3.56 fold reduction of *L. monocytogenes* population on water melon juice when subjected to PEF treatment.

2.6.2.7 High Pressure Processing

As the name implies, High pressure processing (HPP) involves subjecting foods to high pressure (10-1000 MPa) in order to inhibit microbial action (Ramos *et al.* 2013). This process combines varying degrees of pressure and temperatures to attain target antimicrobial effect without adding any deleterious impact on the physicochemical attributes of the food (Guerrero-Beltrán, Barbosa-Cánovas and Swanson 2005). Application of this method reported 4.0 log CFU/g reduction of *L. monocytogenes* in ready-to-eat salad (Stratakos *et al.* 2016).

2.6.2.8 Membrane filtration

The use of membrane filtration (MF) which involves the passage of water through a pore sized aperture membrane has been reported as a means of removing pathogenic contaminants in fresh produce treatment water (Allende *et al.* 2008; Gil *et al.* 2009). This process involves operating units of microfiltration, ultrafiltration, nanofiltration and reverse osmosis depending on membrane pore size, molecular weight cut off of the microorganism and flow pressure of the water (Salehi 2014).

Information on the use of this technique against *L. monocytogenes* is still limited, but it has been reported on *E. coli, Salmonella typhimurium,* and *Enterococcus faecalis* in cantaloupe and jalapeno pepper where higher recovery in wash water was observed (Heredia *et al.* 2015). However, its demerit is linked to high cost of investment and maintenance of components.

2.6.2.9 Nanoparticles

The use of nanomaterials as antimicrobials has received attention in recent times. Nanoparticles of certain metallic oxide such as calcium oxide, zinc oxide, and silver oxide have been reported to exhibit significant antibacterial activity (Premanathan *et al.* 2011). This activity has been linked to the synthesis of oxygen radical species including hydrogen peroxide which disrupts cell membrane functions (Sawai 2003; Brayner *et al.* 2006; Jones *et al.* 2008).

The application of zinc oxide nanoparticles at 5 and 8 mM has been reported to reduce *L. monocytogenes* population in fresh mango juice by 5.91 and 5.66 log CFU/ml (Firouzabadi *et al.* 2014).

2.6.3 Biological method

The use of natural-sourced biocidal antimicrobials has been clamored as a good alternative to the use of chemical-based sanitizers because they do not constitute toxicity risk to food, processor and consumers. Such natural antimicrobials include; Bacteriocin, bacteriophages, enzymes and some phytochemicals (Holah 2014; Meireles, Giaouris and Simões 2016). They can be applied individually or in combination for improved efficacy.

2.6.3.1 Nisin

Nisin is a bacteriocin which contains cationic peptides produced by some species of acid bacteria mostly *Lactococcus lactis* subsp *lactis.* It is known to exhibit bactericidal effect on gram-positive bacteria (Lubelski *et al.* 2008). Nisin demonstrates this effect by creating pore channels within bacterial cell membrane, via charged potential difference between the peptide and the membrane thus disrupting the fusion of peptidoglycan cell walls (Hancock 1997; Nunes 2012).

This low mass peptide is a biological and natural preservative substance which has attained a GRAS status by USA Food Drug and Administration hence its utilization in the food industry (FDA 1998a). It is also commercially available and its successful application stems from its broad range bactericidal action against gram positive bacteria (Settanni and Corsetti 2008). Bari *et al.* (2005), reported 2.20-4.35 log CFU/ml reduction of *L. monocytogenes* population when treated with nisin in combination with some salts and organic acid in mung bean, cabbage, and broccoli. Similarly, Oladunjoye, Ijabadeniyi and Singh (2016) reported 1-3 log fold reduction when nisin, in combination with salts of organic acid, was used to inhibit *L. monocytogenes* in fresh-cut tomato.

2.6.3.2 Enzymes

The use of enzymes to inhibit biofilms of pathogens has been reported (Simões, Simões and Vieira 2010). Studies have shown that proteolytic enzymes are capable of removing biofilms

from target cells by attacking the extracellular polymeric matrix which surrounds the biofilm cells (Lequette *et al.* 2010). However, there has been no report of its application against biofilm of *L. monocytogenes* bacteria in fresh produce.

2.6.3.3 Phytochemicals

Secondary products of metabolism from some plants have been found to possess antimicrobial ability (Belletti *et al.* 2008; El Abed *et al.* 2014). Many of these metabolites which have earned GRAS status include alkaloids, essential oils, phenolic, and lectins (Negi 2012; Borges *et al.* 2013; Negi and Anand 2015). Due to their variability, their antimicrobial activity has been found to vary. But studies have shown that they mostly increase cell permeability leading to leaching of cellular substances (Tiwari *et al.* 2009). Other authors reported the use of chitosan- a phytochemical to inhibit *L. monocytogenes* growth on a solid surface (Knowles and Roller 2001).

2.6.3.4 Probiotics

The use of probiotic bacteria as a natural antimicrobial agent has been reported (Šušković *et al.* 2010; Teplitski *et al.* 2011). They produce certain metabolites which offer prophylactic protection against certain intestinal pathogen of the host. Scolari and Vescovo (2004), reported 2.4 log CFU/g reduction of *L. monocytogenes* population on damaged lettuce when treated with *Lactobacillus casei.* Mechanism of inhibition includes nutrient and space competition, secretion of inhibitory compounds and sometimes entire colonization of the microbiota (Whipps *et al.* 2008).

2.7 Bacteriophage

Bacteriophage or phage is a virus which infects and kills bacteria (Labrie, Samson and Moineau 2010; Mahony *et al.* 2011; Sillankorva, Oliveira and Azeredo 2012). It has been regarded as one of the most abundant and self- replicating biomaterial on the earth (Hagens and Loessner 2015). An estimated 10^{30}-10^{31} number of phage particles have been reported to exist and this usually outnumbers its bacterial host by over a logarithm fold (Brüssow and Hendrix 2002; Weinbauer 2004; Sulakvelidze 2013). Bacteriophage belongs to the *caudovirales* order consisting of three families based on length and tail contraction *myoviridae, siphoviridae* and *podoviridae* (Doffkay *et al.* 2015).

2.7.1 History of Bacteriophage

Historically, bacteriophage was discovered by two independent scientists-Frederick Twort and Felix d`Herelle (Kutateladze and Adamia 2010). They both found that this bacterial parasite holds a therapeutic capacity against certain human infections (Duckworth 1976; Twort 2011). Meanwhile, the development of antibiotics for clinical use reduced further research into its utilization. However, due to evolution of antibiotic-resistant bacteria, the resurgence of bacteriophage was facilitated and has gained increasing applications in various fields including food safety (Chanishvili 2012).

2.7.2 Bacteriophage lytic cycle

The infection cycle of bacteriophage on its host bacteria principally operates in a series of steps. It begins with binding of the phage on specific host bacterial cell wall, after which the phage transfers its DNA genomes into the host system upon adsorption. It replicates itself in the host mechanism severally to synthesize a new set of phage particles which eventually kill the host bacteria (Chan, Abedon and Loc-Carrillo 2013; Schmelcher and Loessner 2014). This replication, however, occurs in two major ways which are lysogenic and lytic cycle. In lysogenic cycle, the phage simply adapts its DNA genomes into host cell and replicates itself as part of host genome which often does not kill the host bacteria. In lytic cycle, which constitutes the present study, the phage invades and replicates itself in an overbearing way for the host cell to contain and eventually leads to death of the bacteria (Dalmasso, Hill and Ross 2014; Hagens and Loessner 2015).

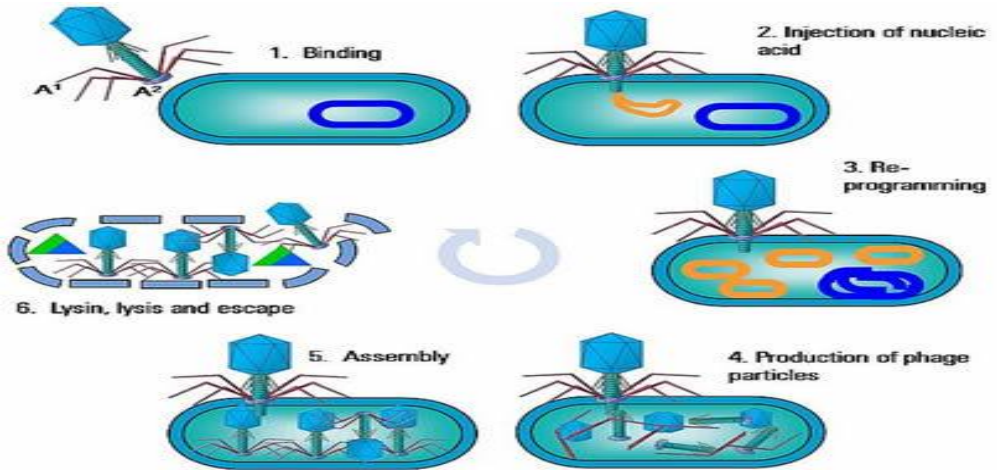


Figure 2.7 Bacteriophage lytic cycle
(Kingwell 2015)

2.7.3 Application of lytic bacteriophage in food safety

The use of lytic bacteriophage in the food safety industry has gained attention in recent times as a result of its excellent attributes. It has been found that bacteriophages are natural antimicrobials which co-exist with their specific bacterial hosts. Hence, it offers the advantage of leaving food microbiota unaffected without causing superinfection (Golkar, Bagasra and Pace 2014). Also, its high rate of self-replication is an economic means of being used in small amounts. Furthermore, its abundant availability in the environment makes isolation quite easy. This important attribute of phage has given rise to its utilization to improve food safety challenges including contamination with common bacterial pathogens such as *Salmonella, Campylobacter*, *E.coli*, and *Listeria* (Schmelcher and Loessner 2014). This recent attention and attraction of phage biocontrol as a means of improving food safety challenges could be linked to sundry reasons. The major factor may be as a result of increased consumer and regulatory demands in reducing environmentally harsh and chemical-based sanitizers (Doffkay *et al.* 2015). Food safety challenges have indeed assumed a global phenomenon with its attendant public health and economic impacts. In the USA, it has been estimated yearly that about 48 million people get sick, with 128,000 hospitalized and 3000 death cases due to foodborne diseases, incurring about $14 billion per year (Mead *et al.* 1999). Although, phages are without their draw backs, but their advantages of rendering environmentally safe, non-deleterious organoleptic impact and significant reduction on their target bacteria in various food makes them excellent interventions in food safety applications (Goodridge and Bisha 2011; Sulakvelidze 2013).

2.7.3.1 As Bio-therapeutic agent

Bacteriophage has been used as a bio-therapeutic agent in animal husbandry to significantly reduce colonization of domesticated livestock with common pathogenic bacteria such as *E. coli* O157:H7, *Salmonella Spp*, *Campylobacter jejuni* and *L. monocytogenes* (Sulakvelidze 2013). Investigation into phage therapy against foodborne pathogens from animal origin primarily targets optimization of pre-harvest interventions where the pathway of phage application has received most attention, and also on the optimization of postharvest strategies (Sillankorva, Oliveira and Azeredo 2012). Pre-harvest strategy involves direct application of phage either orally or by injection to livestock in order to prevent animal diseases and/or also to minimize

the pathogen carriage in the gastrointestinal tract, thereby preventing zoonotic diseases (Goodridge and Bisha 2011).

Phage therapeutic efficacy has been studied in animals such as cattle, sheep, swine and poultry, with more investigation on the use of phage either singly or as cocktail to inhibit food borne pathogens in sheep and cattle (Dykes and Moorhead 2002; Callaway *et al.* 2003). For example, in young cattle which houses considerable amount of *E.coli* O157:H7 due to faecal contamination from water and skin, the use of phage to significantly reduce *E.coli* O157:H7 has been reported (Waddell *et al.* 2000). Result obtained from this author showed that untreated calves shed this pathogen in the feces between 12-16 days while phage treated calves had stopped shedding after day 8 with an increase in phage concentration due to replication. However, one challenge that could affect phage application as biotherapy intervention in ruminants is its efficacy in the acidic medium of the abomasum, digestive enzymes and other compounds such as bile (Smith, Huggins and Shaw 1987). This phenomenon opens more investigation to know the optimum conditions such as phage dosage and delivery approach to maximize the efficacy of phage therapy in food-based animals.

Similarly, the use of phage cocktail to reduce *Salmonella typhimurium* inoculated orally and intramuscularly in swine has been reported (Lee and Harris 2001). Result of investigation showed that samples of analyzed organs after being sacrificed at 9 h of post treatment were significantly reduced which offers phage therapy as short term intervention to control *Salmonella* infection in swine.

Furthermore, phage therapy application against foodborne pathogen in poultry has been reported (Fiorentin, Vieira and Barioni Jr 2005). The author investigated phage cocktail isolated from free range chickens to reduce *Salmonella Enterica* serovar *Enteritidis* in the ceca of some broilers. Result showed log reduction of 3.5 folds after 25 days of treatment.

Postharvest interventions are based on direct application on animal carcasses as a sanitary measure to the final product. However, conflicting results on the phage-inactivation of bacterial pathogens on animal-based foods have been reported. For example, Bach *et al.* (2003) reported no reduction of *E. coli* O157:H7 for over 30 days when a lytic phage-DC 22 was orally administered on the rumen of a sheep in-vitro. Similar finding was also reported in a poultry product when a *Salmonella* phage-SP6 was orally administered (Hurley, Maurer and Lee 2008). On the other hand, 2 fold log reduction was reported in sheep within 2 days when an *E.coli* phage-CEV1 was orally administered (Raya *et al.* 2006). Similar significant reductions have been reported in other animal products to invalidate earlier claims of bacteriophage inefficacy for therapeutic purpose (Carrillo *et al.* 2005; Sheng *et al.* 2006; Zhou *et al.* 2015).

2.7.3.2 As Bio-sanitation agent

Bacteriophage has also been used as a bio-sanitation agent to decontaminate kitchens at home, processing environment and other contact surfaces used in food safety facilities (Gibson *et al.* 1999). Proper cleaning and sanitation of food-contact surfaces is essential to avoid potential contamination by (Gibson *et al.* 1999). Even though a substantial population of foodborne bacterial pathogens is inactivated when foods are properly cooked, some of the contaminating bacteria may survive on the food-contact surfaces prior to cooking. Hence, potential cross-contamination is possible on such surfaces especially in ready-to-eat foods such as fresh produce that are not usually cooked before consumption (Bloomfield and Scott 1997; Kusumaningrum *et al.* 2003). Similarly, the possibility of foodborne bacteria persistence on various surfaces in food-processing facilities could contaminate foods that are being processed or packaged in those facilities. The outcome of some previous investigations suggest that lytic phages can significantly reduce contaminations of pathogens including *L. monocytogenes* on food-contact surfaces such as gypsum board, stainless steel and glass (Roy *et al.* 1993; Hibma, Jassim and Griffiths 1997). The bacteria has been identified to form biofilms in most food processing environments and has raised food safety concerns as they tend to resist common sanitizers (Orgaz *et al.* 2013). The closeness of bacterial cells in a biofilm could enhance phage access for post-lysis infection but they are often surrounded by extracellular polymeric substance (EPS) which are made up of bacterial polysaccharides which offers cell protection from antimicrobial agents (Chan and Abedon 2015). In the same vein, certain lytic phages contains polysacharases or polysaccharide lyases which degrades the EPS thereby providing accessibility to the cells enclosed in the biofilm. Specifically, Listex P100 phage has been reported to destroy biofilms of *L. monocytogenes* on stainless steel coupon resulting in log reduction of 5.4 fold/cm^2 (Soni and Nannapaneni 2010). This development has further defined bacteriophage as a veritable tool to eliminate these pathogens but may be limited in use due to differences in the specie of bacteria that could be found at the same time in such environments.

2.7.3.3 As Bio-pesticide agent

Recently, the use of bacteriophage as bio-pesticide has been reported (Doffkay *et al.* 2015). Bacteriophages were first found to be associated with plant pathogenic bacteria in 1924 where the filtrate of decomposed cabbage inhibited the growth of *Xanthomonas campestris pv.campestris* (Mallmann and Hemstreet 1924). By 2005, the first phage-containing pesticide

(AgriPhage™) was registered with the U.S. Environmental Protection Agency (http://www.omnilytics.com/products/agriphage). This biopesticide contains phages specifically used to control wide spp of plant bacterial pathogens such as *Agrobacterium tumefaciens, Erwinia amylovora, Pseudomonas syringae pv. tomato, Ralstonia solanacearum, Xanthomonas arboricola pv. juglandis, Xanthomonas arbori- cola pv. pruni, Xanthomonas oryzae pv. oryzae, Xanthomonas campestris pv. Vesicatoria* (Dömötör *et al.* 2012; Romero-Suarez, Jordan and Heinemann 2012; Young *et al.* 2012; Chae *et al.* 2014).. Isolation of the phage for bio-pesticidal effect could be done from surrounding soil of the infected plant and its tissue as well. However, the nature of bacterium host specie could help to determine the strategy of phage isolation. Ritchie and Klos (1977), reported the use of a single bacterial strain for phage isolation on *Erwinia amylovora* from the aerial part of apple where lysis only, took place. Conversely, Gill *et al.* (2003) reported that phages isolated from multiple bacterial strains had a broader range. This development has further enhanced the advantages of phage as a bio-pesticide as opposed to most chemical-based pesticides with post treatment toxicity concerns. However, due to climate change effect on plant phyllosphere, difficulty in obtaining a reliable intervention measure void of phage-based approach in pest management will continue to constitute a challenge (Nagy, Király and Schwarczinger 2012).

2.7.3.4 As Biocontrol agent

Importantly, bacteriophage serves as a biocontrol agent against wide strains of bacteria pathogen in foods (Pietracha and Misiewicz 2016). They have been reported to significantly reduce certain species of common bacterial contaminants in both fresh and minimally processed foods such as *Salmonella* (Kocharunchitt, Ross and McNeil 2009; Guenther *et al.* 2012), *Campylobacter* (Goode, Allen and Barrow 2003), *E. coli* (Abuladze *et al.* 2008; Viazis *et al.* 2011) and *Listeria* (Guenther *et al.* 2009). The outbreak of a Shiga toxin *E. coli* isolate linked to bean sprouts in 2011 on a German organic farm further redefines the need to develop improved and safer interventions to control foodborne pathogens in ready-to eat foods (Buchholz *et al.* 2011). Furthermore, organic farming practices speculation that only natural antimicrobials be deplored during processing makes phage a right choice as a biocontrol approach. However, the efficacy of phage treatment appears to vary depending on certain factors or conditions such as the types and state of food used, the levels of contaminating bacteria, the phage concentrations applied (Guenther *et al.* 2009). For example, Spricigo *et al.*

(2013) reported reductions of 3.9 and 2.2 log CFU/g of *S. typhimurium* and *S. enteritis* population on lettuce respectively, when treated with a lytic phage (UAB_Phi20, UAB_Phi78 and UAB_87) stored at room temperature for 60 mins. Also, 1-2 log reduction of *Campylobacter jejuni* has been reported on chicken skin stored at 4^{o}C when Φ2 phage was applied (Wagenaar *et al.* 2005). Furthermore, Abuladze *et al.* (2008) reported 94 and 100% reduction of *E. coli* on fresh tomato and spinach respectively after 120 and 24h when treated with ECP-100 phage cocktail. Similarly Sharma *et al.* (2009) reported significant reduction of *E. coli* O157: H7 on fresh-cut lettuce and cantaloupes stored at 4 and 20^{o}C for 7 days.

Particularly, phage has been experimentally used to reduce the growth population of *L. monocytogenes* in a wide range of fresh produce (Leverentz *et al.* 2004; Oliveira *et al.* 2014; Perera *et al.* 2015). Numerous commercially prepared phages for *L. monocytogenes* have been approved by FDA and USDA. This includes Listshield and Ecoshield (Intralytix Inc., USA) and Listex P100 (Micreos Food Safety, Netherlands). This approval has further elicited the need to evaluate phage application especially in ready-to-eat foods (Oliveira *et al.* 2014) A phage cocktail (LMP-102), significantly reduced *L. monocytogenes* population on fresh-cut melon and apple by 2.0–4.6 and 0.4 log units respectively (Leverentz *et al.* 2003). This efficacy of bacteriophage in practice is linked to its level of diffusion in the test medium, where greater diffusion has been observed in a liquid medium compared to solid matrix where phage immobilization is highly possible (Guenther *et al.* 2009). This assertion was further confirmed in the findings of Oliveira *et al.* (2014) where reduction of 8.00 log CFU/ml *L. monocytogenes* population was obtained on phage treated fresh melon juice compared to 1.50 log CFU/ml on fresh-cut melon.

Furthermore, the acidity of the lytic environment also affects phage-host interaction. This development was confirmed by Oliveira *et al.* (2014) where level of *L. monocytogenes* reduction in three different fresh produce (melon, pears and apple) of varied pH values (5.92, 4.91 and 3.76) determined the effectiveness of phage application. The author found log fold reductions of 1.50, 1.00 and <1 on the fresh-cut produce respectively. Intervention measures to address this seeming limitation of bacteriophage includes; combination with other antimicrobials to enhance lysis. Hong *et al.* (2006), reported one fold log reduction on fresh-cut honey dew melon stored at 10^{o}C for 7 days when treated with LMP-102 lytic phage, but when combined with an antagonistic bacteria- *Gluconobacter asaii,* obtained log reduction of 6 fold. The use of higher phage concentration or cocktails for effective penetration have been reported by other authors as a good measure (Chan, Abedon and Loc-Carrillo 2013). Leverentz *et al.* (2004), further reported that a cocktail of six phages resulted in a higher reduction of *L.*

monocytogenes population on honey dew melon, when applied at higher dosage. Development of acid-adapted phages has also been suggested to help address challenges encountered with low pH foods (Oliveira *et al.* 2014; Strydom and Witthuhn 2015).

2.7.4 Advances in Bacteriophage utilization

The knowledge of adaptation, physiology and morphology of pathogens is very crucial to its detection and control measures. Recent trends in research aimed at tackling this is depicted in the recent production of poly-bacteriophage or poly-phage, which will be able to detect and control many genera of bacteria in a food matrix (Hyman and Abedon 2010). Further, genomic engineering studies have aided the novel development of incorporating phages into biosensor and Nano-based sensor for rapid detection of pathogens in foods especially for on-site assessment (Shen *et al.* 2009; Singh, Poshtiban and Evoy 2013; Billington, Hudson and D'Sa 2014). Application of phage-based magneto-elastic biosensor for detection of *Salmonella* in tomato has been reported (Li *et al.* 2010; Park, Li and Chin 2013). Similarly, other authors have advocated the use of nano sensors in phages for broad range detection of pathogens (Hyman 2012; Lee, Domaille and Cha 2012; Raghu *et al.* 2012).

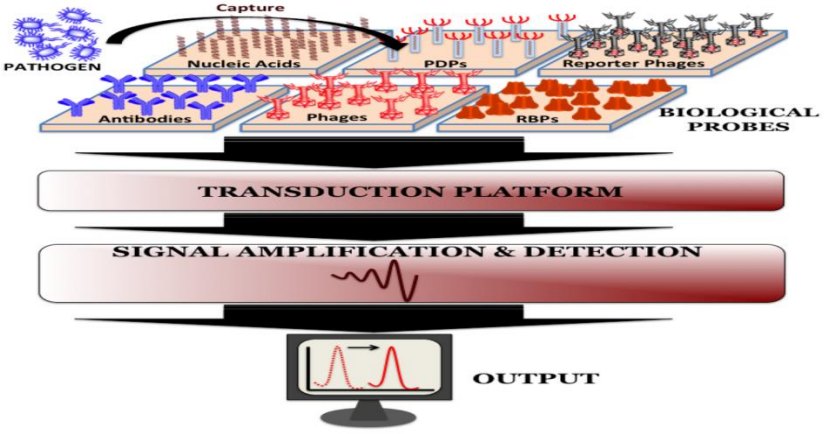


Figure 2.8 Phage-based molecular probes for pathogen detection

(Singh, Poshtiban and Evoy 2013).

2.8 Artificial Neural Network

2.8.1 Background information of neural network

Artificial neural network (ANN) is a mathematical modelling tool which mimics the activity of the human brain (Huang, Kangas and Rasco 2007; Goyal 2013). The basic unit of the human brain in transmission of information is the neuron. The human neuron consists of several

interconnectivities with diverse functions in the body. They receive process and transmit information in a form of electric signals.

Artificial neural network concept was reported to have begun as early as 1943, but its efficacy was criticized as a result of limitations of data availability and application (McCulloch and Pitts 1943). It however gained attention again in the 1980s when these limitations were addressed and a new set of algorithms for learning were found to have developed (Hopfield 1982; Williams and Hinton 1986).

Furthermore, rapid development of learning new set of algorithm, coupled with innovative trends in computer technology, has helped to enhance application of this artificial intelligence tool in different fields and professions of human endeavor. It has quickly gained popularity in the field of medicine, meteorology and financial management. However, its utilization in the field of food science is quite new and upcoming (Huang, Kangas and Rasco 2007).

In the human brain, the neuron comprises four basic components for information transmission which are the dendrites, soma, axon and synapses. The dendrites receive signals in the form of impulses from other neurons, the soma is the neural portion that processes the signal, the axon helps to transmit the processed information while the synapse are the connecting gaps between each neurons which regulates the transmission output (Figure 2.9).

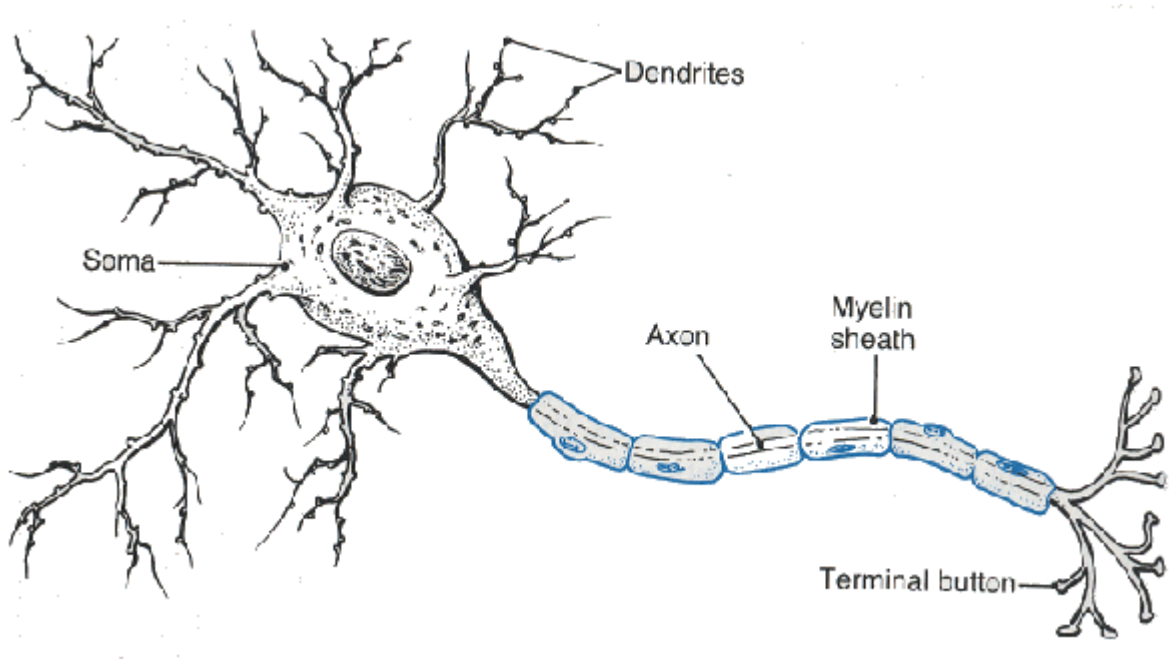


Figure 2.9 The Human neuron

(Funes *et al.* 2015)

Similarly, ANN which is a simulated form of neuron activity, comprises series of information which are received as inputs, (X_1, X_2, $X_3 \ldots X_n$), connected to a weight (Wi) for onward transmission into output signal. This output signal can subsequently become an input variable for another network or as final output (Figure 2.10).

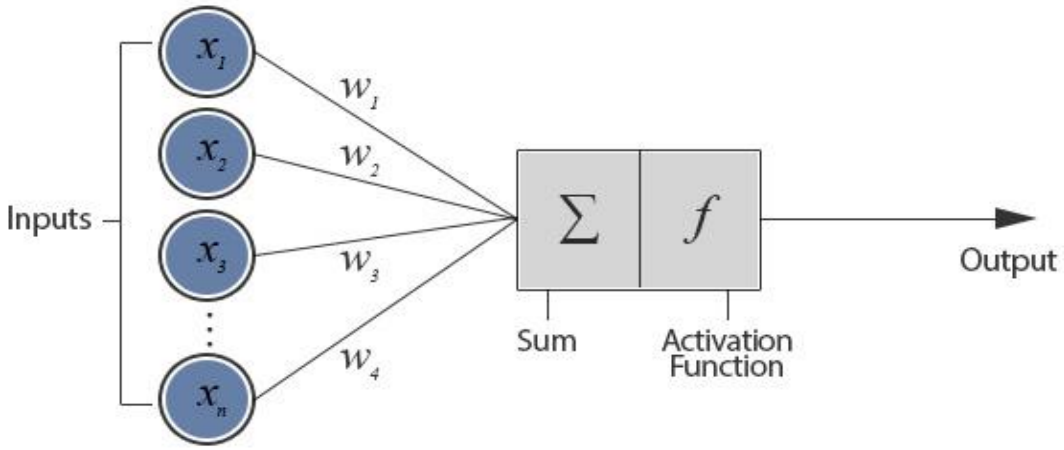


Figure 2.10 Representation of ANN model functions

(Matich 2001c)

2.8.2 Components of neural networks

The basic components or architecture of artificial neural networks involves development of models, learning or training and validation processes. Development of models for ANN topology is dependent on the number of input variables, target output, and the number of hidden nodes in the hidden layer (Funes *et al.* 2015). The distributions of data set for ANN must be void of variation, hence the introduction of normalization techniques such as min-max which normalizes any type of data from their real range [a, b] to [0 1] and others such as z-score, z-score standard deviation, decimal scaling normalization technique.

An Artificial Neural Network (ANN) is an interconnected group of artificial neurons that uses computational models to simulate the structure and function of biological nervous system (Huang, Li and Wang 2004; Rahbari 2014). In ANN, the pattern of connections between the neurons and the propagation of data is called network topology. The network topology consists of input, hidden and output layer. The two main types of ANN topology are the feed-forward and recurrent topologies.

In the feed-forward network, the input layer receives information which is transferred to the hidden layer where the information is being trained and transferred to the output layer. One

hidden layer is generally adequate for a network training to take place, but more than one hidden layer may be applied depending on the complexity of the application. Increasing the number of hidden layer enhances the learning ability of the data set and could sometimes lead to poor network (Knight 1990).

The flow of data in a feed-forward neural network is in a forward direction from input to output with no feedback of connections, while recurrent networks contain feedback connections. One of the most commonly used feed-forward network topology is Multi-Layer Perceptron (MLP). The MLP is a nonlinear neural network, which is primarily composed of orderly interconnected artificial neurons that are arranged in layers. Typically, MLP is composed of a minimum of three layers, which comprises an input layer, one or more hidden layers, and an output layer which represents the building block of all ANN-MLP systems (Delashmit and Manry 2005). An MLP-ANN topology is illustrated pictorially in Figure 2.11 below.

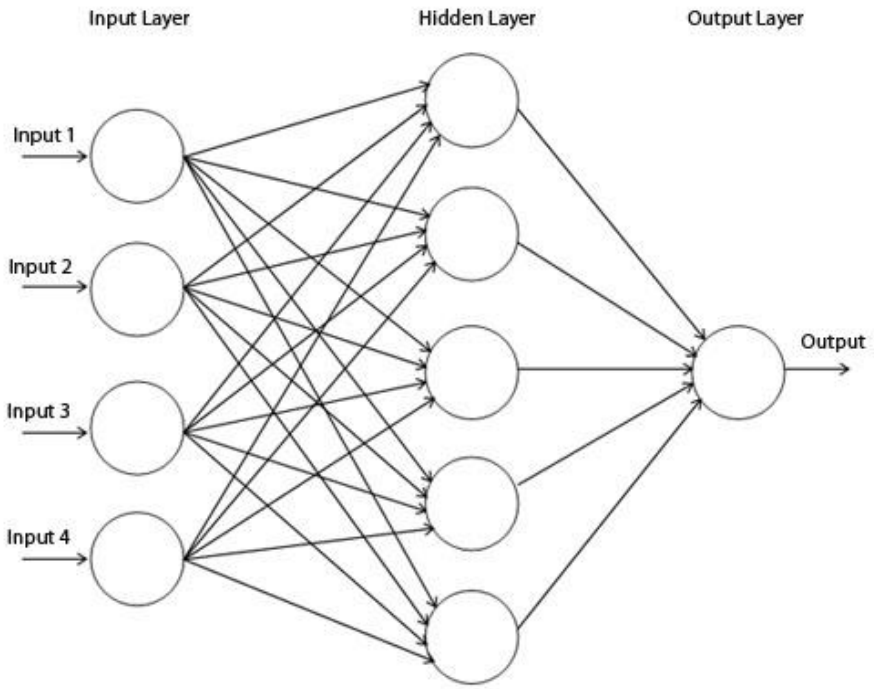


Figure 2.11 Topology of back propagation (BP) artificial neural network (Agatonovic-Kustrin and Beresford 2000)**.**

Learning ANN involves obtaining the weighted values for effective training into target output. This is usually achieved by adjustment of weights in the connection using activation functions, and final output is correlated with values obtained from real experimental values.

The accuracy of the final output is however dependent on certain factors such as size of data set, learning rate, training cycle or epochs, and error limit.

The rate of learning helps to determine the speed required for learning the algorithm data set. Matich (2001b), however, reported two different types of learning patterns which are supervised and unsupervised learning. The supervised learning is controlled by an external factor which ensures correlation and adjustment in network output. This is done to reduce differences between network output and expected values. Example includes Adaline, MPL, Associative Memory Bi-directional and Back propagation (BP) type used in the present study. It has the advantage of learning data sets easily.

This learning pattern has been reported in the processing operations of some fresh produce such as drying and ultrafiltration process in apple and tomato samples formulating models (Movagharnejad and Nikzad 2007; Gokmen *et al.* 2009; Khoshhal *et al.* 2010). The unsupervised learning does not require external factors. Large sets of data have been found to increase the accuracy of learning but extremely large data makes longer learning time. The main drawback with unsupervised learning is space creation within groups or categories. Examples includes Hopfield, Machines Boltzmann and Cauchy and networks with competitive learning, such as SOM and ART. Verification is intended to confirm the capability of the ANN based model to respond accurately to examples never used in network development. The validation process which is the final stage during ANN helps to check if the developed model used to train the data set corresponds to the actual value obtained from other concepts. This stage involves correlation of the ANN based models to other approaches. Here, the trained weights must be conditioned or enhanced in their individual process of learning. This is usually done by comparing the real values via statistical tool to the trained values.

2.8.3 Nature of Artificial neural networks

Artificial neural networks comprise numerous attributes which are quite similar to brain activities as a result of its structural basis and composition (Matich 2001a). These networks are gaining useful application in various technological concepts and offers laudable advantages. This includes: Adaptive learning capacity which involves the ability to continuously learn certain task using descriptive examples. Due to the number of process units involved, they could change and adapt themselves to any condition. Furthermore, it has been found that they could generate their own weight via learning and during post-learning period. However, this can be enhanced when appropriate learning structure with good algorithm for training is put in place (Ben and van der Smagt 1996; Matich 2001a).

Furthermore, ANN employ their ability to adaptive learning to arrange information received during learning thereby giving response to a new condition, and solution to unclear or incomplete input information. They can also be flexible to drawbacks emanating from incomplete or distorted data sets.

Neural computations can be carried out without interference, and most of the networks can function in real-time conditions since the need for change in weight or training is very small.

In a more practical term, a neural network can be rapidly trained, validated and applied to a hardware for a technology drive (Karwe 2001).

2.8.4 Application of ANN prediction in food science

ANNs have been deplored as useful tools in food science parlance especially in the concept of food safety and quality analyses, such as growth modeling of microorganism, hence predicting food safety, interpreting spectroscopic data, and predicting physicochemical and functional properties of food products during processing and distribution (Huang, Kangas and Rasco 2007). ANNs holds a great potential for modeling complex tasks in quality management concepts such as process control and simulation, and in applications of machine perception such as machine vision and the electronic nose (Table:2.2) (Arshak *et al.* 2003; Goyal 2013; Funes *et al.* 2015). In microbial safety and quality of fresh produce, the use of ANNs to predict the effect of sanitizer against coliforms and *E. coli* on tomato and lettuce has been reported (Keeratipibul, Phewpan and Lursinsap 2011). Similarly, storage quality of fresh-cut green pepper using ANN prediction has also been reported (Meng, Zhang and Adhikari 2012). Noteworthy, Ozturk *et al.* (2012) reported application of some data-driven neural models to predict impact of some plant hydrosols on *L. monocytogenes* inactivation on inoculated fresh–cut apple. Recently, prediction of risk associated with *L. monocytogenes* growth on fresh-cut tomato and carrot treated with bacteriophage and sucrose monolaurate by using Artificial neural network has been reported (Oladunjoye *et al.* 2017).

Table 2.2 Applications of ANN prediction on certain fresh produce

Fresh produce	Neural Prediction objective	Type of ANN	References
Apple	ANN modeling of apple drying process.	MLPFF	(Khoshhal *et al.* 2010)
Lime	ANN modeling of mass transfer during osmotic dehydration of kaffir lime peel	MLPFF	(Lertworasirikul and Saetan 2010)
Tomato	Modeling of tomato drying using ANN	MLP	(Movagharnejad and Nikzad 2007)
Cherries	Use of genetic artificial neural networks and spectral imaging for defect detection on cherries	MLPFF, BP	(Guyer and Yang 2000)
Fruit	Pattern recognition of fruit shape quantitatively with ANN	SOM	(Morimoto *et al.* 2000)
Grape	Determination of anthocyanin concentration in whole grape skins using hyperspectral imaging and adaptive boosting ANN	MLPFF	(Fernandes *et al.* 2011)
Banana	Determination of ripening stages of banana using electronic nose-based system	ARTMAP, LVQ, MLP	(Llobet *et al.* 1999)
Carrot	Predicting properties of dried carrot based on composition, drying technique, and microstructural features.	BP	(Kerdpiboon, Kerr and Devahastin 2006)
Corn	Artificial neural networks to predict corn yield	MLPFF, BP	(Uno *et al.* 2005)
Orange, peach and pear	Predicting of viscosity of fruit juice as a function of concentration and temperature	MLP	(Rai *et al.* 2005)
Guava	Prediction of respiration model of guava using enzyme-chemical kinetics and ANN	LVQ	(Wang, Duan and Hu 2009)
Broccoli	Grading of broccoli using computer vision and ANN	BP, LVQ	(Tu *et al.* 2007)
Spinach	Harnessing of spinach growth parameters using microwave-sensor and ANN	BP	(Prasad *et al.* 2012)

(Goyal 2013; Funes *et al.* 2015).

2.9 Aim

The aim of this research is to control *Listeria monocytogenes* ATCC 7644 growth in selected fresh produce using *Listeria monocytogenes* bacteriophage and to predict the risk involved using artificial neural network predictions.

2.10 Hypothesis

1. The use of a lytic bacteriophage to control *Listeria monocytogenes* in fresh produce will bring a significant log reduction due to its ability to penetrate and integrate into bacterial host genome to cause lysis (Hagens and Loessner 2015; Strydom and Witthuhn 2015). However, the pathogen will show increased resistance to antimicrobials when subject to stress conditions. *L. monocytogenes* will `survive stress conditions because it contains certain proteins which are expressed to change the morphology and physiology of the bacteria against any stress condition (Lou and Yousef 1997; Gandhi and Chikindas 2007).

2. The synergistic combination of bacteriophage with other control techniques will bring about higher log reduction of *L. monocytogenes* population. Hurdle techniques will attack the pathogen in different ways. Bacteriophage will bring about lysis by adsorption through the cell wall, while sugar esters will exhibit surface active property (Guenther *et al.* 2009; Zhao *et al.* 2015).

3. Kinetic growth models in predictive microbiology often generate large sets of data to predict microbial growth under a wide range of experimental conditions. Artificial neural network as a data driven model will offer a more accurate approach in learning such large sets of data (McKellar & Lu, 2001).

4. *Listeria monocytogenes* will survive a wide range of environmental conditions because bacteria has been found to utilize basic elemental nutrients in their environment to survive harsh conditions (Bochner 2009).

2.11 Objectives

1. To determine the safety of fresh produce (tomato and carrot) artificially inoculated with stress-adapted *L. monocytogenes* ATCC 7644 when treated with bacteriophage.

2. To determine the inhibition level of *Listeria monocytogenes* ATCC 7644 on the selected fresh produce when treated with bacteriophage in combination with sucrose monolaurate.

3. To predict the risk assessment of inhibiting *L. monocytogenes* ATCC 7644 growth on the fresh produce when treated with bacteriophage and sucrose monolaurate using an artificial neural networks tool.

4. To investigate the energy utilization profile of *L. monocytogenes* ATCC 7644 on inoculated fresh produce when treated with bacteriophage using biolog phenotype micro-array method.

CHAPTER THREE

3.0 Susceptibility of stress-adapted *Listeria monocytogenes* ATCC 7644 to bacteriophage control on artificially inoculated fresh produce

Abstract

The aim of this work is to investigate the susceptibility of stress adapted *Listeria monocytogenes* ATCC 7644 to bacteriophage control in fresh-cut produce. Fresh-cut carrot and tomato inoculated with 10^8 CFU/ml of *L. monocytogenes* (acid-adapted-AA, chlorine-adapted-CA, heat-adapted-HA) in comparison with non-adapted strain (NA) were treated with 10^8 PFU/ml of Listex P100 bacteriophage and stored at 4, 10 and 25^{o}C for 10 days. Effect of treatment on quality attributes (pH, total soluble solids, titratable acidity and color), and morphology was also investigated. All stress-adapted and non-adapted *L. monocytogenes* were ($p \leq 0.05$) susceptible to bacteriophage control. With phage treatment, the population of *L. monocytogenes* on fresh-cut tomato reduced by less than one fold log cycle, while greater log reductions (2 fold) were observed for fresh-cut carrot. Phage treatment averagely reduced NA, AA, CA and HA *L. monocytogenes* population by 0.57, 0.81, 0.86 and 0.95 log CFU/ml on fresh-cut tomato and by 2.26, 2.41, 2.49 and 2.54 log CFU/ml on fresh-cut carrot respectively. Morphological changes due to stress adaptation were observed. Quality attributes of both fresh-cut produce were not significantly affected by the phage treatment. Bacteriophage biocontrol can offer eco-friendly means of addressing possible *L. monocytogenes* contamination under different post-harvest conditions in fresh produce.

3.1 Introduction

Food-borne listeriosis caused by *Listeria monocytogenes* is a life- threatening disease of public health concern in the food safety industry. It is a gram-positive bacterium, belonging to one of the six known *Listeria* genus (Farber and Peterkin 1991). Two new additional species have also been reported (Graves *et al.* 2010; Leclercq *et al.* 2010). Recently, seven novel species have recently been reported but might not be widely distributed like others (Orsi and Wiedmann 2016). The pathogen is widely distributed in nature, and can be found in several ecological niche such as water, soil, and animal dungs (Liu 2006; Wulff *et al.* 2006).

Ready-to-eat (RTE) foods such as fresh produce have also been reported as a medium of contamination during pre-harvest and post-harvest processes (Beuchat 2002; Guenther *et al.* 2009). In the USA, it has been rated the third leading cause of death in foodborne diseases, which makes approximately 19% of total death cases reported annually (Hoffmann, Maculloch and Batz 2015). The United States Department of Food and Agricultural has enforced a "zero tolerance" measure to check the occurrence of *L. monocytogenes* pathogen contamination in RTE foods (Klima and Montville 1995).

Despite this approach, food recalls due to listeriosis outbreaks in RTE foods, particularly in fresh produce, continues to persist (Dey *et al.* 2013). A recent multistate recall of frozen fruit and vegetable products due to possible contamination of *L. monocytogenes* has been reported (CDC 2016b). In another development, Tasara *et al.* (2015) also reported the outbreak of similar disease in the European nation of Switzerland. This pathogen has been found to develop adaptive mechanism to survive certain food processes such as acid shock-during (fermentation), heat shock (during pasteurization) and chlorine (during chlorine wash) of fresh produce (Yousef and Juneja 2002).

Based on this discovery, it is pertinent to decode the mode of action by which this pathogen perseveres under different post-harvest and processing conditions of fresh produce (such as low pH, sanitizers, high osmotic and temperature conditions) so as to come up with effective control measures to be adopted. This resilient nature among other factors has however been linked to its adaptation to various stress conditions, which increases the bacterial resistance to other antimicrobial treatments (Doyle *et al.* 2006; Shen, Soni and Nannapaneni 2015). It responds to stress by inducing certain regulatory proteins in order to repair injured cells, enhance resistance to stress factors, change status (viable to dormant), circumvent host macrophages, and create adaptive mutants (Yousef and Juneja 2002; Gandhi and Chikindas 2007).

This phenomenon is a safety concern in the fresh produce industry as measures to control this development become more important. One such measure includes use of bacteriophage control. Bacteriophages or phages are viruses which invade and kill bacteria. They operate basically through a lytic process on their bacterial host and are available in the environment (Simões, Simões and Vieira 2010; Ackermann and Węgrzyn 2014). It has been found that bacteriophages are natural antimicrobials which co-exist with their specific bacterial hosts. Hence, it offers the advantage of leaving food microbiota intact (Veiga-Crespo and Villa 2010). Also, bacteriophage replicates very fast in the host genome, hence small quantities are required to bring about lysis. Furthermore, its abundant availability in the environment makes its isolation quite easy (Hughes *et al.* 1998; Sillankorva, Oliveira and Azeredo 2012).

These exceptional attributes of phage to control growth of *L. monocytogenes* contamination on fresh produce has been reported (Leverentz *et al.* 2003; Olaimat and Holley 2012; Sulakvelidze 2013). Furthermore, Oliveira *et al.* (2014) also reported approximately 1.5 and 1 fold reductions after using a *Listeria* bacteriophage to control three strains of *L. monocytogenes* on melon and conference pear slices respectively. Furthermore, previous work on increased resistance of stress-adapted *L. monocytogenes* to certain antimicrobial has been reported (Al-Nabulsi *et al.* 2015; Shen, Soni and Nannapaneni 2015).

However, as phage efficacy continues to receive more attention in fresh produce decontamination, there is a need to investigate the fate of stress-adapted *L. monocytogenes* to phage treatment. Therefore the aim of this study is to investigate susceptibility of stress-adapted *L. monocytogenes* on fresh-cut tomato and carrot which represents commonly used fresh produce.

3.2 Materials and Methods

3.2.1 Fresh produce

Fresh produce: Tomato (*Lycopersicon esculentum*) and carrot (*Daucus carota* subsp. *sativus*) were obtained from a local grocery supermarket (Woolworth supermarket, Durban. South Africa). Prior to experimental studies, each of the fresh produce was washed in running tap water and surface disinfected with 70% ethanol (Lichro chemical and laboratory supplies. Malvern, 4055 Queensburgh, Durban, South Africa), and allowed to dry at room temperature before cutting into 10 mm thick slices.

3.2.2 Bacteria and preparation of inoculum

Listeria monocytogenes ATCC 7644 serovar 1/2c was used in this study (Merck, South Africa). Working culture was prepared from frozen stock culture kept in glycerol (-80^{o}C) by streaking on *Listeria* oxford medium slant kept at 4^{o}C (LOM, Sigma-Aldrich Inc. USA) using Oxford *Listeria* selective supplement (Fluka 75806 Sigma-Aldrich Switzerland) for 24 h at 37^{o}C. Bacteria colonies were transferred into 50 ml Fraser broth base (F6672 FB, Sigma-Aldrich Switzerland) using Fluka Fraser selective supplement (F18038 FSS, Sigma-Aldrich, Switzerland) Bacterial culture was prepared using McFarland standard solution of approximately 8 $\log_{10}$ CFU/ml before use (Ijabadeniyi and Naidoo 2014).

3.2.3 Preparation of stress adapted bacteria

3.2.3.1 Acid adapted bacteria

Acid adapted bacteria was prepared by modifying the method reported by Al-Nabulsi *et al.* (2015). Briefly, 10 ml of bacteria cells from the supplemented Fraser broth culture incubated for 18 to 20 h at 37^{o}C were harvested via centrifugation (Eppendorf centrifuge 5810R Hamburg, Germany) at 3000 x *g* for 15 min at 4^{o}C. Pellets were re-suspended in 10 ml sterile peptone water buffered (PWB, 10 g L^{-1} peptone, 5 g L^{-1} sodium chloride, 3.5 g L^{-1} disodium hydrogen phosphate, 1.5 g L^{-1} potassium dihydrogen phosphate) and the pH was decreased from 5.5 to 4.7 using 0.2 M lactic acid (Sigma Aldrich Inc., USA) and held at room temperature for 30 mins. The cycle was repeated twice at which point the surviving *L. monocytogenes* cells were classified as acid adapted. A final working concentration of approximately 8 $\log_{10}$ CFU/ml using McFarland standard solutions was prepared before use.

3.2.3.2 Heat adapted bacteria

A modified method of Shen *et al.* (2014) was used in this study. *L. monocytogenes* bacteria cell from Fraser broth was centrifuged (Eppendorf centrifuge 5810R Hamburg, Germany) at 3000 x *g* for 15 min re-suspended in 5 ml sterile peptone water buffered. Cell culture was maintained at 8 log CFU/ml using McFarland standard solution and 100 ml of each culture was transferred to Eppendorf tubes into a hot water bath (Foss tecator technology, Sweden) at 60^{o}C for 10 min containing 900 ml of Fraser broth with selective supplement (Fluka FB 75806 Sigma-Aldrich). The surviving cells were enumerated after 10 min at 60^{o}C on *Listeria* oxford medium (LOM, Sigma-Aldrich Inc. USA) using Oxford *Listeria* selective supplement (Fluka 75806 Sigma-Aldrich Switzerland) for 24 h at 37^{o}C. Process cycle was repeated twice and

recovered cells grown on the medium after incubation was transferred on the Fraser broth as heat-stressed cells

3.2.3.3 Chlorine adapted bacteria

Chlorine adapted bacteria was prepared by the method reported by Gao and Liu (2014). Briefly, 90 µL of bacteria culture obtained from previous standard solution were transferred into 9 ml supplemented Fraser broth (F6672 FB Sigma-Aldrich) containing sodium hypochlorite at three different minimum inhibitory concentration (MIC) and incubated at 37ºC for 24 h to ascertain level of adaptation. This procedure was repeated for about 10 to 20 cycles and 90 µl of cultures obtained from these cycles were transferred into 9 ml Fraser broth at 37ºC for 24 h. Bacteria cells were tested against the different MICs to determine their chlorine tolerance ability.

3.2.4 Phage

Listex P100 bacteriophage (Micreos food safety 6709, Netherlands) hallmarked with a wide range lytic effect against *Listeria monocytogenes* was used in this study. The concentration of the phage was ~ 10^{11} plague forming units (PFU/ml) in a buffered saline and kept at refrigerated temperature.

3.2.5 Preparation of fresh-cut produce and sample inoculation

Each of the fresh produce was cut thinly into an average of 10 mm thickness and a 6 mm diameter wedge was made into the center of each produce to contain the inoculum. These were all placed into sterile bags with air passage to discourage modified atmosphere creation prior to inoculation. The fresh-cut produce were inoculated with the control and stress adapted *L. monocytogenes* by pipetting 1 ml of the inoculum to the wedges and allowed for a retention time of 30 min for attachment. Bacteriophage concentration containing 10^8 PFU/ml was later applied. Each of the fresh-cut produce samples was stored at three different temperatures (4, 10 and 25ºC) for 10 days (Oliveira *et al.* 2014).

3.2.6 Microbiological analysis

Bacteria recovery population was performed at intervals of 2 days for 10 days in all the storage temperatures (Oliveira *et al.* 2014). Fresh-cut produce were placed into sterile stomacher bags (Tuffy Brands Pty Ltd. South Africa) and homogenized in a stomacher blender (Stomacher laboratory blender 400 BA 6021 Seward Lab UAC House Blackfriars Road London SE 19UG)

for 120 s with 10 ml of sterile peptone water buffer (PWB, Biolab). Aliquots of mixtures obtained were serially diluted in saline peptone water (SP; 8.5 g L^{-1} Nacl and 1 g L^{-1} peptone) and 1 ml spread plated on Oxford agar. Inoculated plates were incubated at 37^{o}C for 48 h in order to obtain log reduction values as log CFU/ml.

3.2.7 Phage titration

Phage titration was carried out by the method described by Leverentz *et al.* (2003). Briefly, aliquots from phage treated samples were homogenized and filtered through 0.45-um-pore size membrane (Acrodisk; Pall Gelman, Ann Arbor, Mich). Phage titer was then determined using the soft agar overlay method using Brain heart infusion agar (BHI Biolab). Resulting plaques were counted as expressed as log PFU/ml.

3.2.8 Bacterial morphology of adapted and non-adapted strain

This procedure was carried out by modifying the method reported by Gao and Liu (2014). Bacterial cells in broth were centrifuged (Dragon Lab D 2012 Shunyi District, Beijing 101318 China.) at 10000 *x g* (4^{o}C, 10 mins). Pellets obtained were rinsed three times with phosphate buffer saline (PBS pH 7.2, Sigma Aldrich, South Africa) and fixed with 2.5% (vol/vol) glutaraldehyde at room temperature for one hour. Nitrocellulose membrane filters (13 mm diameter; 0.22-µm GV pore size) were used to filter the cells. Subsequent post-fixing of the cells was done using 1% tetroxide at room temperature for one hour, and dehydrated using 96% ethanol. The critical point of the samples was dried using CO_2 in a critical point drying apparatus. Finally, samples were gold-coated and examined in scanning electron microscope (SEM, Zeiss Ultra Pus. Germany)

3.2.9 Quality parameters

Quality parameters (pH, soluble solids, titratable acidity and color) were performed on the sample ten days of storage with the untreated sample as control. pH was determined using pH meter (Jenway 3510 pH meter. Bibby scientific Ltd, stone staffs, UK ST15 OSA). Soluble solids (SS, ^{o} Brix) were measured at 20^{o}C using digital refractometer (Atago Co. Ltd., Tokyo, Japan). Titratable acidity (TA) was determined by diluting 10 ml of sample with 10 ml distilled water and titrated against 0.1 N NaOH up to 8.1. Results were expressed as g of citric acid /L for both fresh produce. Color parameters were carried out using the hunter lab colorimeter

(Color flex EZ, CFEZ 0840 Virginia USA). All determinations were carried out three times per parameter.

3.2.10 Statistical analysis

All experiments were replicated three times with each of the treatments. Data obtained were subjected to analysis of variance and means compared using Duncan multiple range test ($p \leq 0.05$).

3.3 Results

3.3.1 Effect of phage treatment on growth population of *L. monocytogenes* in fresh-cut produce.

In the present study, choice of stress factor was informed by common post-harvest processing conditions encountered by most fresh produce. Generally, *L. monocytogenes* grew on both fresh-cut tomato and carrot regardless of the storage period and temperature (Figure 3.1 and 3.2). Along these storage temperatures, the pathogen significantly ($p<0.05$) grew from ~ 3.40 to 5.36, 6.13 and 6.28 log CFU/ml in fresh-cut tomato and from ~4.50 to 6.36, 6.33, and 6.38 log CFU/ml in fresh-cut carrot respectively. With phage treatment, the population of *L. monocytogenes* on fresh-cut tomato stored at 4, 10 and 25^{o}C was significantly ($p<0.05$) reduced on acid adapted (AA) by 0.87, 0.96 and 0.54 log CFU/ml, chlorine adapted (CA) by 0.89, 0.98, and 0.52 log CFU/ml, heat adapted (HA) by 0.88, 0.98 and 0.52 log CFU/ml) and non-adapted (NA) by 0.55, 0.68 and 0.50 log CFU/ml respectively. On fresh-cut carrot stored in same temperature conditions, phage treatment significantly ($p<0.05$) reduced the bacterial population on AA by 3.23, 3.18 and 3.19 log CFU/ml, CA by 3.20, 3.22 and 3.31 log CFU/ml, HA by 3.23, 3.25 and 3.55 log CFU/ml, and NA by 3.13, 3.04 and 3.09 log CFU/ml respectively. Furthermore, phage treatment on stress adapted *L. monocytogenes* resulted in slightly higher log reductions than the non-adapted pathogen. In fresh-cut tomato, phage treatment averagely reduced bacterial population by 0.57, 0.81, 0.86 and 0.95 log CFU/ml in non-adapted, acid-adapted, chlorine-adapted and heat-adapted *L. monocytogenes* respectively. Similarly, in fresh-cut carrot, phage treatment resulted in average bacterial reduction of 2.26, 2.41, 2.49 and 2.54 log CFU/ml in non-adapted, acid-adapted, chlorine-adapted and heat-adapted *L. monocytogenes* respectively.

Similarly, acid-adapted *L. monocytogenes* inoculated into milk and carrot juice showed increased resistance of 2 log CFU/ml over its non-adapted form against lauric arginate antimicrobial treatment (Shen, Soni and Nannapaneni 2015).

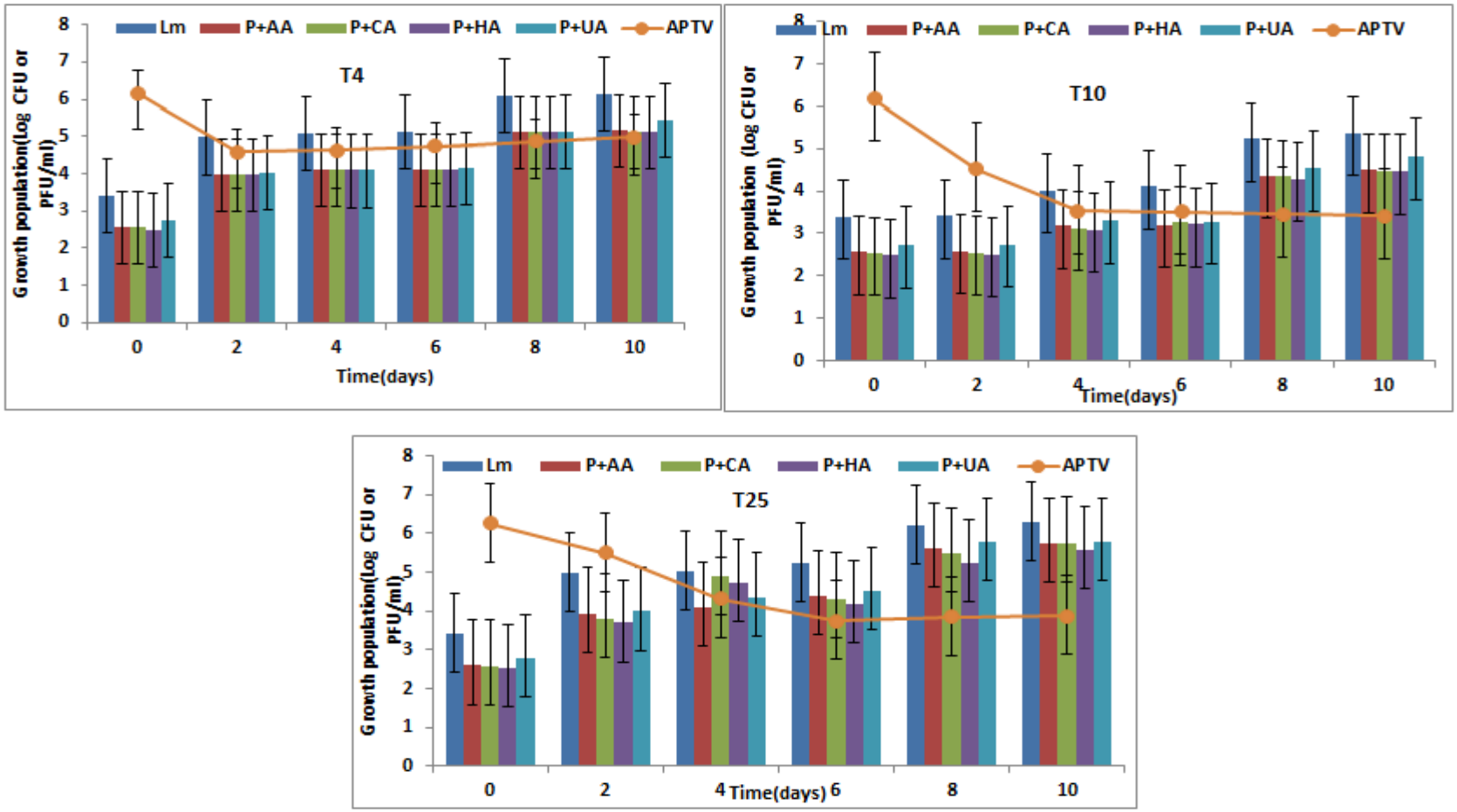


Figure 3.1 Effect of phage treatment on growth population of stress-adapted *L. monocytogenes* bacteria in fresh cut tomato stored at 4 (T4), 10 (T10) and 25°C (T25) for 10 days. Lm (*L. monocytogenes*), P+AA (phage & acid-adapted bacteria), P+CA (phage & chlorine-adapted bacteria), P+HA (phage& heat-adapted bacteria), P+UA (phage & Non-adapted bacteria), APTV (average phage titre value).

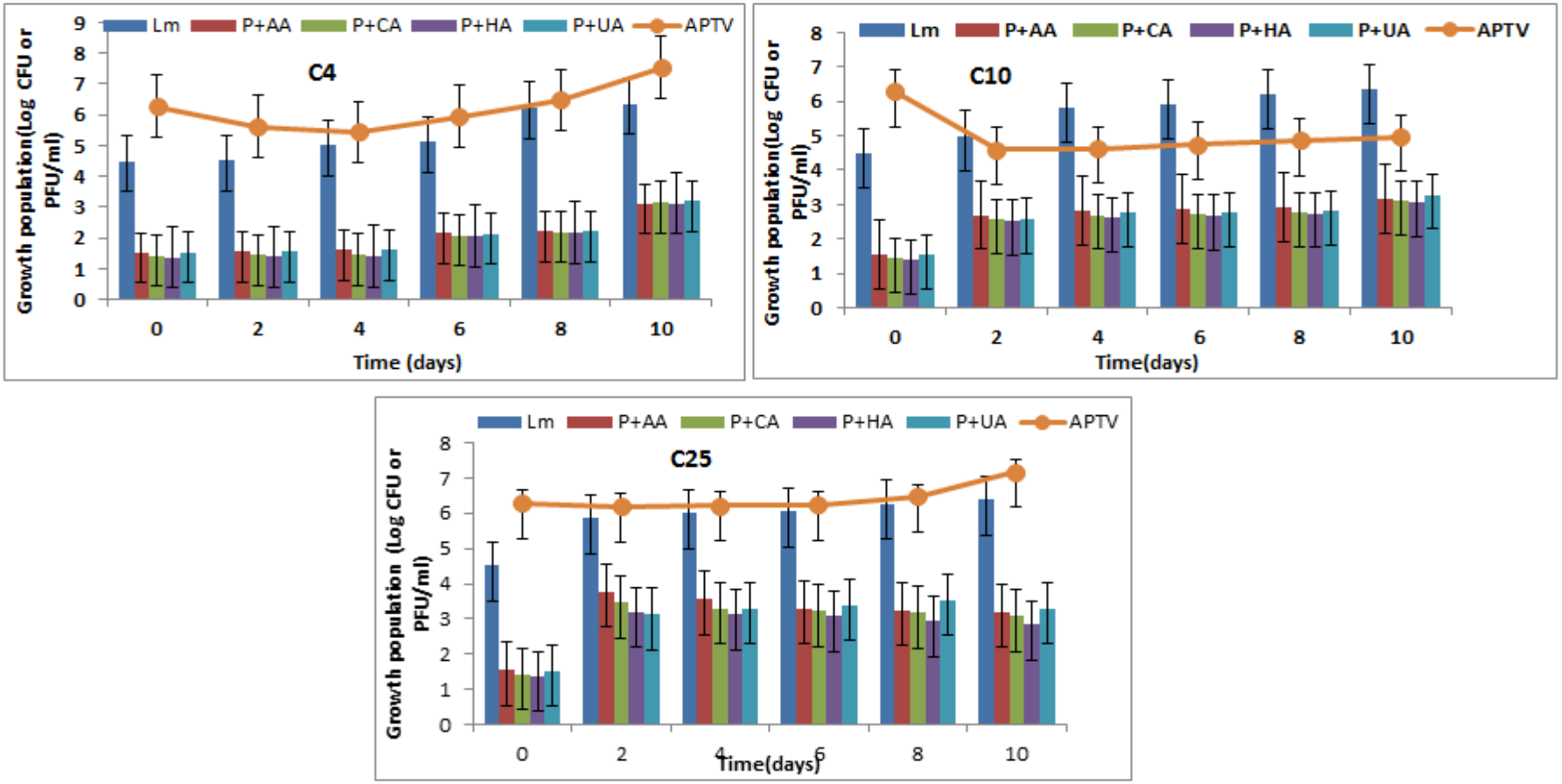


Figure 3.2 Effect of phage treatment on growth population of *L. monocytogenes* bacteria in fresh- cut carrot stored at 4, (C4) 10 (C10) and 25ºC (C25)

Lm (*L .monocytogenes*), P+AA (phage & acid-adapted bacteria), P+CA (phage & chlorine-adapted bacteria), P+HA (phage & heat-adapted bacteria), P+UA (phage & Non-adapted bacteria), APTV(average phage titre value),

3.3.2 Effect of phage treatment on morphological changes on stress adapted bacteria

Figure 3.3 shows the morphological changes of phage treatment in adapted and non-adapted *L. monocytogenes* cells. Generally, background of SEM pictures showed lysed cells due to phage treatment. Non-adapted bacteria cell showed normal and typical morphology with a smooth rod-like shape. Effect of stress conditions revealed alterations in the shape of the cells. Chlorine-stress treatment resulted in clustered cells of coccoid shape, while treatment with heat and acid stress showed rough-edged and stretched configuration.

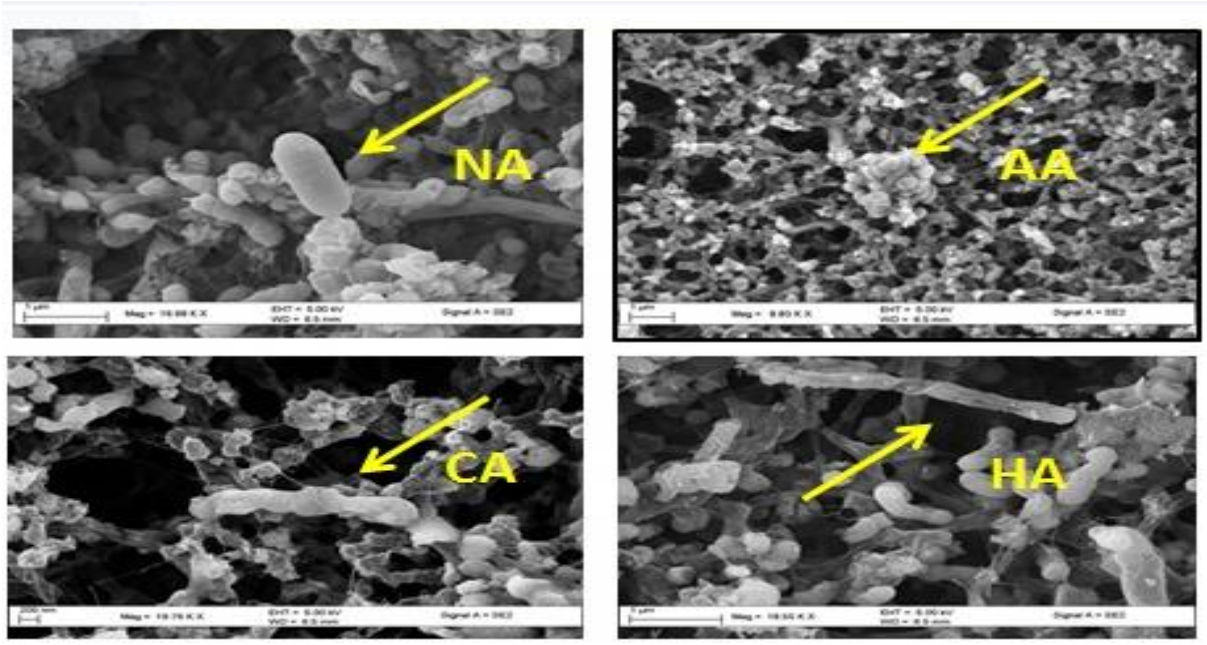


Figure 3.3 Scanning electron micrographs of phage treatment on *L. monocytogenes* ATCC 7644.

NA: Non-adapted, AA: acid-adapted, CA: chlorine adapted, HA: heat-adapted.

3.3.3 Effect of phage treatment on physicochemical parameters of fresh produce

Effect of phage treatment on physicochemical attributes of pH, total soluble solids (TSS ^{o}brix), total titratable acidity (TTA g citric acid/L) and chroma values (CIE *L* a* b*)* on both fresh-cut produce stored for 10 days is represented in Tables 3.1 and 3.2 below. Generally, with increasing storage temperature, slight variations ($p \leq 0.05$) on the physicochemical parameters of the phage- treated adapted sample compared to the control were observed. The pH values increased ($p<0.05$) slightly with increase in storage temperature. The pH values at 4, 10 and 25^{o}C on untreated fresh-cut tomato increased ($p<0.05$) from 4.32- 4.57, 4.33-4.58 and 4.43-4.94 respectively, while similar trend of 5.89-5.98, 5.91-6.01, and 5.96-6.07 was observed on

untreated fresh-cut carrot. Adapted and non-adapted samples also showed similar observation. On fresh-cut tomato, the slight ($p<0.05$) increase in pH values of phage-treated adapted samples on AA were 4.43-4.71, 4.55-4.72 and 4.55-5.00, CA: 4.40-4.54, 4.41-4.57 and 4.51-5.00, HA: 4.49-4.62, 4.51-4.65 and 4.61-5.02, and NA: 4.53-4.64, 4.53-4.67 and 4.63-5.64 respectively. Similarly, on fresh-cut carrot stored at same temperature condition, the pH values of phage treated adapted samples on AA were 5.90-6.00, 5.92-6.03 and 5.94-6.07, CA: 5.97-6.03, 5.99-6.07 and 6.02-6.10, HA: 5.99-6.06, 6.01-6.08 and 6.02-6.12, and NA: 5.93-5.99, 5.95-6.05 and 5.97-6.00.

The TSS showed slight ($p<0.05$) increase in value with increasing storage temperature. On fresh-cut tomato, TSS range of values along the storage temperatures were of 5.60-7.80, 5.80-7.33 and 5.97-7.90. While on fresh-cut carrot, TSS values were 8.61-8.68, 8.63-8-72, and 8.68-8.97 respectively. A similar trend was observed on the adapted samples. On fresh-cut tomato, the TSS values of phage-treated adapted samples were AA: 5.53-7.40, 5.77-7.20 and 6.20-7.97, CA: 5.40-7.80, 5.60-7.33 and 6.60-8.07, HA: 5.40-7.50, 5.60-7.00 and 6.60-8.43 and NA: 5.80-7.60, 6.00-7.10 and 7.03-8.63. On fresh-cut carrot, the TSS values of phage-treated adapted samples were AA: 8.51-8.59, 8.53-8.62 and 8.63-9.04, CA: 8.72-8.84, 8.74-8.86 and 8.82-9.11, HA: 8.91-9.01, 8.93-9.04 and 8.99-9.28 and NA: 8.62-8.69, 8.64-8.74 and 8.72-9.07.

TTA values of fresh-cut tomato stored at same conditions were 0.75-0.69, 0.72-0.63 and 0.67-0.53 while that of fresh-cut carrot were 0.05-0.04, 0.04 and 0.05-0.04. However, the TTA values slightly ($p<0.05$) decreased as the storage temperature increases. Phage treatment on the adapted samples resulted in slight variation. On fresh-cut tomato, the TTA values of phage-treated adapted samples were AA: 0.78-0.73, 0.74-0.63 and 0.67-0.47, CA: 0.75-0.69, 0.73-0.63 and 0.68-0.49, HA: 0.76-0.70, 0.73-0.61 and 0.67-0.49 and NA: 0.73-0.67, 0.71-0.61 and 0.68-0.47. On fresh-cut carrot, the TSS values of phage-treated adapted samples were AA: 0.06-0.05, 0.05 and 0.06-0.05, CA: 0.06, 0.05 and 0.06-0.05, HA: 0.07-0.06, 0.06 and 0.07-0.06 and NA: 0.06-0.05, 0.05 and 0.05-0.04.

The L^* a^* b^* chroma values showed some significant ($p<0.05$) variations along storage temperatures on both fresh cut produce. Noteworthy, the a^* values obtained from fresh-cut tomato which slightly ($p<0.05$) increased along the storage temperatures were 15.20-15.31, 16.33-16.69, 16.01-18.63 respectively, while that of fresh-cut carrot were 31.53-31.92, 28.99-30.24, 22.30-26.12 respectively. Adapted samples showed some variations. On fresh-cut tomato, slight ($p<0.05$) decrease on a^* values was observed on phage-treated adapted samples of AA: 24.17-18.97, 23.25-23.95 and 19.68-18.01, CA: 20.05-17.59, 20.87-19.37 and 22.89-21.77, HA: 23.10-15.40, 20.53-23.31 and 17.60-17.87 and NA: 15.89-18.54, 25.75-28.88 and

16.75-19.71. On fresh-cut carrot, similar ($p<0.05$) decrease on b^* values was observed on phage-treated adapted samples which are AA: 52.72-30.14, 45.71-33.44 and 38.13-31.78, CA: 49.53-32.40, 39.16-38.49 and 36.09-34.20, HA: 51.94-40.76, 29.99-36.83 and 19.34-38.17 and NA: 46.67-38.76, 44.39-40.89 and 20.13-34.49.

Table 3.1 Effect of phage treatment on physicochemical parameters of fresh-cut tomato stored at 4,10 and 25ºC (pH), total soluble solids (TSS, ^{O}Brix), total titratable acidity (TTA, g citric acid/L), Color (CIE L* a*b*)

Day	0						10					
Parameter	pH	TSS	TTA	*L**	*a**	*b**	pH	TSS	TTA	*L**	*a**	*b**
Tomato (4ºC)												
Control	$4.32^{a}\pm0.01$	$5.60^{ab}\pm0.20$	$0.75^{a}\pm0.01$	$44.28^{d}\pm0.26$	$15.20^{a}\pm0.04$	$18.92^{a}\pm0.04$	$4.57^{a}\pm0.03$	$7.80^{b}\pm0.20$	$0.69^{ab}\pm0.01$	$40.96^{c}\pm0.01$	$15.31^{a}\pm0.03$	$10.02^{a}\pm0.07$
P+AA	$4.43^{b}\pm0.02$	$5.53^{a}\pm0.15$	$0.78^{c}\pm0.01$	$34.69^{a}\pm0.03$	$24.17^{e}\pm0.05$	$19.27^{b}\pm0.06$	$4.71^{c}\pm0.01$	$7.40^{a}\pm0.20$	$0.73^{b}\pm0.04$	$39.36^{b}\pm0.11$	$18.97^{d}\pm0.06$	$23.90^{d}\pm0.06$
P+CA	$4.40^{b}\pm0.02$	$5.40^{a}\pm0.10$	$0.75^{b}\pm0.01$	$35.59^{b}\pm0.02$	$20.05^{c}\pm0.16$	$19.24^{b}\pm0.03$	$4.54^{a}\pm0.02$	$7.80^{b}\pm0.20$	$0.69^{a}\pm0.02$	$36.89^{a}\pm0.11$	$17.59^{b}\pm0.14$	$17.26^{b}\pm0.17$
P+HA	$4.49^{c}\pm0.02$	$5.40^{a}\pm0.10$	$0.76^{bc}\pm0.01$	$38.20^{c}\pm0.03$	$23.10^{d}\pm0.05$	$19.21^{b}\pm0.02$	$4.62^{b}\pm0.01$	$7.50^{ab}\pm0.10$	$0.70^{ab}\pm0.02$	$39.69^{b}\pm0.03$	$15.40^{a}\pm0.01$	$21.65^{c}\pm0.04$
P+UA	$4.53^{d}\pm0.02$	$5.80^{b}\pm0.10$	$0.73^{a}\pm0.01$	$58.64^{e}\pm0.01$	$15.89^{b}\pm0.05$	$24.42^{c}\pm0.03$	$4.64^{b}\pm0.03$	$7.60^{ab}\pm0.00$	$0.67^{a}\pm0.02$	$39.53^{b}\pm0.36$	$18.54^{c}\pm0.44$	$22.02^{c}\pm0.04$
Tomato(10ºC)												
Control	$4.33^{a}\pm0.01$	$5.80^{ab}\pm0.10$	$0.72^{ab}\pm0.01$	$45.22^{a}\pm0.05$	$16.33^{a}\pm0.05$	$19.63^{a}\pm0.03$	$4.58^{a}\pm0.04$	$7.33^{a}\pm0.25$	$0.63^{a}\pm0.03$	$46.58^{d}\pm0.03$	$16.69^{a}\pm0.04$	$12.09^{a}\pm0.08$
P+AA	$4.55^{c}\pm0.03$	$5.77^{a}\pm0.15$	$0.74^{c}\pm0.01$	$55.60^{c}\pm0.01$	$23.25^{d}\pm0.02$	$32.38^{e}\pm0.04$	$4.72^{c}\pm0.03$	$7.20^{a}\pm0.20$	$0.63^{a}\pm0.03$	$32.98^{b}\pm0.40$	$23.95^{c}\pm0.37$	$24.03^{c}\pm0.59$
P+CA	$4.41^{b}\pm0.02$	$5.60^{a}\pm0.10$	$0.73^{bc}\pm0.01$	$57.53^{d}\pm0.00$	$20.87^{c}\pm0.01$	$24.54^{c}\pm0.04$	$4.57^{a}\pm0.03$	$7.33^{a}\pm0.15$	$0.63^{a}\pm0.03$	$36.63^{c}\pm0.02$	$19.37^{b}\pm0.02$	$16.26^{b}\pm0.05$
P+HA	$4.51^{d}\pm0.02$	$5.60^{a}\pm0.10$	$0.73^{bc}\pm0.01$	$57.98^{e}\pm0.05$	$20.53^{b}\pm0.19$	$28.25^{d}\pm0.09$	$4.65^{b}\pm0.02$	$7.00^{a}\pm0.20$	$0.61^{a}\pm0.01$	$35.28^{c}\pm2.60$	$23.31^{c}\pm1.11$	$29.41^{d}\pm3.57$
P+UA	$4.53^{d}\pm0.01$	$6.00^{b}\pm0.10$	$0.71^{a}\pm0.01$	$49.49^{b}\pm0.04$	$25.75^{e}\pm0.09$	$22.37^{b}\pm0.30$	$4.67^{a}\pm0.02$	$7.10^{a}\pm0.20$	$0.61^{a}\pm0.03$	$27.73^{a}\pm0.33$	$28.88^{d}\pm0.31$	$24.36^{c}\pm0.58$
Tomato(25ºc)												
Control	$4.43^{a}\pm0.02$	$5.97^{a}\pm0.21$	$0.67^{a}\pm0.01$	$62.13^{e}\pm0.03$	$16.01^{a}\pm0.01$	$27.73^{b}\pm0.05$	$4.94^{a}\pm0.03$	$7.90^{a}\pm0.10$	$0.53^{a}\pm0.02$	$41.90^{b}\pm0.03$	$18.63^{a}\pm0.05$	$28.58^{c}\pm0.04$
P+AA	$4.55^{b}\pm0.03$	$6.20^{a}\pm0.10$	$0.67^{a}\pm0.02$	$53.53^{b}\pm0.29$	$19.68^{c}\pm0.42$	$27.33^{b}\pm0.44$	$5.00^{a}\pm0.04$	$7.97^{a}\pm0.12$	$0.47^{a}\pm0.01$	$46.51^{d}\pm0.32$	$18.01^{a}\pm0.50$	$26.43^{d}\pm0.50$
P+CA	$4.51^{b}\pm0.02$	$6.60^{b}\pm0.10$	$0.68^{a}\pm0.01$	$42.24^{a}\pm0.92$	$22.89^{d}\pm1.02$	$23.58^{a}\pm1.35$	$5.00^{a}\pm0.03$	$8.07^{a}\pm0.15$	$0.49^{a}\pm0.01$	$37.25^{a}\pm0.02$	$21.77^{c}\pm0.07$	$23.51^{a}\pm0.05$
P+HA	$4.61^{c}\pm0.02$	$6.60^{b}\pm0.10$	$0.67^{a}\pm0.01$	$59.85^{d}\pm0.04$	$17.60^{a}\pm0.04$	$24.68^{a}\pm0.02$	$5.02^{a}\pm0.02$	$8.43^{b}\pm0.15$	$0.49^{a}\pm0.05$	$42.95^{c}\pm1.02$	$17.87^{a}\pm0.82$	$25.62^{b}\pm1.52$
P+UA	$4.63^{c}\pm0.01$	$7.03^{c}\pm0.12$	$0.68^{a}\pm0.04$	$57.58^{c}\pm0.14$	$16.75^{ab}\pm0.31$	$23.51^{a}\pm0.22$	$5.64^{b}\pm0.55$	$8.63^{b}\pm0.25$	$0.47^{a}\pm0.05$	$42.09^{bc}\pm0.25$	$19.71^{b}\pm0.41$	$23.21^{a}\pm0.42$

Values are means ± standard deviations of three replicate experiments

Mean values in the same column with the same superscripts are not significantly different ($p \leq 0.05$)

Table 3.2 Effect of phage treatment on physicochemical parameters of fresh-cut carrot at 4, 10 and 25ºC
(pH), total soluble solids (TSS, ^{O}Brix), total titratable acidity (TTA, g citric acid/L), color (CIE L* a*b*) of fresh-cut carrot stored for 10 days at 4, 10 and C

	Day			0						10		
Parameter	pH	TSS	TTA	*L**	*a**	*b**	pH	TSS	TTA	*L**	*a**	*b**
Carrot(4ºC)												
Control	5.89^{a}±0.01	8.61^{b}±0.02	0.05^{a}±0.00	50.19^{c}±0.02	31.53^{a}±0.02	41.28^{a}±0.03	5.98^{a}±0.01	8.68^{b}±0.01	0.04^{a}±0.00	51.88^{d}±0.04	31.92^{d}±0.04	42.17^{d}±0.01
P+AA	5.90^{a}±0.01	8.51^{a}±0.02	0.06^{b}±0.00	51.53^{e}±0.03	36.39^{e}±0.02	52.72^{e}±0.13	6.00^{ab}±0.05	8.59^{a}±0.02	0.05^{b}±0.00	48.55^{c}±0.01	19.70^{a}±0.04	30.14^{a}±0.06
P+CA	5.97^{c}±0.01	8.72^{c}±0.02	0.06^{c}±0.00	49.63^{b}±0.04	35.73^{d}±0.09	49.53^{c}±0.19	6.03^{bc}±0.01	8.84^{c}±0.02	0.06^{c}±0.00	54.89^{e}±0.04	22.46^{b}±0.32	32.40^{b}±0.56
P+HA	5.99^{c}±0.01	8.91^{d}±0.02	0.07^{c}±0.00	50.98^{d}±0.01	34.80^{c}±0.04	51.94^{d}±0.13	6.06^{d}±0.01	9.01^{d}±0.01	0.06^{c}±0.00	47.19^{b}±0.06	26.88^{c}±0.13	40.76^{d}±0.06
P+UA	5.93^{b}±0.02	8.62^{b}±0.01	0.06^{d}±0.00	48.60^{a}±0.17	31.89^{b}±0.24	46.67^{b}±0.63	5.99^{ab}±0.01	8.69^{b}±0.01	0.05^{c}±0.00	45.83^{a}±0.04	26.87^{c}±0.01	38.67^{c}±1.73
Carrot(10ºC)												
Control	5.91^{a}±0.01	8.63^{b}±0.02	0.04^{a}±0.00	50.63^{c}±0.05	28.99^{c}±0.04	39.19^{b}±0.05	6.01^{a}±0.03	8.72^{b}±0.02	0.04^{a}±0.00	55.41^{d}±0.05	30.24^{e}±0.03	36.87^{b}±0.05
P+AA	5.92^{a}±0.01	8.53^{a}±0.01	0.05^{b}±0.00	46.36^{a}±0.30	31.87^{e}±0.56	45.71^{c}±1.53	6.03^{ab}±0.04	8.62^{a}±0.02	0.05^{b}±0.00	56.73^{e}±0.12	19.25^{a}±0.24	33.44^{a}±0.14
P+CA	5.99^{c}±0.01	8.74^{c}±0.01	0.05^{c}±0.00	49.58^{b}±0.44	26.43^{b}±0.91	39.16^{b}±0.96	6.07^{bc}±0.01	8.86^{c}±0.03	0.05^{c}±0.00	51.93^{a}±0.44	24.03^{c}±0.60	38.49^{b}±0.24
P+HA	6.01^{c}±0.01	8.93^{d}±0.02	0.06^{d}±0.00	49.40^{b}±0.04	21.81^{a}±0.16	29.99^{a}±0.25	6.08^{d}±0.02	9.04^{d}±0.02	0.06^{d}±0.00	52.80^{b}±0.21	20.95^{b}±0.14	36.83^{b}±0.44
P+UA	5.95^{b}±0.02	8.64^{b}±0.01	0.05^{a}±0.00	52.61^{d}±0.07	30.61^{d}±0.18	44.39^{c}±0.57	6.05^{abc}±0.03	8.74^{b}±0.02	0.05^{c}±0.00	53.49^{c}±0.15	26.72^{d}±0.57	40.89^{d}±0.73
Carrot(25ºC)												
Control	5.96^{a}±0.04	8.68^{ab}±0.02	0.05^{a}±0.00	57.27^{e}±0.05	22.30^{c}±0.04	26.40^{c}±0.04	6.07^{a}±0.04	8.97^{a}±0.02	0.04^{a}±0.00	51.15^{d}±0.04	26.12^{a}±0.30	33.39^{b}±0.04
P+AA	5.94^{a}±0.01	8.63^{a}±0.06	0.06^{b}±0.00	50.37^{b}±0.01	28.16^{e}±0.01	38.13^{e}±0.04	6.07^{a}±0.01	9.04^{b}±0.02	0.05^{b}±0.00	48.14^{b}±0.05	20.30^{b}±0.05	31.78^{a}±0.01
P+CA	6.02^{b}±0.01	8.82^{c}±0.01	0.06^{bc}±0.00	46.16^{a}±0.02	24.18^{d}±0.02	36.09^{d}±0.03	6.10^{a}±0.03	9.11^{c}±0.03	0.05^{b}±0.00	46.52^{a}±0.05	28.17^{e}±0.06	34.20^{c}±0.04
P+HA	6.02^{b}±0.01	8.99^{d}±0.01	0.07^{c}±0.00	53.39^{d}±0.04	14.15^{a}±0.02	19.34^{a}±0.05	6.12^{a}±0.03	9.28^{d}±0.03	0.06^{c}±0.00	49.23^{c}±0.04	19.20^{a}±0.02	38.17^{d}±0.02
P+UA	5.97^{a}±0.02	8.72^{b}±0.02	0.05^{b}±0.00	53.17^{c}±0.06	18.58^{b}±0.06	20.13^{b}±0.11	6.10^{a}±0.04	9.07^{b}±0.04	0.04^{a}±0.00	53.11^{e}±0.02	22.11^{c}±0.08	34.49^{e}±0.06

Mean values in the same column with the same superscripts are not significantly different ($p \leq 0.05$)
Values are means± standard deviations of three replicate experments

3.4 Discussion

In the present study, variation in phage efficacy on the fresh produce could be linked to differences in their pH values. The pH of tomato (4.3±0.1) used in this study was much lower than that of carrot (6.0±0.1). Similar results on fresh-cut melon (pH: 5.77-5.92), pear (pH: 4.61-4.91) and apple (pH: 3.70-3.76) where phage efficacy reduced with increasing acid condition (pH) of food matrix have been previously reported (Leverentz *et al.* 2003; Oliveira *et al.* 2014; Hong *et al.* 2015). For example, (Oliveira *et al.* 2014) reported log CFU/ml of 1.50, 1.00 and no reduction on the fresh-cut produce of melon, pear and apple used. In order to improve phage lysis, increase in phage concentration or dosage, and/or combination with other reported natural antimicrobial compounds such as bacteriocin, antagonistic bacteria and essential oils has been suggested by certain authors (Leverentz *et al.* 2003; Hong *et al.* 2015). This could significantly reduce bacterial level to the 5 log CFU/ml reduction requirement for preventing listeriosis outbreak on ready-to-eat foods such as fresh or minimally processed produce. Storage temperature did not significantly affect the efficacy of phage on both fresh produce. This development could be due to the ability of bacteriophage to adapt with its bacterial host as a process of co-evolution (Soni, Nannapaneni and Hagens 2010; Sulakvelidze 2013). Previous work with Listex P100 at the same concentration observed a similar trend in raw catfish fillets where storage temperature (4, 10 and 22^{o}C) did not significantly influence phage efficacy (Soni, Nannapaneni and Hagens 2010). Application of bacteriophage to fresh produce with low pH can be enhanced by increasing the phage concentration and combining with other natural antimicrobials. The level of reduction observed for stress-adapted and non- adapted *L. monocytogenes* population on each of the fresh produce was similar with slight variations showing no increased resistance. This result could signal non-resistance of the stress-adapted bacterial pathogen to phage treatment. *L. monocytogenes* genome principally contains a sigma (σ^{B}) factor that is induced during stress conditions where certain stress protein such as F_0F_1 ATPase, GroEL and DnaK are expressed. Even though phage-resistance by bacteria during stress condition has been reported, bacteriophage has been found to also mutate and evolve to still lyse resistant bacteria (Labrie, Samson and Moineau 2010; Golkar, Bagasra and Pace 2014; Strydom and Witthuhn 2015). As part of the resistance process, *L. monocytogenes* has been found to release a restriction endonuclease. However, increased synthesis of endolysin by the phage which are peptidoglycan hydrolases has been found to be particularly active against

gram-positive bacteria during adaptation process phage (Kim and Kathariou 2009; Zhang *et al.* 2012; Schmelcher *et al.* 2015).

Due to the highly conservative and target-specific nature of endolysin, development of endolysin-resistance by gram-positive compared to antibiotics has been rarely reported (Schmelcher and Loessner 2016). This development further enhances the use of bacteriophage as a potential solution to limitations facing the use of antibiotics and other natural compounds as antimicrobials against *L. monocytogenes* contamination in the fresh produce industry when adapted to stress or postharvest treatments and conditions.

On the morphological changes of the bacterial cells, investigation into the use of chlorine sanitizer on *L. monocytogenes* cells reported similarly a type of shape, while cell elongation was observed when subjected to low acid (pH 5.0) condition (Bereksi *et al.* 2002; Gao and Liu 2014). Similar elongation of *L. monocytogenes* cells was reported when treated to temperature condition of 30^{o}C for 24 hours (Ratti *et al.* 2010). Response of bacterial cells to stress conditions have been reported to alter their overall morphology (Hengge-Aronis 1999). The physicochemical parameters help to assess level of phage treatment on the quality, safety and acceptability of the food commodity after storage. Storage temperature remains an important factor in determining the keeping quality of fresh or minimally processed produce as most biochemical reactions that occur are temperature-dependent (Lana, Tijskens and Van Kooten 2005).

The slight pH increase observed on both samples with increase in storage temperature could be associated with decrease in acidity of the fresh-cut samples, as they have been identified to be rich sources of organic acid. Similar observation has been reported on mango, pawpaw and guava when treated with some antimicrobial compounds and stored at 4 to 28^{o}C (Mgaya-Kilima *et al.* 2014).

The pH contributes to acidity which is an important factor for fresh produce flavor and microbial safety. Minimal increase in TSS value of fresh produce with storage temperature has been reported to be desirable to enhance good quality (Bhardwaj and Pandey 2011). Increase in TSS values with storage temperature on both fresh produce as supported by Nath *et al.* (2012) could be associated with loss of moisture which increases the concentration and breakdown of carbohydrate to soluble sugars. TSS represents the ratio of sugar to acid of the fresh produce and it's an inverse representation of the fruit size. The decrease TTA values observed could be linked to loss of the organic acid content with increasing storage temperature which agrees with similar findings of Beckles (2012). The TTA helps to know breakdown of acid level to sugar which indicates degree of sweetness and maturity index.

The slight variation in a* values which defines the degree of redness can be linked to loss of lycopene biosynthesis and chlorophyll degradation. Similar work showing the inhibitory effect of antimicrobial treatment on shelf life and quality of stored tomatoes at different maturities and temperatures have been reported (Yanuriati, Savage and Rowe 1999). Redness of tomato is an important quality attribute for consumer appeal and acceptability. On fresh-cut carrot, the decrease in b* observed could be link to loss of carotenoid pigment which further supported similar observation by Sharma *et al.* (2012). This chroma value represents yellowness which correlates to the carotenoid coloration of carrot. It is an important precursor of vitamin A which helps to improve body immunity against certain degenerative diseases

Colour is an important factor of maturity and quality index, economic value and consumer perception in fresh produce. Meanwhile, Perera *et al.* (2015) reported that physicochemical qualities of some ready-to-eat foods including colour of apples and lettuce were not affected when treated with phage. The slight variations observed in all these physicochemical parameters showed that phage application significantly reduced *L. monocytogenes* on both fresh–cut produce. Furthermore, this application will not constitute quality and safety challenge, as phage has been identified to principally target bacteria and not the food substrates which often remain unaffected.

CHAPTER FOUR

4.0 Combination of Listex P100 Bacteriophage with sucrose monolaurate to inactivate *Listeria monocytogenes* ATCC 7644 growth on fresh cut produce

Abstract

The aim of this study is to investigate the combination of bacteriophage and sucrose monolaurate inactivate *Listeria monocytogenes* on fresh-cut produce (tomato and carrot). Fresh produce inoculated with *L. monocytogenes* (10^8 Log CFU/ml) was subjected to antimicrobial treatment of bacteriophage (10^8 PFU/ml) and sucrose monolaurate (SML) at 100, 250 and 400 ppm. Chlorine (sodium hypochlorite at 200 ppm) was used as control. Treated samples were stored at 4, 10 and 25^{o}C for 6 days and physicochemical qualities (pH, soluble solids, titratable acidity and CIE L* a* b*) and microscopic structures were further evaluated.

After storage at 4, 10 and 25^{o}C, phage treatment ($p \leq 0.05$) alone reduced bacterial population by 0.48, 0.53 and 0.49 log CFU/ml on fresh-cut tomato, and 2.88, 2.85 and 2.02 log CFU/ml on fresh-cut carrot respectively. Phage combination with SML at 100 and 250 ppm had no additive effect in reducing *L. monocytogenes* population, but its combination with SML at 400 ppm resulted in higher reductions of 1.25, 1.39 and 1.00 log CFU/ml in fresh cut tomato and 3.05, 3.00 and 3.0 in fresh-cut carrot at the same trend of storage temperatures respectively. Physicochemical qualities were substantially retained. Treated cells showed morphological changes of smooth lysed cells to wrinkled and coarse shapes.

Combination of bacteriophage with sucrose monolaurate can be used in the produce industry for the reduction of *L. monocytogenes* and other pathogens.

4.1 Introduction.

Food borne listeriosis is a disease caused by *Listeria monocytogenes* contamination in foods (Farber and Peterkin 1991). It is a gram positive, bacterium broadly distributed in the environment and can be isolated from natural sources such as soil, water and animal dungs (Oliveira *et al.* 2014). Notably, a good number of food varieties such as dairy, meat, sea and fresh produce have been identified as medium of contamination (Colburn *et al.* 1990; Bell and Kyriakides 2012; Strawn *et al.* 2013). A recent multistate outbreak of listeriosis in frozen vegetables involving 8 cases of hospitalization and 2 deaths has been reported. The largest multistate endemic invasion of listeriosis in the USA was linked to cantaloupe contamination, where 147 illnesses, 33 deaths and 1 miscarriage was reported (CDC 2011; CDC 2016b).

The use of inactivating compounds such as chlorine sanitizer at 50 -200 ppm have been used to reduce microbial loads of any adhering pathogens in fresh produce industry (Joshi *et al.* 2013). However, its continuous application has been associated with toxicity concerns both to the food microbiota and processor. Also, the development of halogen-based carcinogenic by-products have been identified, leading to the clamor for natural antimicrobials (Gopal *et al.* 2010; Meireles *et al.* 2015).

Bacteriophages or phages are bacteria-killing viruses. They operate majorly through a lytic process which leaves micro biota of the food substrate unaffected. They have been considered a veritable biocontrol tool in foods generally (Sillankorva, Oliveira and Azeredo 2012). Furthermore, in order to eliminate possible resistance by this pathogen on the phage treatment previously reported, an additive antimicrobial with a surface-active ability such as sucrose monolaurate was used as hurdle (Hyman and Abedon 2010; Labrie, Samson and Moineau 2010).

Sucrose monolaurate (SML) is a sugar fatty acid ester extensively used as emulsifier both in food and pharmaceutical industries (Gumel *et al.* 2011). It is a food grade bio-surfactant generally recognised as safe (GRAS). This antimicrobial property has been widely explored with divergent views. Some authors reported its inhibitory nature against gram negative bacteria (Ferrer *et al.* 2005; Nobmann *et al.* 2009; Xiao *et al.* 2011), while others reported for gram positive bacteria (Devulapalle *et al.* 2004; Piao *et al.* 2006).

SML exhibit both bacteriostatic and bactericidal attributes depending on type of sugar, nature of fatty acid, and degree of esterification (Chen, Nummer and Walsh 2014; Zhao *et al.* 2015). SML at 250 or 10,000 ppm was reported to significantly inactivate *E. coli* O157: H7 on spinach when combined with sodium hypochlorite at 200 ppm (Xiao *et al.* 2011). However, SML at

400 ppm has been reported to be lethal on *L. monocytogenes* and *Staphylococcus aureus* when combined with some natural antimicrobials (Monk, Beuchat and Hathcox 1996).

Hence, with limited information available on this hurdle, it is envisaged that combining the strength of sugar ester and bacteriophage will further help to significantly reduce *L. monocytogenes* in fresh produce, such as tomato and carrot, which are extensively consumed alone or as an ingredient in many ready-to-eat food recipes.

4.2 Materials and Methods

4.2.1 Fresh produce

Fresh tomato and carrot was obtained from a local produce store in Durban, South Africa and were used in the present study. Before the start of the experiment, produce was rinsed with distilled water, surface sanitized with 70% ethanol (Lichro chemicals) and dried at ambient temperature prior to cutting into 10 mm thickness.

4.2.2 Bacteria preparation

The serotype 1/2c of the bacterial pathogen was used in the present study (Merck, South Africa). Experimental culture obtained from stock culture stored at -80^{o}C in glycerol was streaked on *Listeria* oxford medium (Sigma-Aldrich Inc. USA) with *Listeria* supplement (Sigma-Aldrich) and incubated for 24 h at 37^{o}C. Colonies of bacteria populations were transferred into 50 ml broth base (FB, Sigma-Aldrich Switzerland) containing Fraser selective supplement (F18038, Sigma-Aldrich Switzerland). Final concentration of the cultures was prepared to give 8 $\log_{10}$ CFU/ml using McFarland standard solutions before use

4.2.3 Antimicrobials

Listex P100 bacteriophage obtained from Micreos (Wageningen, Netherlands) was used in the present study. Phage population of about 10^{11} plagues forming units (PFU/ml) contained in a buffered saline at 4^{o}C was prepared for the present study. Sucrose monolaurate (99.4% purity) was obtained from Sigma-Aldrich (South Africa) while chlorine (Sodium hypochlorite) obtained from (Merck chemicals) South Africa.

4.2.4 Fresh-cut produce preparation and inoculation

This was done my modifying the method of Xiao *et al.* (2011). Inhibition of *L. monocytogenes* on the fresh-cut samples was evaluated. Each of the fresh produce used were thinly cut into an average of 10 mm thickness and a 6 mm diameter wedge was made into the centre of each produce to contain the inoculum. They were all placed in perforated sterile plastic bags (Tuffy Brands Pty. Gulfstream Avenue, Airport City, South Africa) to prevent modified atmosphere creation. Inoculation was done by pipetting 1 ml of bacteria inoculum at 10^8 CFU/ml into the wedges for a retention time of 30 min for attachment. Fresh-cut samples treated with no antimicrobial served as control, while treated samples were inoculated with phage alone and in combination with SML at 100, 250 and 400 ppm. Samples were stored at three different temperatures (4, 10 and 25^{o}C) for 6 days.

4.2.5 Microbiological enumeration

Enumeration of bacterial population was performed at intervals of 2 days for 6 days in all the storage temperatures (Oliveira *et al.* 2014). Fresh-cut produce from plastic bags were homogenized in a stomacher blender (Stomacher laboratory blender London, UK) for 120 s mixed with 10 ml of sterile peptone water buffer (PWB, Biolab). Small portion of mixtures obtained were diluted in series with saline peptone water (SP; 8.5 g L^{-1} Nacl and 1 g L^{-1} peptone) and 1 ml spread plated on listeria media. Incubation of inoculated plates were evaluated at 37^{o}C for 48 h values recorded as log CFU/ml.

4.2.6 Effect of treatment on bacteria morphology

This procedure was carried out by modifying the method reported by (Zhao *et al.* 2015) Briefly, experimental culture of *L. monocytogenes* treated with phage and SML were incubated at 37^{o}C for 6h. Incubated samples were later centrifuged (Dragon Lab D 2012 Shunyi District, Beijing 101318 China.) at 10000 *x g* (4^{o}C, 10 mins). Pellets obtained were rinsed thrice with 0.1M phosphate buffer saline (PBS pH 7.2, Sigma Aldrich, South Africa). Fixing of the cells was done using 2.5% (vol/vol) glutaraldehyde at ambient temperature for one hour. Filtration of the cells was done using Nitrocellulose membrane filters (13 mm diameter; 0.22-µm GV pore size) subsequently followed by 1% tetroxide at room temperature for one hour. Further dehydration was carried out with serial grades of ethanol (30, 50, 70, 80, 90 and 96%). The critical point of the samples was dried using CO_2 in a critical point drying apparatus. Finally,

samples were gold-coated and examined in scanning electron microscope (SEM, Zeiss Ultra Pus. Germany).

4.2.7 Phage titration

Phage titration was performed following the method reported by Leverentz *et al.* (2003). Homogenized samples treated with phage were filtered via 0.45 um pore-size aperture membrane (Acrodisk; USA). Phage titers were obtained by soft agar overlay method using Brain-heart infusion agar (BHI Biolab). Resulting plaques were enumerated in log PFU/ml.

4.2.8 Quality parameters

Physicochemical qualities (pH, soluble solids, titratable acidity and color) of the treated and inoculated fresh produce were performed after the experiment (Oliveira *et al.* 2014). The pH was evaluated using a pH meter (Jenway 3510 pH meter. UK). Soluble solids (SS) measured in percentage brix with digital refractometer at 20^{o}C using digital refractometer (Atago Co. Ltd., Tokyo, Japan). Titratable acidity (TA) was obtained by diluting 10 ml of aliquot sample with 10 ml distilled water and titrated against 0.1 N NaOH up to 8.1. Results were expressed as g of citric acid /L for tomatoes and carrots. Color parameters (CIE L^* a^* b^*) were carried out using the hunter lab colorimeter (Color flex EZ, CFEZ 0840 Virginia USA). All determinations were carried out in triplicate.

4.2.9 Statistical analysis

Data obtained in triplicates were subjected to analysis of variance (SPSS) and means separated using Duncan multiple range tests ($p \leq 0.05$).

4.3 Results

4.3.1 Effect of phage and sucrose monolaurate treatment on fresh-cut produce

Generally, the bacteria grew on both samples in all the storage conditions (Figures 4.1 and 4.2). On fresh-cut tomato, the bacteria grew ($p < 0.05$) from 3.42 to 4.27, 4.64 and 7.17 log CFU/ml and grew from 4.42 to 4.97, 5.07 and 7.27 log CFU/ml on fresh-cut carrot.

After six days of storage, inoculated samples treated with phage alone at 4, 10 and 25^{o}C showed ($p \leq 0.05$) bacteria reductions of 0.48, 0.52, and 0.49 log CFU/ml in fresh-cut tomato and 2.88, 2.85 and 2.02 log CFU/ml in fresh-cut carrot respectively.

Phage treatment resulted in higher log reductions in carrot samples than in tomato.

The additive effect of phage with sucrose monolaurate at 100 and 250 ppm maintain <1fold log reductions in fresh-cut tomato and 2 fold log reductions in fresh-cut carrot. Addition of SML at 100 ppm reduced the bacterial population by 0.89, 0.55 and 0.49 log CFU/ml on fresh cut tomato and by 2.89, 2.85 and 2.04 log CFU/ml on fresh cut carrot respectively. Similarly, addition of SML at 250 ppm reduced the pathogen population by 0.93, 0.56 and 0.50 log CFU/ml and by 2.9, 2.88 and 2.07 log CFU/ml on fresh cut carrot respectively.

Meanwhile, an enhanced reduction was observed with phage treatment combined with SML at 400 ppm. *L. monocytogenes* population was reduced by 1.25, 1.39, and 1.00 log CFU/ml in fresh-cut tomato and 3.05, 3.00 and 3.01 log CFU/ml in fresh cut carrot respectively.

Chlorine control was observed to maintained 1- 2 fold log reduction along the storage temperature on both fresh produce.

.

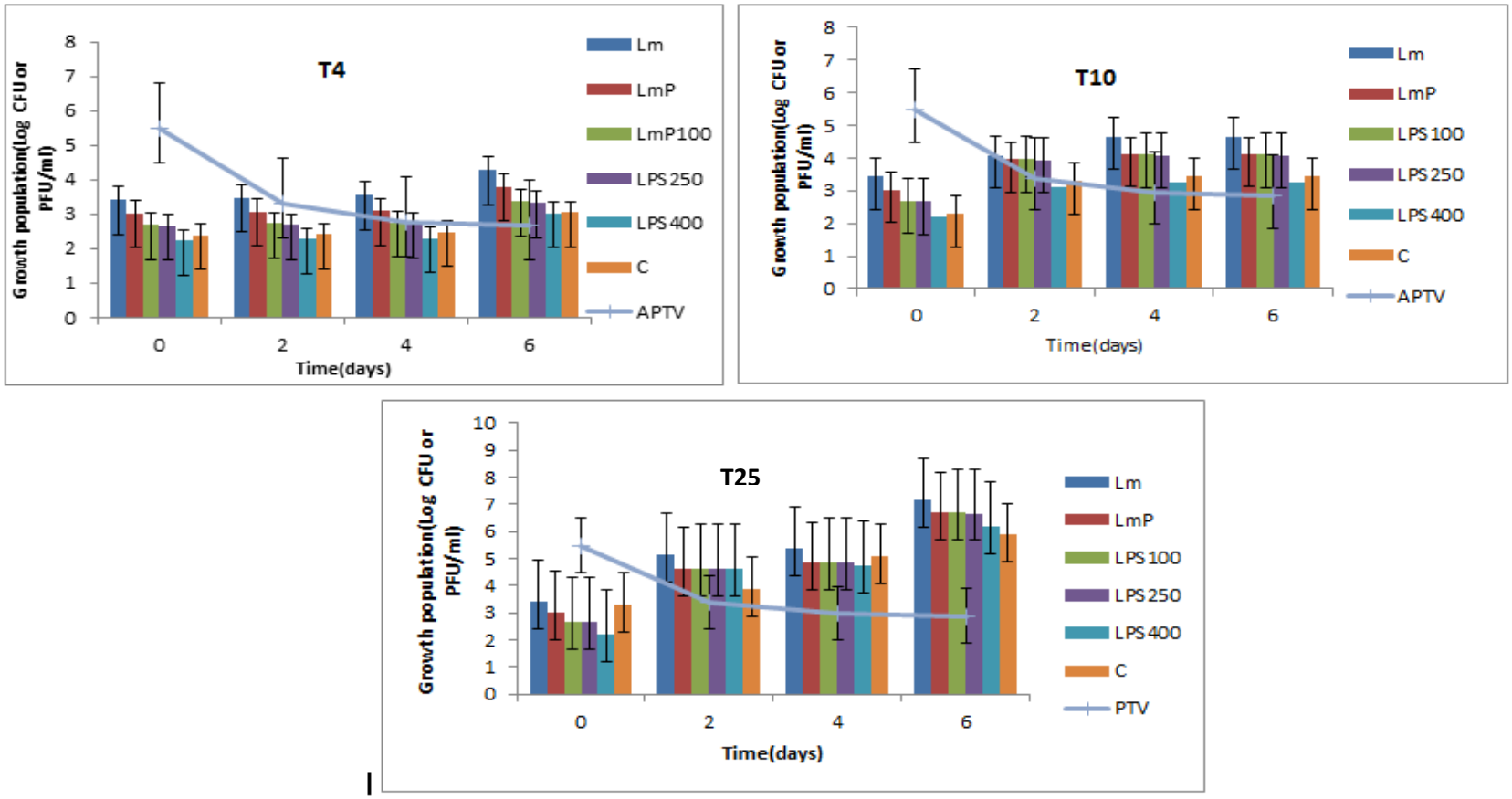


Figure 4.1 Effect of phage and sucrose monolaurate treatment on growth population of *L. monocytogenes* on fresh cut tomato stored at 4 (T4), 10 (T10), and 25ºC (T25) Lm: *L. monocytogenes*, LmP*: L. monocytogenes* plus phage, LPS100: *L. monocytogenes* plus sucrose monolaurate at 100 ppm, LPS250: *L. monocytogenes* plus sucrose monolaurate at 250 ppm, LPS400: *L. monocytogenes* plus sucrose monolaurate at 400 ppm. C (Chlorine control at 200 ppm) APTV: Average phage titre value.

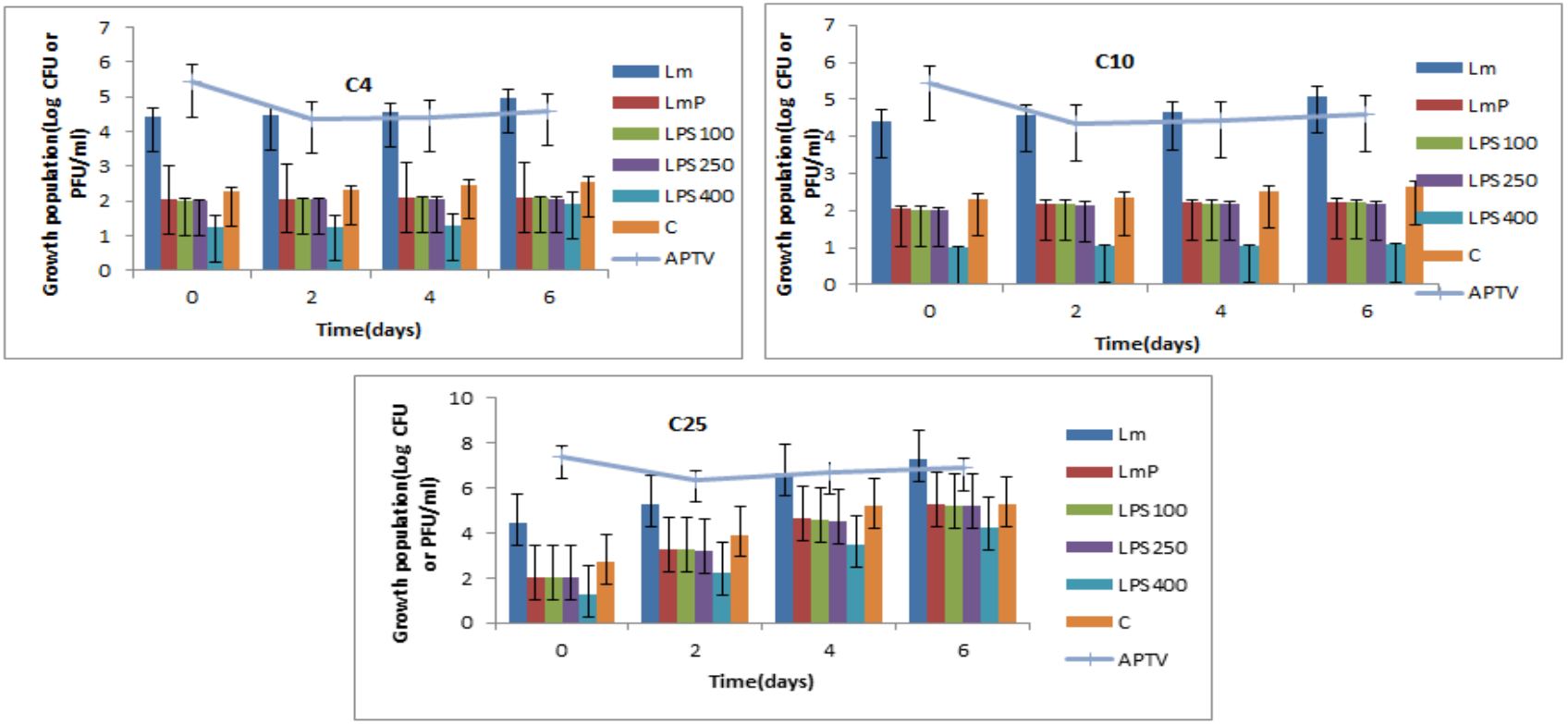


Figure 4.2 Effect of phage and sucrose monolaurate treatment on growth population of *L. monocytogenes* on fresh cut carrot stored at 4, 10, 25ºC

Lm: *L. monocytogenes*, LmP*: L. monocytogenes* plus phage, LPS100: *L. monocytogenes* plus sucrose monolaurate at 100 ppm, LPS250: *L. monocytogenes* plus sucrose monolaurate at 250 ppm, LPS400: *L. monocytogenes* plus sucrose monolaurate at 400 ppm. C (Chlorine control at 200 ppm) APTV: Average phage titre value.

4.3.2 Scanning electron microscopy analysis

The effect of bacteriophage and SML treatments on morphological changes of *L. monocytogenes* was evaluated using SEM analysis (Figure 4.3) Phage treatment alone showed lysed cells with some irregular shapes. Phage combined with SML at 100 ppm showed similar trend of shape. Noticeable impact on the morphology of the cells which showed wrinked, coarsed, and stretched shapes were observed in phage combined with SML at 250 and 400 ppm respectively.

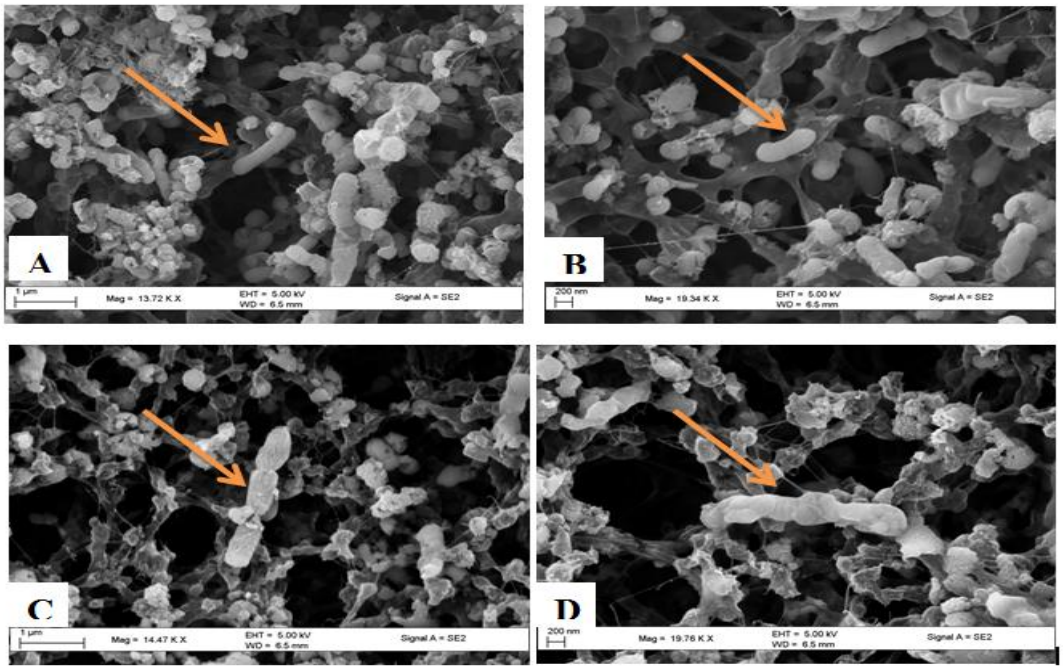


Figure 4.3 Scanning electron micrograph of *L. monocytogenes* treated with phage and SML

Phage alone (A), phage and SML at 100 ppm (B), phage and SML at 250 ppm (C), phage and SML at 400 ppm (D).

4.3.2 Effect of phage and SML treatment on physicochemical parameters of the fresh-cut produce

Antimicrobial combination of phage and sucrose monolaurate on physicochemical parameters of the fresh-cut produce are shown in Table 4.1 & 4.2 below. These parameters help to further elucidate the quality, safety and overall perception natural antimicrobial treatment on fresh-cut produce. In general, minimal variation ($p<0.05$) on these parameters with increase in storage temperatures were observed. After storage at 4, 10 and 25^{o}C for six days, the pH values of fresh-cut tomato slightly increased ($p<0.05$) from 4.33-4.61, 4.43-4.71 and 4.43-4.74 respectively while on fresh-cut carrot, pH values similarly increased from 5.89-5.95, 5.91-5.97 and 5.96-6.02 respectively. Treated samples showed similar trend with phage combination with SML at 400 ppm showing the lowest range of values. On fresh-cut tomato stored at 4,10 and 25^{o}C, phage-treatment alone resulted in slight ($p<0.05$) increase in pH from 4.45-4.58, 4.55-4.85 and 4.73-4.77. Addition of SML at 100 pm showed pH values of 4.41-4.55, 4.51-4.76 and 4.66-4.76. SML addition at 250 ppm showed pH values of 4.51-4.53, 4.61-4.78 and 4.65-4.77, while SML addition at 400 ppm showed pH values of 4.34-4.39, 4.43-4.72 and 4.35-4.71. On fresh cut carrot, phage treatment alone resulted in pH values of 5.90-5.97, 5.92-6.01, and 5.94-6.03. Addition of SML at 100 ppm resulted in pH values of 5.97-6.00, 5.99-6.03 and 6.02-6.07. SML addition at 250 ppm showed pH values of 5.99-6.03, 6.01-6.04 and 6.02-6.07. SML addition at 400 ppm showed pH values of 5.91-5.97, 5.93-5.98 and 5.96-6.02.

Increase in TSS values on both fresh produce was observed with increasing storage temperature. On fresh-cut tomato, TSS value slightly ($p<0.05$) increased from 4.20-4.80, 5.80-7.33 and 5.97-6.90 while on fresh cut carrot, TSS values increased 8.61-8.67, 8.63-8.69 and 8.68-8.82. Phage treatment on fresh-cut tomato stored in the three temperature conditions resulted in TSS value of 4.70-5.10, 5.77-7.20 and 6.20-7.13. Addition of SML at 100 ppm showed TSS values of 4.50-4.87, 5.60-7.33 and 6.60-7.47. SML addition at 250 ppm showed TSS values of 4.27-4.77, 5.60-7.00 and 6.60-7.80, while SML addition at 400 ppm showed TSS values of 4.30-4.80, 6.00-7.10 and 7.03-8.03. On fresh-cut carrot, phage treatment on the TSS values showed TSS values of 8.51-8.55, 8.53-8.59 and 8.63-8.88. Addition of SML at 100 ppm resulted in TSS values of 8.72-8.81, 8.74-8.83 and 8.82-8.99. SML 250 ppm showed TSS values of 8.91-8.98, 8.93-9.01 and 8.99-9.17, while SML at 400 ppm resulted in TSS values of 8.62-8.67, 8.64-8.68 and 8.72-8.94. The TTA values slightly ($p<0.05$) decreased on both fresh-cut samples along the storage conditions. On fresh-cut tomato, TTA values observed were 0.74-0.70, 0.72-0.63, 0.67-0.58 respectively, while on fresh-cut carrot, it showed 0.05-0.04 in all the

storage temperatures. Phage treatment of fresh-cut tomato resulted in TTA values of 0.76-0.74, 0.74-0.63 and 0.67-0.53. Addition of SML at 100 ppm resulted in TTA values of 0.74-0.72, 0.73-0.63 and 0.68-0.56. SML at 250 ppm resulted in 0.76-0.71, 0.73-0.61 and 0.67-0.55 while SML at 400 ppm resulted in 0.73-0.70, 0.71-0.61 and 0.68-0.56.

L a* b** chroma values showed some variations on both fresh produce samples. On fresh-cut tomato, the values of *a** which shows degree of redness were 18.11-18.12, 22.12-19.23 and 23.91-27.24. Treatment with phage resulted in values of 22.97-20.17, 23.13-23.81 and 24.19-19.39. Addition of SML at 100 ppm resulted in 18.56-18.47, 25.35-18.49 and 28.19-17.9. SML at 250 ppm showed 18.16-16.75, 23.14-26.49 and 19.17-18.52, while SML at 400 ppm showed 18.66-18.04, 22.13-19.26 and 23.96-27.30. The *b** value of fresh-cut carrot stored in 4, 10 and 25^{o}C were 45.90-35.34, 36.14-35.01 and 26.36-37.69. Phage treatment resulted to 44.20-33.04, 37.00-34.90 and 33.20. Addition of SML at 100 ppm showed values of 42.68-26.32, 35.13-36.05, and 26.31-34.43. SML at 250 ppm showed 43.74-31.16, 36.13-35.90 and 32.16-36.00, while SML at 400 ppm showed values of 45.32-35.33, 36.09-35.44 and 26.19-37.58.

Table 4.1Effect of phage and SML treatment on physicochemical parameters of fresh-cut tomato at 4, 10 and 25°C (pH), total soluble solids (TSS, °Brix), total titratable acidity (TTA, g citric acid/L), Color (CIE L* a*b*)

Day	0						6					
Parameter	pH	TSS	TTA	L*	a*	b*	pH	TSS	TTA	L*	a*	b*
Tomato(4°C)												
Control	$4.33^{a}\pm0.11$	$4.20^{a}\pm0.10$	$0.74^{ab}\pm0.01$	$57.01^{d}\pm0.02$	$18.11^{a}\pm0.02$	$18.74^{b}\pm0.02$	$4.61^{e}\pm0.10$	$4.80^{b}\pm0.10$	$0.70^{a}\pm0.01$	$58.00^{c}\pm0.05$	$18.12^{b}\pm0.03$	$20.70^{ab}\pm0.03$
LP	$4.45^{b}\pm0.03$	$4.70^{c}\pm0.10$	$0.76^{ab}\pm0.01$	$53.42^{c}\pm0.58$	$22.97^{c}\pm0.05$	$24.19^{e}\pm0.11$	$4.58^{d}\pm0.10$	$5.10^{b}\pm0.10$	$0.74^{b}\pm0.03$	$40.63^{a}\pm1.69$	$20.17^{c}\pm1.47$	$21.18^{ab}\pm2.32$
LPS_{100}	$4.41^{b}\pm0.02$	$4.50^{b}\pm0.10$	$0.74^{ab}\pm0.01$	$44.78^{a}\pm0.09$	$18.56^{ab}\pm0.01$	$23.59^{d}\pm0.02$	$4.55^{c}\pm0.01$	$4.87^{a}\pm0.06$	$0.72^{ab}\pm0.02$	$42.94^{b}\pm0.25$	$18.47^{b}\pm0.19$	$22.51^{b}\pm0.37$
LPS_{250}	$4.51^{c}\pm0.02$	$4.27^{a}\pm0.06$	$0.76^{b}\pm0.02$	$60.84^{e}\pm0.03$	$18.16^{a}\pm0.01$	$19.06^{c}\pm0.01$	$4.53^{b}\pm0.01$	$4.77^{a}\pm0.06$	$0.71^{ab}\pm0.01$	$41.78^{ab}\pm0.42$	$16.75^{a}\pm0.27$	$19.21^{a}\pm0.45$
LPS_{400}	$4.34^{a}\pm0.03$	$4.30^{a}\pm0.10$	$0.73^{a}\pm0.02$	$52.34^{b}\pm0.20$	$18.66^{b}\pm0.23$	$18.40^{a}\pm0.31$	$4.39^{a}\pm0.01$	$4.80^{b}\pm0.10$	$0.70^{a}\pm0.01$	$59.48^{d}\pm0.01$	$18.04^{b}\pm0.01$	$20.76^{ab}\pm0.04$
Tomato(10°C)												
Control	$4.43^{a}\pm0.02$	$5.80^{ab}\pm0.10$	$0.72^{ab}\pm0.01$	$56.20^{d}\pm0.01$	$22.12^{a}\pm0.01$	$24.16^{a}\pm1.99$	$4.71^{a}\pm0.03$	$7.33^{a}\pm0.25$	$0.63^{a}\pm0.03$	$47.91^{e}\pm0.03$	$19.23^{a}\pm0.04$	$26.89^{c}\pm0.02$
LP	$4.55^{b}\pm0.03$	$5.77^{a}\pm0.15$	$0.74^{c}\pm0.01$	$38.21^{b}\pm0.02$	$23.13^{a}\pm0.02$	$24.11^{a}\pm0.02$	$4.85^{d}\pm0.02$	$7.20^{a}\pm0.20$	$0.63^{a}\pm0.03$	$30.21^{a}\pm0.19$	$23.81^{c}\pm0.10$	$23.23^{a}\pm0.15$
LPS_{100}	$4.51^{b}\pm0.02$	$5.60^{a}\pm0.10$	$0.73^{bc}\pm0.01$	$35.34^{a}\pm0.01$	$25.35^{d}\pm0.02$	$23.13^{a}\pm0.02$	$4.76^{b}\pm0.01$	$7.33^{a}\pm0.15$	$0.63^{a}\pm0.03$	$36.38^{c}\pm0.13$	$18.49^{a}\pm0.07$	$25.18^{b}\pm0.17$
LPS_{250}	$4.61^{c}\pm0.02$	$5.60^{a}\pm0.10$	$0.73^{bc}\pm0.01$	$56.16^{e}\pm0.02$	$23.14^{b}\pm0.02$	$27.17^{b}\pm0.02$	$4.78^{c}\pm0.02$	$7.00^{a}\pm0.20$	$0.61^{a}\pm0.01$	$31.83^{b}\pm0.67$	$26.49^{d}\pm0.61$	$23.85^{a}\pm0.92$
LPS_{400}	$4.43^{a}\pm0.01$	$6.00^{b}\pm0.10$	$0.71^{a}\pm0.01$	$55.15^{c}\pm0.04$	$22.13^{c}\pm0.02$	$23.51^{a}\pm0.60$	$4.72^{a}\pm0.03$	$7.10^{a}\pm0.20$	$0.61^{a}\pm0.03$	$47.13^{d}\pm0.29$	$19.26^{b}\pm0.03$	$26.85^{c}\pm0.55$
Tomato(25°c)												
Control	$4.43^{b}\pm0.04$	$5.97^{a}\pm0.21$	$0.67^{a}\pm0.01$	$41.13^{b}\pm0.02$	$23.91^{b}\pm0.03$	$25.15^{b}\pm0.02$	$4.74^{ab}\pm0.03$	$6.90^{a}\pm0.10$	$0.58^{a}\pm0.02$	$34.12^{c}\pm0.03$	$27.24^{d}\pm0.04$	$24.08^{b}\pm0.06$
LP	$4.73^{e}\pm0.01$	$6.20^{a}\pm0.10$	$0.67^{a}\pm0.02$	$42.14^{d}\pm0.02$	$24.19^{c}\pm0.02$	$28.02^{d}\pm0.02$	$4.77^{c}\pm0.02$	$7.13^{b}\pm0.21$	$0.53^{a}\pm0.03$	$34.84^{c}\pm0.22$	$19.39^{c}\pm0.27$	$26.16^{d}\pm0.35$
LPS_{100}	$4.66^{d}\pm0.02$	$6.60^{b}\pm0.10$	$0.68^{a}\pm0.01$	$36.18^{a}\pm0.02$	$28.19^{d}\pm0.02$	$26.14^{c}\pm0.02$	$4.76^{b}\pm0.01$	$7.47^{c}\pm0.06$	$0.56^{a}\pm0.02$	$33.87^{b}\pm0.20$	$17.91^{a}\pm0.35$	$19.15^{a}\pm0.36$
LPS_{250}	$4.65^{c}\pm0.03$	$6.60^{b}\pm0.10$	$0.67^{a}\pm0.01$	$41.12^{b}\pm0.02$	$19.17^{a}\pm0.02$	$23.14^{a}\pm0.02$	$4.77^{c}\pm0.02$	$7.80^{d}\pm0.10$	$0.55^{a}\pm0.04$	$32.27^{a}\pm0.10$	$18.52^{b}\pm0.10$	$24.58^{bc}\pm0.14$
LPS_{400}	$4.35^{a}\pm0.03$	$7.03^{c}\pm0.12$	$0.68^{a}\pm0.04$	$41.21^{c}\pm0.02$	$23.96^{b}\pm0.04$	$25.51^{b}\pm0.58$	$4.71^{a}\pm0.04$	$8.03^{e}\pm0.12$	$0.56^{a}\pm0.05$	$35.02^{c}\pm0.96$	$27.30^{d}\pm0.07$	$25.09^{c}\pm0.82$

Values are means ± standard deviations of three replicate experiments

Mean values in the same column with the same superscripts are not significantly different ($p \leq 0.05$)

Table 4.2 Effect of phage and SML treatment on physicochemical parameters of fresh-cut carrot at 4, 10 and 25°C (pH), total soluble solids (TSS, °Brix), total titratable acidity (TTA, g citric acid/L), Color (CIE L* a*b*)

Day	0						6					
	pH	TSS	TTA	L*	a*	b*	pH	TSS	TTA	L*	a*	b*
Carrot(4°C)												
Control	$5.89^{a}\pm0.01$	$8.61^{b}\pm0.02$	$0.05^{a}\pm0.00$	$54.32^{d}\pm0.02$	$36.35^{e}\pm0.04$	$45.90^{d}\pm0.02$	$5.95^{a}\pm0.02$	$8.67^{b}\pm0.01$	$0.05^{a}\pm0.00$	$57.74^{c}\pm0.03$	$29.39^{d}\pm0.04$	$35.34^{d}\pm0.54$
LP	$5.90^{ab}\pm0.01$	$8.51^{a}\pm0.02$	$0.06^{b}\pm0.00$	$48.93^{c}\pm0.01$	$31.29^{c}\pm0.02$	$44.20^{bc}\pm0.05$	$5.97^{a}\pm0.06$	$8.55^{a}\pm0.01$	$0.06^{c}\pm0.00$	$54.99^{b}\pm0.03$	$24.29^{c}\pm0.70$	$33.04^{c}\pm1.19$
LPS_{100}	$5.97^{c}\pm0.01$	$8.72^{c}\pm0.02$	$0.07^{c}\pm0.00$	$48.09^{b}\pm0.03$	$30.10^{b}\pm0.13$	$42.68^{a}\pm0.40$	$6.00^{ab}\pm0.01$	$8.81^{c}\pm0.02$	$0.06^{c}\pm0.00$	$51.90^{a}\pm0.02$	$18.45^{a}\pm0.30$	$26.32^{a}\pm0.45$
LPS_{250}	$5.99^{c}\pm0.01$	$8.91^{d}\pm0.02$	$0.07^{d}\pm0.00$	$47.30^{a}\pm0.27$	$27.66^{a}\pm0.55$	$43.74^{ab}\pm1.51$	$6.03^{b}\pm0.01$	$8.98^{d}\pm0.01$	$0.07^{d}\pm0.00$	$53.53^{b}\pm1.87$	$21.33^{b}\pm1.10$	$31.16^{b}\pm0.02$
LPS_{400}	$5.91^{b}\pm0.01$	$8.62^{a}\pm0.01$	$0.06^{c}\pm0.00$	$54.79^{c}\pm0.04$	$36.35^{d}\pm0.67$	$45.32^{cd}\pm0.25$	$5.97^{a}\pm0.02$	$8.67^{b}\pm0.02$	$0.05^{b}\pm0.00$	$57.71^{c}\pm0.07$	$28.39^{d}\pm0.22$	$35.33^{d}\pm0.34$
Carrot(10°C)												
Control	$5.91^{a}\pm0.01$	$8.63^{b}\pm0.02$	$0.05^{a}\pm0.00$	$53.11^{a}\pm0.02$	$30.19^{c}\pm0.04$	$36.14^{b}\pm0.02$	$5.97^{a}\pm0.02$	$8.69^{b}\pm0.01$	$0.04^{a}\pm0.00$	$57.74^{c}\pm0.03$	$29.40^{c}\pm0.02$	$35.01^{a}\pm0.04$
LP	$5.92^{ab}\pm0.01$	$8.53^{a}\pm0.01$	$0.06^{b}\pm0.00$	$57.20^{e}\pm0.02$	$31.12^{e}\pm0.01$	$37.00^{c}\pm0.03$	$6.01^{bc}\pm0.04$	$8.59^{a}\pm0.02$	$0.06^{d}\pm0.00$	$60.61^{d}\pm0.14$	$27.04^{b}\pm0.57$	$34.90^{a}\pm0.46$
LPS_{100}	$5.99^{c}\pm0.01$	$8.74^{c}\pm0.01$	$0.06^{b}\pm0.00$	$54.00^{d}\pm0.03$	$28.16^{b}\pm0.02$	$35.13^{a}\pm0.02$	$6.03^{c}\pm0.01$	$8.83^{c}\pm0.02$	$0.06^{c}\pm0.00$	$56.77^{b}\pm0.06$	$27.42^{b}\pm0.17$	$36.05^{c}\pm0.19$
LPS_{250}	$6.01^{c}\pm0.01$	$8.93^{d}\pm0.02$	$0.07^{c}\pm0.00$	$53.21^{b}\pm0.02$	$28.03^{a}\pm0.02$	$36.13^{b}\pm0.04$	$6.04^{c}\pm0.02$	$9.01^{d}\pm0.01$	$0.06^{c}\pm0.00$	$52.13^{a}\pm0.01$	$21.00^{a}\pm0.02$	$35.90^{a}\pm0.02$
LPS_{400}	$5.93^{b}\pm0.02$	$8.64^{b}\pm0.01$	$0.06^{b}\pm0.00$	$53.42^{c}\pm0.04$	$30.52^{d}\pm0.04$	$36.09^{b}\pm0.58$	$5.98^{ab}\pm0.01$	$8.68^{b}\pm0.01$	$0.05^{b}\pm0.00$	$57.04^{b}\pm0.57$	$29.57^{c}\pm0.01$	35.44b±0.05
Carrot(25°C)												
Control	$5.96^{a}\pm0.04$	$8.68^{ab}\pm0.02$	$0.05^{a}\pm0.00$	$57.24^{d}\pm0.03$	$20.22^{c}\pm0.04$	$26.36^{b}\pm0.03$	$6.02^{a}\pm0.04$	$8.82^{a}\pm0.01$	$0.04^{a}\pm0.00$	$56.72^{d}\pm0.05$	$28.30^{d}\pm0.03$	$37.69^{c}\pm0.03$
LP	$5.94^{a}\pm0.01$	$8.63^{a}\pm0.06$	$0.06^{b}\pm0.00$	$55.12^{b}\pm0.03$	$23.15^{d}\pm0.02$	$33.20^{d}\pm0.02$	$6.03^{a}\pm0.01$	$8.88^{b}\pm0.01$	$0.05^{b}\pm0.00$	$50.76^{b}\pm0.18$	$24.42^{c}\pm0.45$	$34.31^{a}\pm0.41$
LPS_{100}	$6.02^{b}\pm0.01$	$8.82^{c}\pm0.01$	$0.07^{c}\pm0.00$	$54.15^{a}\pm0.02$	$16.31^{a}\pm0.03$	$26.31^{b}\pm0.03$	$6.07^{a}\pm0.02$	$8.99^{a}\pm0.02$	$0.05^{b}\pm0.00$	$49.42^{a}\pm0.04$	$22.20^{a}\pm0.08$	$34.43^{a}\pm0.10$
LPS_{250}	$6.02^{b}\pm0.01$	$8.99^{d}\pm0.01$	$0.07^{c}\pm0.00$	$58.08^{e}\pm0.03$	$25.05^{e}\pm0.03$	$32.16^{c}\pm0.02$	$6.07^{a}\pm0.01$	$9.17^{e}\pm0.01$	$0.06^{c}\pm0.00$	$51.24^{c}\pm0.02$	$23.30^{b}\pm0.04$	$36.00^{b}\pm0.03$
LPS_{400}	$5.96^{a}\pm0.03$	$8.72^{b}\pm0.02$	$0.06^{b}\pm0.00$	$57.15^{c}\pm0.02$	$20.02^{b}\pm0.04$	$26.19^{a}\pm0.05$	$6.02^{a}\pm0.02$	$8.94^{c}\pm0.02$	$0.05^{b}\pm0.00$	$58.80^{e}\pm0.00$	$28.01^{d}\pm0.06$	$37.58^{c}\pm0.17$

Values are means ± standard deviations of three replicate experiments

Mean values in the same column with the same superscripts are not significantly different ($p \leq 0.05$)

4.4 Discussion

Minimal processing such as slicing has been found to increase proliferation of microorganism in fresh produce due to release of nutrient from internal tissues (Muriel-Galet *et al.* 2013; Ramos *et al.* 2013). This development constitutes quality and economic loss in fresh produce. Hence, the practice of necessary quality management along the food chain will go a long way to address any possible safety challenge (Olaimat and Holley 2012). Differences in the lytic output on the fresh produce could be as a result of inherent factors such as ionic and pH condition of the two fresh-cut samples. From preliminary findings, tomato had a lower pH value (4.3±0.1) compared to carrot (6.0±0.1). Phage efficacy has been reported to decline with increased acid condition of food matrix (Leverentz *et al.* 2003; Guenther *et al.* 2009). This phenomenon is also in agreement with findings of other authors where pH values above 5 resulted in greater inactivation by phage treatment (Oliveira *et al.* 2014; Perera *et al.* 2015).

Effect of bacteriophage to inactivate inoculated fresh-cut produce (especially on solid matrix) with low pH can be enhanced by increasing the phage concentration (Hagens and Loessner 2010). Also, combination with other natural antimicrobials has been suggested by other authors (Oliveira *et al.* 2014; Hong *et al.* 2015; Perera *et al.* 2015). Hence, development of acid-tolerant phages will be a welcome improvement in phage biocontrol especially in fresh produce that are acidic in nature.

Sucrose monolaurate (SML) is a food grade surfactant, whose antimicrobial ability is influenced among other factors by its critical micelle concentration (CMC) reported to be around 184-210 ppm (Makino, Ogimoto and Koga 1983; Husband *et al.* 1998). Therefore, insignificant antibacterial effect of SML at 100 ppm on phage lysis could be linked to its low CMC where sufficient interfacial tension on the thick peptidoglycan cell wall could not be attained. Meanwhile, the inactive antibacterial effect of SML at 250 ppm combined with phage could be linked to certain unexamined factors such as time-kill assay which is opened to further investigation. Similar application of SML at 250 ppm in combination with sodium hypochlorite (200 ppm) to inactivate *Escherichia coli* O157:H7 on spinach stored at 4 and 20^{o}C has been reported (Xiao *et al.* 2011). Authors however found significant reductions of bacteria population than when the sample was treated with sodium hypochlorite alone regardless of treatment temperatures. This variation could be linked to differences in peptidoglycan cell wall of the two microorganisms. *E. coli* O157:H7 is a gram negative bacterium with thin cell walls compared to gram positive *L. monocytogenes* with

a thicker cell wall. Also, the variation can be further linked to the compatibility of SML with different antimicrobials. Improved inactivation at 400 ppm may be linked to better access for lysis via reduction of interfacial tension of the bacteria peptidoglycan cell wall. Similar findings by Monk, Beuchat and Hathcox (1996) reported the lethality of SML at 400 ppm which is above the CMC when combined with Ethylenediaminetetraacetic (EDTA) against the broth of this pathogen. Increasing the concentration of SML is envisaged to bring higher reductions of *L. monocytogenes* population on both fresh produce to an appreciable level within regulatory requirement of controlling listeriosis outbreak during fresh produce contamination. Although, report by Hoelzer *et al.* (2014) of chlorine wash on fresh produce showed 1-3 fold reduction. However, its comparative effectiveness has also been found to be partially determined by the nature (intrinsic and extrinsic factors) of the fresh produce (Parish *et al.* 2003; Hirneisen *et al.* 2010; Pfunter 2011). Practically speaking, the use of chlorine sanitizers in recent times had been criticized in the food safety industry going by development of health-based challenges it constitutes to workers and toxicity concerns in food quality. Hence, a clamor for more eco-friendly and natural antimicrobials (Nieuwenhuijsen, Toledano and Elliott 2000; Joshi *et al.* 2013).

The results from SEM analysis showed that the treatments had a damaging impact on the morphological configuration of the cells. Similar report of a sugar fatty ester on morphology of three different bacteria have been reported (Zhao *et al.* 2015).

Storage temperature represents an important factor in evaluating retention attribute of fresh or fresh-cut produce as this affects most biochemical activities (Lana, Tijskens and Van Kooten 2005). Slight decrease in pH with increase in storage temperature could be due to dissociation of hydrogen ion concentration of the organic acid. Similar work on the use of SML in combination with ethylenediaminetetraacetic acid (EDTA) against *L. monocytogenes* growth reported slight changes in the pH condition of the test medium (Monk, Beuchat and Hathcox 1996). The pH contributes to acidity which plays a vital role in flavour development and microbial safety of the fresh produce. Increase in TSS value along storage temperature could be linked to moisture loss and breakdown of complex sugars to simple ones. Previous work showing an increase in TSS value of a citrus fruit and fresh-cut pineapple when treated with a sucrose-based polymer and methyljasmonate antimicrobial compound has been reported (Martinez-Ferrer and Harper 2005; Tao *et al.* 2012). Decrease in TTA value could be associated with loss of organic acid in the fresh produce via dissociation of weak ions from the acid molecules. Similar work by Tao *et al.* (2012)

reported reduced TTA when treated with a sugar-based polymer. The TTA is a measure of sweetness and maturity index of the fresh produce. Variation in a* and b* with storage temperature could be linked to differences in biosynthesis of pigment compounds of lycopene and carotenoids in tomato and carrot respectively.

The use of similar surface-active compound with no deleterious visual quality on spinach has been reported (Zhang *et al.* 2016). Also, other authors reported that certain natural antimicrobials treatment on apple cubes and minimally processed mango showed no significant impact on their chromatic and physicochemical qualities (Barbosa *et al.* 2013; Siroli *et al.* 2014). The use of bacteriophage with food grade surfactant such as SML will further add to eco friendly strategies to control *L. monocytogenes* contamination in the fresh produce industry. Minimal variations observed on the physicochemical parameters of the treated sample showed that phage application when combined with sucrose monolaurate will significantly reduce *L. monocytogenes* and this combination will not cause substantial reduction in the quality of the fresh produce, because phage does not affect the microbiota. Also, SML have been amply used both in food and pharmeceutical industry.

CHAPTER FIVE

5.0 Predicting the effect of artificially inoculated *Listeria Monocytogenes* ATCC 7644 on fresh-cut produce after treatment with bacteriophage and a Bio-surfactant using artificial neural networks

Abstract**.**

Combination of bacteriophage and sucrose monolaurate (SML) against *Listeria monocytogenes* growth on fresh-cut produce and prediction of relationship among initial bacterial load, fresh-produce type, antimicrobial concentration and residual bacteria using Artificial Neural Networks (ANNs) was investigated. Inoculated samples (tomato and carrot) containing 10^8 log cfu mL^{-1} *L. monocytogenes*, treated with bacteriophage (10^8 pfu mL^{-1}), SML (100, 250 and 400 ppm) and chlorine control (200 ppm) were stored at 4, 10 and 25^{o}C for 6 days. Mathematical models were developed using a linear regression and sigmoid (hyperbolic and logistic) activation functions. Data sets (120) were trained using Back propagation ANN containing one hidden layer with four hidden neurons. Phage treatment on tomato and carrot showed ($p<0.05$) < 1 and 2 fold bacterial reductions respectively. Addition of SML at 100 and 250 ppm was ($p>0.05$) ineffective, but showed significantly ($p<0.05$) higher log reductions on both fresh produce at 400 ppm. Control treatment resulted in 1-2 log reductions on both fresh produce. Prediction with logistic activation function showed the highest positive correlation relationship between predicted and observed values with ~ 0.99 R^2-value and MSE of 0.0831. ANN offered better prediction of risk in phage biocontrol of pathogens in fresh produce.

5.1. Introduction

The demand for fresh or minimally-processed produce has notably increased in the last few decades (Abadias *et al.* 2008). Ready-to-eat (RTE) fruits and vegetables contribute to a healthy lifestyle in a more convenient way as they contain nutritious and health-stabilizing constituents (James, Ngarmsak and Rolle 2011). "Five servings per day" recommendation have been canvassed by some global organization as nutritional practice to reduce certain diseases such as cancer, diabetes, and some cardiovascular disorder (Allende *et al.* 2006; Warriner *et al.* 2009).

Meanwhile, contamination by pathogens among other factors in the food value chain, both at pre and post-harvest stage often compromise the eating, storage and economic value of this food commodity. Furthermore, usual post-harvest operations such as washing, cooling and peeling holds little or no antimicrobial effect against the proliferation of these pathogens (Tauxe 1997; Beuchat 2002). Prominent among these pathogens of safety challenge in fresh produce includes *Salmonella* Spp., *Escherichia coli*, and *Listeria monocytogenes* (Potter *et al.* 2012).

L. monocytogenes is, however, of particular importance because of its high mortality rate (20-30 %) relative to other pathogens, and its ability to survive wide-range of environmental and stress conditions raises more concerns (Farber and Peterkin 1991; Gandhi and Chikindas 2007). It is a gram positive, adaptable and recalcitrant bacterium which causes human listeriosis disease. Symptoms of this disease include septicemia, encephalitis, meningitis and premature abortion during pregnancy (Vázquez-Boland *et al.* 2001). It can be found across a wide range of locations in the environment including soil, effluent water and animal excreta (Strawn *et al.* 2013). Ready to-eat foods such as fresh produce have also been spotted as a major vehicle of microbial proliferation in several developed countries of the world (Scallan *et al.* 2011).

Recent multi-state outbreak of listeriosis in salad has been reported by Centre for Disease and Prevention involving 18 Hospitalization, and 1 death case (CDC 2016a). In an effort to address this development, the United State Food and Drug Administration (FDA) and the United States Department of Agriculture (USDA), Food Safety and Inspection Service (FSIS) enacted "a zero tolerance" policy against this opportunistic bacterium (Donnelly 2001). One such biocontrol agent endorsed to control this contamination especially in fresh produce is the use of Bacteriophage.

Bacteriophages or phages are regarded as a natural anti-microbial with an approved "GRAS" status (Sulakvelidze 2013). They naturally co-exist as commensals with specific bacteria in their natural

habitat over time, making them to be host-specific; they are environment-friendly with no deleterious impact on food microbiota (Guenther and Loessner 2011; Bueno *et al.* 2012). These inherent attributes make them an excellent tool for control of recalcitrant pathogen such as *L. monocytogenes*. A catalogue of phage application on various RTE and food commodities have been reported by Sillankorva, Oliveira and Azeredo (2012).

However, as effective as this biocidal agent may suffice, development of phage resistance mechanism by bacteria have been reported (Labrie, Samson and Moineau 2010). This development among other factors tends to limit its efficacy in a long range ecological niche. Therefore, the use of other natural antimicrobials has become a suggestive hurdle to reduce this draw back. Application of natural, surface-active antimicrobial compounds such as sucrose monolaurate (SML) with a bactericidal characteristic will be a welcome development in addressing this phage limitation (Nobmann *et al.* 2009; Zhao *et al.* 2015).

SML biocidal attribute is dependent on sugar type, nature and strength of fatty acid, and also degree of esterification (Chen, Nummer and Walsh 2014; Zhao *et al.* 2015). SML have been identified as strong inhibitor to *L. monocytogenes* and *Staphylococcus aureus* when combined with certain natural antimicrobials (Monk, Beuchat and Hathcox 1996). As a result, the synergy of phage and SML will offer a potential means of significantly inactivating *L. monocytogenes* on fresh produce as no investigation on this combination has been reported.

Investigation into the use of this synergistic combination in our laboratory against this pathogen showed reduction on growth population to some extent. Incidentally, results obtained from these studies have shown that the antimicrobials only reduce the growth of *L. monocytogenes* in the fresh produce and not total eradication. There is therefore a need to predict the actual risk involved after treatment of the inoculated fresh produce with these combined antimicrobials as a real time assessment check.

Predictive microbiology describes the influence of environmental factors on the growth and survival of microorganism via a mathematical model (Pérez-Rodríguez and Valero 2013; Huang 2014). This concept has been reviewed over time and its application in food safety has proven successful. The practical application of this package in risk assessment has led to the development of relevant quantitative models among pre-existing ones. The use of Artificial Neural Networks (ANN) have been found to offer better approach of modelling as it helps to deal with uncertainty and inexactness associated with microbial growth (Jeyamkondan, Jayas and Holley 2001). The

use of this technique has been reported on some fresh produce after using sanitizers (Keeratipibul, Phewpan and Lursinsap 2011; Ozturk *et al.* 2012).

Due to increased attraction and relevance of phage biocontrol against pathogens of health importance in the fresh produce industry, the need for prediction as a risk assessment tool becomes more imminent. Therefore, this study seeks to predict the risk assessment of controlling *L. monocytogenes* ATCC 7644 growth on the fresh produce when treated with bacteriophage and sucrose monolaurate using artificial neural networks tool.

5.2 Materials and Methods

5.2.1 Fresh produce preparation

Randomly picked, matured, ripe and organically grown tomato (*Lycopersicon esculentum*) and carrot (*Daucus carota* subsp. *sativus*) of approximately 1 kg was obtained from local grocery supermarket (Woolworth supermarket, Durban. South Africa). Each of the fresh produce surfaces were cleaned under running tap water disinfected with 70% ethanol and allowed to dry at room temperature before transferred into a clean plastic bowl

5.2.2 Bacteria and preparation of inoculum

L. monocytogenes ATCC 7644 serovar 1/2c (Human isolate) was used in this study (Merck, South Africa). Following a modified method of Singh, Mnyandu and Ijabadeniyi (2014), working culture was obtained from frozen stock culture kept in glycerol (-80^{o}C) by thawing in water bath (WB 1024, Foss tecator technology, Hoganas. Sweden) at 25^{o}C for 3 mins and streaked on *Listeria* oxford media [LOM 75805, Sigma-Aldrich Inc. St Louis, MO 63103 USA] containing Oxford *Listeria* selective supplement (Fluka 75806 Sigma-Aldrich Inc. Buch, Switzerland) for 24 h at 37 ± 1^{o}C. Bacteria colonies were transferred into 50 mL fraser broth base (F6672 FB, Sigma-Aldrich Buch, Switzerland) using Fluka Fraser selective supplement (F18038 FSS, Sigma-Aldrich Buch, Switzerland). A final bacterial concentration of approximately 8 $\log_{10}$ cfu mL^{-1} was obtained using McFarland standard solutions before inoculation.

5.2.3 Antimicrobials

Antimicrobial compounds used in the present study includes; bacteriophage (Listex P100, Micreos food safety. Wageningen, Netherlands) stored at 4^{o}C in a buffered saline containing ~ 10^{11} plaque forming units (pfu mL^{-1}), sucrose monolaurate (SML 84110, Sigma Aldrich Pty Ltd, Aston Manor.

1630 South Africa) and chlorine-sodium hypochlorite (105614 EMPLURA, Merck Pty Ltd, Gauteng. South Africa).

5.2.4 Preparation of fresh-cut produce and sample inoculation

This was done by modifying the method of Xiao *et al.* (2011). Samples were cut thinly into average of 10 mm thickness and a 6 mm diameter wedge made into centre of each produce using a manual fruit corer (FCS 0020 Prestige Pty Ltd, Somerset West 7130, South Africa) to contain the inoculum. 100 µL of bacterial inoculum containing 10^8 cfu mL^{-1} were spot-inoculated (Chen and Zhu 2011) into the wedges for a contact time of 30 min to attach under aeration at ambient temperature in a bio-safety cabinet (BSC-1500IIB2-X, Labotech, Midrand 1685. South Africa). The inoculated fresh-cut samples (25 g) were all placed into sterile bags (Tufflock 170 x 150 mm. Tuffy Brands Pty. Cape Town, South Africa) with perforations to avoid modified atmosphere creation prior to inoculation.

5.2.5 Antimicrobial treatment procedure

This was carried out by following a modified method reported by Oliveira *et al.* (2014). Bacteriophage concentration was diluted from ~ 10^{11} pfu mL^{-1} to ~ 10^8 pfu mL^{-1} using laboratory sterile water (pH 6.5-7.0), from which 10 ppm were inoculated into the wedges. Simultaneously, 20 mL each of SML at 100, 250 and 400 ppm prepared using sterile water (pH 6.5-7.0) at room temperature was later applied and allowed for 10 mins (Ono, Miyake and Yamashita 2005). Chlorine dip (sodium hypochlorite at 200 ppm at 25^{o}C) for 3 mins contact time was carried out as control. Each of the treated samples was allowed to stand for 30 mins in the sterile bags under bio-safety cabinet condition before storage at 4, 10 and 25^{o}C for 6 days in a controlled chamber (LTIM 10 Lab design Engr Pty, Maraisburg. South Africa).

5.2.6 Microbiological analysis

Bacterial colony count was performed every 48 hours for 6 days in all the storage temperatures (Oliveira *et al.* 2014). Samples from the sterile bags were mixed with 10 mL of sterile peptone water buffer (PWB HG00C134.500. Biolab Merck, Modderfontein. South Africa) and the mixture was homogenised in a Stomacher laboratory blender (Model No BA 6021; Seward Lab. London SE 19UG UK) for 120 s. Aliquots from the mixtures were serially diluted in saline peptone water

(SP 1405, Conda Lab., Madrid, Spain), and 1 mL was spread-plated on a sterile petri plates containing Oxford *Listeria* agar. Inoculated plates were incubated at 37^{o}C for 48 h in order to obtain bacterial population as log cfu mL^{-1}.

5.2.7 Phage titration and pH

Phage titration was carried out by the method described by Leverentz *et al.* (2003). Briefly, aliquots from phage treated samples were homogenised and filtered through 0.45-um-pore size membrane (Acrodisk; Pall Gelman, Ann Arbor, Mich). Phage titer was then determined using the soft agar overlay method using Brain heart infusion agar (BHI Biolab). Resulting plaques were counted as expressed as log pfu mL^{-1}. The pH was determined using pH meter (Jenway 3510 pH meter. UK).

5.2.8 Statistical analysis

All experiments were replicated three times in each of the treatments. Data obtained were subjected to analysis of variance (SPSS Version 24) and means separated using Duncan multiple range test ($p \leq 0.05$).

5.2.9 Artificial Neural Networks (ANNs) Modelling

ANN was applied to provide a non-linear relationship between the input (temperature, pH, initial bacterial load, antimicrobial concentration, storage period, type of fresh produce, and antimicrobial) and the output variables (residual level of *L. monocytogenes* after control). Over time, statistical regression models have been applied as a well-established method for data analysis and prediction such as (such as ANOVA) and ranking the importance of independent variables. The outcome of residual bacterial level in the present study was modelled using ANNs, trained and simulated using a commercial software package (Matlab R2012a. The Mathworks, Inc., Natick, Massachusetts) and non-linear multiple polynomial regression (MPR) model in WEKA (Hall *et al.* 2009).

Supervised back propagation (BP-ANN) that is widely used to learn and approximate complex non-linear function was used (Govindaraju and Rao 2000). Also, its ease of learning makes it suitable for numerous applications in food safety (Keeratipibul, Phewpan and Lursinsap 2011; Meng, Zhang and Adhikari 2012; Funes *et al.* 2015). Artificial neural network (ANN) is a

mathematical model that simulates the structure and function of the biological nervous system. One of the most commonly used artificial neural networks is the multilayer perceptron (MLP) composed of orderly interconnected neurons arranged in layers (Figure 5.1). This comprises the input layer, one or more hidden layers, and an output layer which represents the building block of all ANN-MLP systems (Delashmit and Manry 2005).

It consists of n synapses associated with the inputs $(x_1, x_2 \ldots\ldots x_n)$ and each input has an associated weight (w). A signal at input i is multiplied by the weight wi, the weighted inputs are added together, and a linear combination of the weighted inputs is obtained. A bias (w_0), which is not associated with any input, is added to the linear combination and a weighted sum X is obtained as $X = w_0 + w_1 x_1 + w_2 x_2 \ldots\ldots + w_n x_n$. Subsequently, a nonlinear activation function f is applied to the weighted sum and this produces an output y shown in $y = f(X)$.

At the input layer, the values of the input variables were used to determine the residual level of the bacterial population, and approximate errors encountered during the supervised learning process. The hidden layer helps to transform the inputs into what the output layer can use, as one hidden layer has been found to approximate any continuous and multivariate function, within any degree of accuracy on compact subsets of real number (R^n) in a close interval of [-∞, ∞] (Cybenko 1989; Guliyev and Ismailov 2016). Furthermore, the flexibility and ability of an artificial neuron to approximate functions to be learned depend on its activation function. At the input layer, a linear activation function was used. This function was deemed fit for this layer since the input neurons only need to transmit the input dataset directly to the next layer with no transformation (Funahashi 1989). The hidden layer transforms these inputs dataset into non-linear form using activation function. Sigmoid function was employed at the hidden layer, because this function is continuous and differentiable, which makes learning the weights of a neural faster than others (Rahman *et al.* 2012; Guliyev and Ismailov 2016). The sigmoid activation functions are *S*-shaped functions, and the ones that are mostly used are the logistic and hyperbolic tangent as represented in (1) and (2), respectively.

$$f(x) = \frac{1}{1 + e^{-x}}, \qquad (1)$$

$$f(x) = \frac{1 - e^{-x}}{1 + e^{-x}}, \qquad (2)$$

Likewise, at the output layer choice of activation function depends on the problem being solved. Considering the size of dataset used in the present study and the rate at which the size of datasets continues to increase exponentially in food safety domain, the speed of processing is of utmost significance. Hence, scaled conjugate gradient back propagation (SCG-BP) with a reportedly high speed of convergence was used to train the designed MLP-ANN model at the output layer (Gopalakrishnan 2010; Dogra, Hasan and Dogra 2013). The appropriate number of neurons in the hidden layer of MLP-ANN varies based on the nature of the problem being solved and most of the time determined via experimentations (Delashmit and Manry, 2005). In summary, the present study used a BP-ANN topology which consists of seven inputs (Storage temperature, pH, initial load, concentration of antimicrobial, storage period, types of fresh produce, and type of antimicrobial), one hidden layer with four neurons and one output layer which represent residual level of the bacteria. The experimental procedure used in the present study followed similarly work reported by Keeratipibul, Phewpan and Lursinsap (2011).

5.2.10 Normalization and Encoding of Dataset

This was carried out on an Intel Core i5-3210MCPU@2.50GHz speed with 6.00GB RAM and 64-bit Windows 7 operating system. A total number of 120 input datasets were obtained from laboratory for model prediction of two different fresh produce- tomato and carrot. Data sets for training and testing were compared at ratio 70% training, 15% validation and 15% testing sub-dataset in the model design as previously reported by some authors (Prechelt 1994; Powers 2012; Powers and Atyabi 2012). Trained data sets were used to fix the learning rate (LR) to 0.001; and the maximum amount of epoch iterations was fixed at 1000 (Figure 5.3). However, due to variability in the data set, normalization (scaling) of data within a uniform range was essential to prevent larger numbers from overriding smaller ones, and to prevent premature saturation of hidden nodes, which impedes the learning process. Depending on the task objectives, for example; in neural networks, min-max normalization was recommended for activation functions. In this study, normalization of the input values was done using a min-max normalization equation by Saranya and Manikandan (2013).

$$X_i = \frac{(x_i - x_{\min})}{(x_{\max} - x_{\min})} * (b - a) + a \qquad (3)$$

Where x_i represents the data range of seven input values $1 \leq i \leq 7$, a and b represents the normalized range, x_{min} and x_{max} represents minimum and maximum values of the activated sigmoid functions in (1) respectively. Chosen values depend on the task objectives. In the present study, dataset were normalised between -0.9 to 0.9 for hyperbolic tangent function (2), and 0.1 to 0.9 for the sigmoid function in (1) as recommended by Basheer and Hajmeer (2000), and to avoid saturation of the sigmoid function leading to slow or no learning (Masters and Schwartz 1994; Hassoun 1995). The mean square error and linear regression error (R^2) which serves as validation indices for the ANN training in this work are:

$$MSE = \frac{1}{n}\sum (x_d - x_t)^2 \tag{4}$$

$$R^2 = 1 - \left[\frac{\sum (x_d^{'} - x_t)^2}{\sum (x_d^{'} - x^{'})^2}\right] \tag{5}$$

Where x_t is the observed value of the testing pattern t, and x is the average of the observed values. Since the type of vegetable, storage days and type of antimicrobial are considered as nominal data they cannot be used as the inputs for the neural network. These parameters were encoded to facilitate easy recognition during training (Table 5.1).

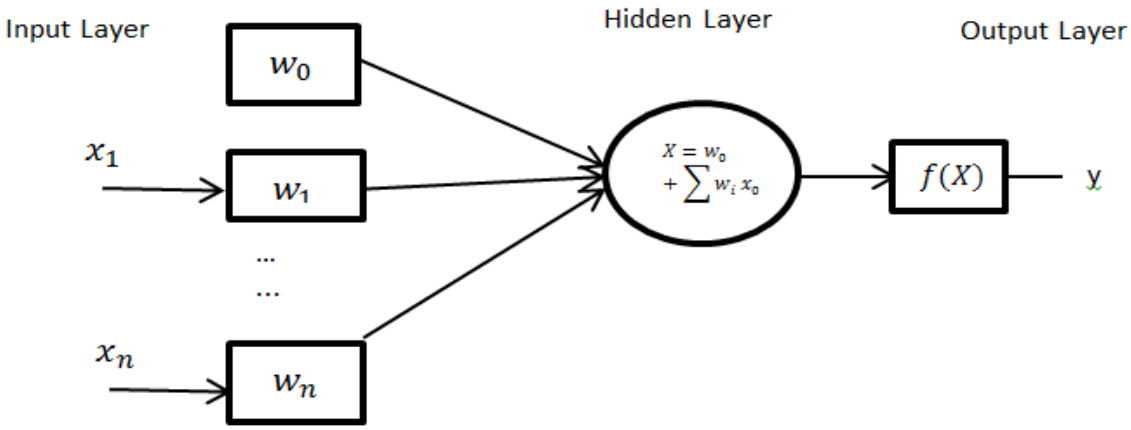


Figure 5.1 The structure of artificial neurons with the functional elements (Mehrotra, Mohan and Ranka 1997).

From the figure above, an artificial neuron is composed of n synapses connected to inputs (x_1, $x_2 \dots\dots x_n$) which are connected to weight (w). A signal at input i multiplied by the weight wi are summed up together to form a linear combination. A bias (wi), unconnected with any input, is added to this combination to obtain a weighted sum X as multiplied by the weight wi. An additional bias (wo), not linked to any input, is also combined to the linear combination resulting in a weighted sum as $X = W_0 + W_1X_1 + W_2X_2 \dots\dots + W_nX_n$ A nonlinear activation function f is eventually applied to the weighted sum to generate an output y shown in $y = f(x)$.

Multi-layer perceptron (MLP) neural networks are typically trained with back propagation (BP) algorithm. BP is an application of the gradient method or other numerical optimization methods to feed-forward ANN so as to minimize the network errors. This algorithm identifies minimum error function in weight space via method of gradient descent. The combination of weights which minimizes the error function is regarded as a solution of the learning problem. It is the most recognized method for performing supervised learning in ANN research (Rehman and Nawi 2011). The present study leverages on the above ANN-BP topology to train group of considered numeric factor obtained from the laboratory that are related to the residual level of micro-organism on fresh fruits. The multilayer feed-forward ANN architecture also consists of three layers. At the input layer, the values of the input variables were used to determine the residual level of the bacterial population, and approximate errors encountered during the supervised learning process. In the ANN-BP topology the choice of the number of hidden layers is crucial to this learning process.

It has been reported that a neural network architecture with one hidden layer can approximate any continuous function (Cybenko 1989). Hence, a hidden layer for the MLP was used in this study. Also, the ease of ANN to approximate functions to be learned is dependent on its activation function. Choice of activation function for the learning process is very vital for performance of the networks in different layers of MLP. Some of the frequently used activation functions for neural network applications are hyperbolic, linear and sigmoid functions.

At the input layer, linear activation function that uses the input neurons to transmit the input dataset directly to the next layer with no transformation was used while sigmoid activation function was used at the hidden layer. The sigmoid activation functions are S-shaped functions, where the popularly used types are the logistic and hyperbolic tangent as represented in (1) and (2), respectively.

$$f(x) = 1/1+e^{-x} \dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots(1)$$

$$f(x) = 1\text{-}e^{-x}/1+e^{-x} \quad \text{.....(2)}$$

Performance of the above-mentioned functions takes place at the hidden layer. The choice of activation function for the output layer neurons is a function of the problem being solved. Scaled conjugate gradient in the Back Propagation (SCG-BP) to train the designed MLP-ANN was used in the present study so as to leverage on its well acclaimed speed of convergence (Gopalakrishnan 2010; Dogra, Hasan and Dogra 2013). In summary, the present study used a BP-ANN topology which consists of seven inputs (Storage temperature, pH, initial load, concentration of antimicrobial, storage period, types of fresh produce, and type of antimicrobial), one hidden layer with three neurons and one output layer which represents the residual level of the bacteria. The experimental procedure used in this work followed that of Keeratipibul, Phewpan and Lursinsap (2011).

Table 5.1 Encoding parameters for ANNs

Type of fresh produce	ANN Encoding
Tomato	1 0
Carrot	0 1
Type of antimicrobial	**ANN Encoding**
Chlorine	00001
Phage	00010
Phage + SML100	00100
Phage + SML 250	01000
Phage + SML 400	10000
Storage days	**ANN Encoding**
0	000
2	010
4	100
6	110

5.3 Results

5.3.1 Effect of antimicrobial treatment on *L. monocytogenes* growth on the fresh-cut produce

Generally, the pathogen grew on both fresh-cut produce regardless of the storage condition (Table 5.2). However, phage treatment significantly ($p<0.05$) reduced *L. monocytogenes* bacteria population on both fresh produce. On fresh-cut tomato, phage treatments alone reduced bacterial population by 0.48-0.53 log cfu mL^{-1} and by 2.02-2.88 log cfu mL^{-1} on fresh-cut carrot. Meanwhile, the additive effect of phage with SML at 100 and 250 ppm maintained <1 fold log reduction in fresh-cut tomato and 2 fold log reductions in fresh-cut carrot. Chlorine control was observed to maintain 1-2 fold log reduction along the storage temperature on both fresh produce. Although, report by Hoelzer *et al.* (2014) of chlorine wash on fresh produce showed 1-3 fold reduction.

Table 5.2 Log reduction and residual population of *Listeria monocytogenes* on fresh-cut tomato and carrot stored at 4, 10 and 25°C after using phage and in combination with sucrose monolaurate (SML).

	Microorganism (Initial load; log_{10} 8 cfu/mL)		Tomato		Carrot	
	Antimicrobial	Concentration (ppm)	Residual (log_{10} cfu/mL)	Log reduction (log_{10} cfu/mL)	Residual (log_{10} cfu/mL)	Log reduction (log_{10} cfu/mL)
Temperature (4°C)	Control	200	6.78^{a}±0.05	1.22^{c}±0.06	5.58^{c}±0.13	2.42^{a}±0.16
	Phage	10	7.52^{c} ±0.26	0.48^{a}±0.05	5.12^{b} ±0.03	2.88^{b}±0.24
	Phage+ SML	100	7.10^{b}±0.25	0.90^{b}±0.06	5.11^{b}±0.03	2.89^{b}±0.24
	Phage+ SML	250	7.06^{b} ±0.30	0.94^{b}±0.07	5.10^{b} ±0.03	2.90^{b}±0.24
	Phage+ SML	400	6.75^{a} ±0.07	1.25^{c}±0.05	4.35^{a} ±0.03	3.65^{c}±0.22
Temperature (10°C)	Control	200	6.76^{b}±0.11	1.24^{c}±0.04	5.56^{c}±0.15	2.44^{a}±0.14
	Phage	10	7.47^{c} ±0.34	0.53^{a}±0.03	5.15^{b} ±0.04	2.85^{b}±0.22
	Phage + SML	100	7.45^{c}±0.30	0.55^{b}±0.03	5.14^{b}±0.03	2.86^{b}±0.23
	Phage + SML	250	7.44^{c} ±0.29	0.56^{b}±0.03	5.12^{b} ±0.03	2.88^{b}±0.23
	Phage + SML	400	6.61^{a} ±0.24	1.39^{c}±0.05	4.00^{a} ±0.03	4.00^{c}±0.23
Temperature (25°C)	Control	200	6.71^{a}±0.14	1.29^{c}±0.15	6.48^{c}±1.45	1.52^{a}±0.41
	Phage	10	7.51^{b} ±0.20	0.49^{a}±0.15	5.98^{b} ±1.17	2.02^{b}±0.02
	Phage + SML	100	7.51^{b}±0.21	0.49^{b}±0.17	5.95^{b}±1.18	2.05^{b}±0.01
	Phage +SML	250	7.51^{b} ±0.12	0.49^{b}±0.16	5.92^{b} ±1.20	2.08^{b}±0.05
	Phage + SML	400	6.41^{a} ±0.21	1.59^{d}±0.14	5·83a ±1.28	2.17^{c}±0.20

Control= Chlorine at 200 ppm, SML= sucrose monolaurate

Values are means ± standard deviations of three replicate experiments

Mean values in the same column with the same superscripts are not significantly different ($p \leq 0.05$)

5.3.2 Neural network prediction

The training set obtained from the two vegetables was used to train the network while the validation set was used to measure the error. The network training stops when the error starts to increase for the validation dataset. The mean square error (MSE) and accuracy for each trial was observed and the best was recorded. Tables 5.3 and 5.4 showed the computed values of R^2, and MSE for the developed ANN models considering different network structures, while their predicted validation curves are as shown in Figure 5.4.

Regression analyses function in WEKA tool was used to build a model by combining attributes and generated weight values from the pH, type of antimicrobial, temperature, type of vegetable, and the storage day. The linear regression model generated is given as:

$$Target = 3.3768 * \text{Temperature} + 1.5852 * \text{Initial}_{\text{Load}} + 0.5235 * \text{EncodingforDay} =$$

$$010{,}100{,}110 + 0.6232 * \text{EncodingforDay} = 100{,}110 + 0.3749 * \text{EncodingforDay} =$$

$$110 + 1.2682 * \text{Encoding}_{\text{forVeg}} = 10 + 0.7043 * \text{Encoding}_{\text{for}_{\text{AntiMic}}} =$$

$$00001, 01000, 00100, 00010 + 1.1982$$

The inputs to a node, are simply the products of the output of preceding nodes with their associated weights, this then summed and passed through an activation function before being sent out from the node. Thus, we have the following:

$$K_j = \sum_i w_{i,j}.x_i \quad \text{and} \quad a_j = f(K_i) \qquad (6)$$

For instance*:*

$$\begin{aligned} K1 = {} & (2.4688 * \text{Temperature}) + (0.0741 * \text{pH}) + (-0.7710 * \text{initial Load}) + \\ & (2.8078 * \text{AntimicrobialConcentration}) + (1.3324 * \text{Storage day value}) + \\ & (0.3237 * \text{Code for Veg Type}) + (-2.5281 * \\ & \text{Code for Antimicrobial Type}). \end{aligned}$$

where K_j is the sum of all relevant products of weights and outputs from the previous layer i, $w_{i,j}$ represents the relevant weights connecting layer i with layer j, a_j represents the activations of the nodes in the previous layer i, a_j is the activation of the node at hand, and f is the activation function. ($w_{i,j}$) i represents node in the current layer and j represents node in the next layer.

Table 5.3: Experimental results of the MLP-ANN-based logistic model with varying number of neurons in the hidden layer

Neurons in the hidden layer	2		3		4		5	
	R-Value	MSE	R-Value	MSE	R-Value	MSE	R-Value	MSE
MLP-ANN performance (Logistic)	0.9721	0.2166	0.9752	0.1927	0.9863	0.1073	0.9857	0.1154
	0.9723	0.2152	0.9811	0.1490	0.9834	0.1332	0.9865	0.1059
	0.9732	0.2089	0.9671	0.2554	0.9894	0.0831	0.9647	0.2740
	0.9653	0.2723	0.9850	0.1180	0.9834	0.1270	0.9811	0.1539
	0.9720	0.2169	0.9806	0.1518	0.9767	0.1831	0.9829	0.1340
Average	0.9709	0.2260	0.9778	0.1734	**0.9838**	**0.1267**	0.9802	0.1566

When 2 neurons were activated at the hidden layer (Table 5.3), an average R^2-Value of 0.9709 was realised with MSE range of 0.2-0.3. Similarly, with 5 neurons an average R^2-Value of 0.9802 was realised with MSE range of 0.1-0.3. Generally, the highest R^2-Value of 0.9894 was realised with 4 neurons at the hidden layer with the lowest MSE of 0.0831.

Similar performance trend was repeated with 4 neurons at the hidden layer with hyperbolic tangent function. Table 5.4 show both the R^2-Value and the MSE results for varying hidden neurons from 2 to 5 using hyperbolic tangent.

Table 5.4: Experimental results of the MLP-ANN-based hyperbolic tangent model with varying number of neurons in the hidden layer

Neurons in the hidden layer	2		3		4		5	
	R-Value	MSE	R-Value	MSE	R-Value	MSE	R-Value	MSE
MLP-ANN performance (Hyperbolic)	0.9693	0.2396	0.9724	0.2170	0.9777	0.1750	0.9851	0.1173
	0.9661	0.2625	0.9819	0.1870	0.9798	0.1591	0.9830	0.1330
	0.9712	0.2251	0.9798	0.1576	0.9823	0.1463	0.9583	0.3238
	0.9702	0.2315	0.9803	0.1534	0.9843	0.1231	0.9703	0.2307
	0.9345	0.5017	0.9732	0.2141	0.9846	0.1216	0.9854	0.1145
Average	0.9623	0.2921	0.9763	0.1858	**0.9817**	**0.1450**	0.9764	0.1839

Notably, the average R^2-Value increased progressively with 2, 3 and 4 neurons but declined when the number of hidden neurons was increased to 5.

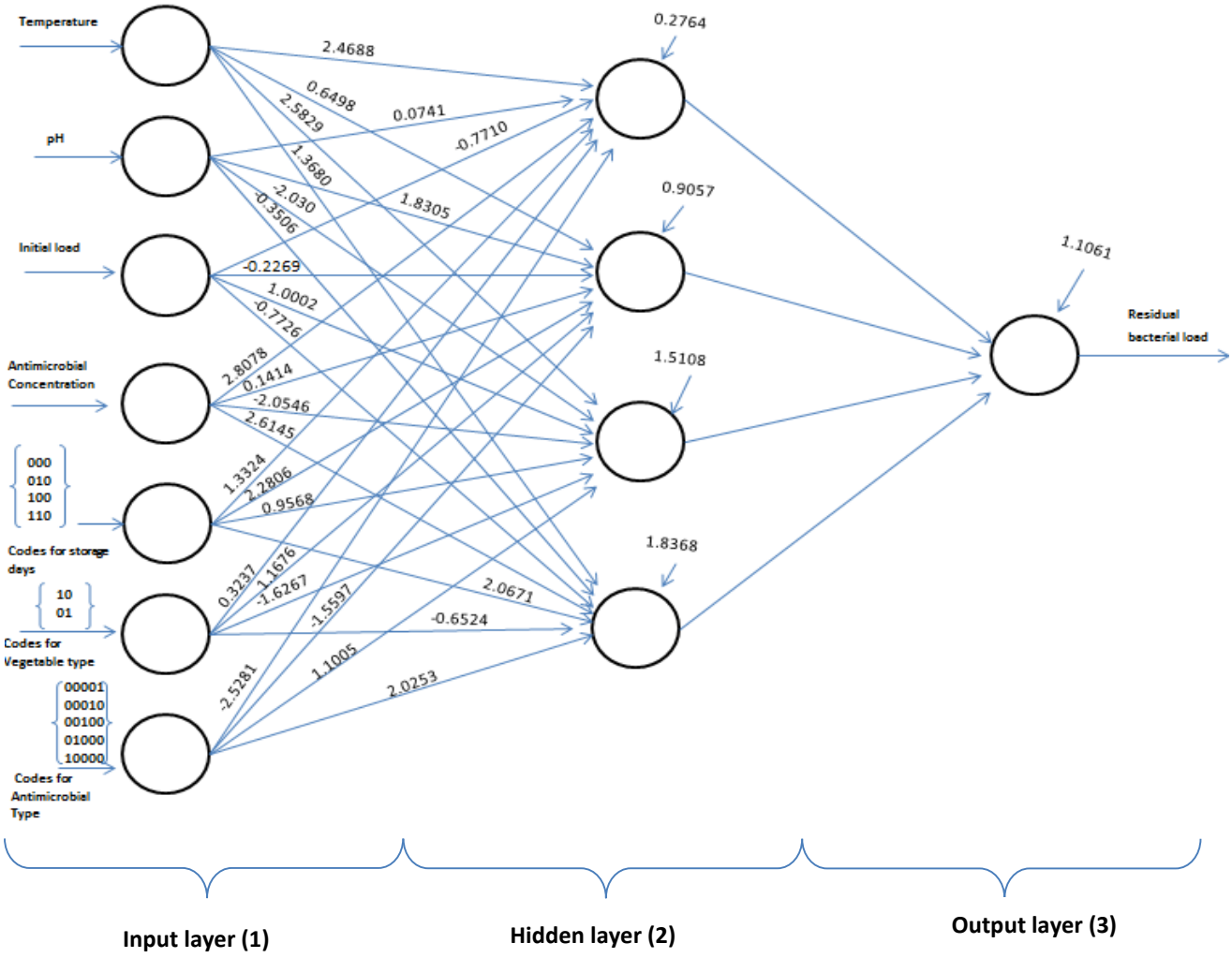
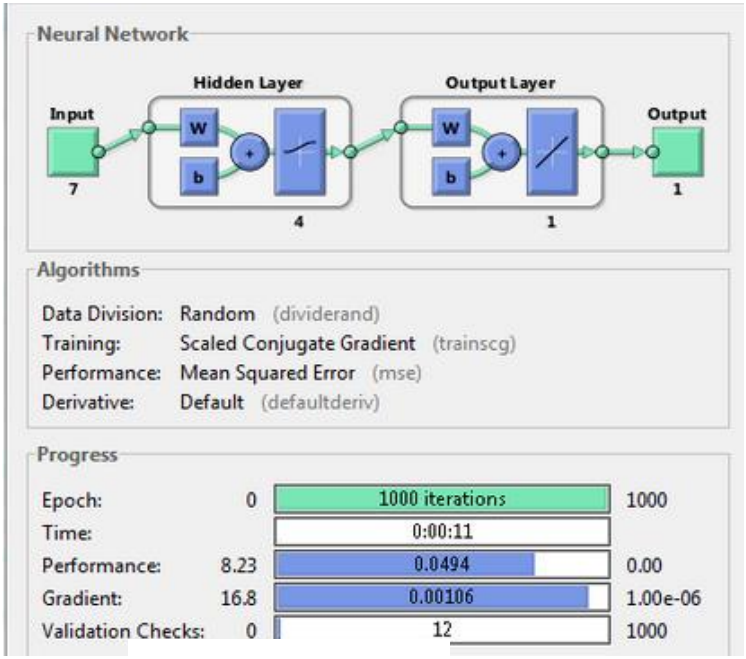


Figure 5.2 Schematic ANN model diagram for prediction of residual *Listeria monocytogenes* bacteria levels on fresh-cut tomato and carrot after antimicrobial control.

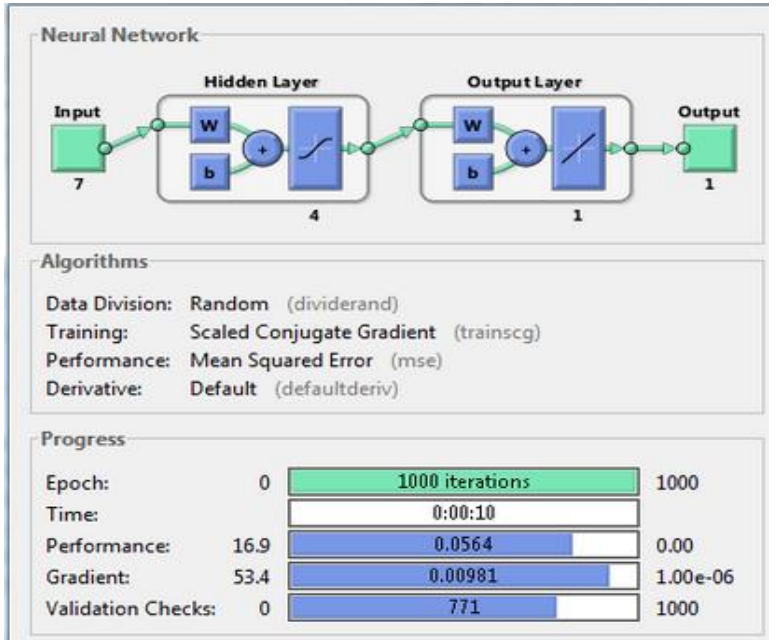
5.3.3 Network Validation

In the linear regression model 10-fold cross validation was used. These results showed that predicted values of the regression models were positive correlated to the observed values (Figure 3). However, there was low positive correlation at the beginning and towards the end of the prediction model. This could be seen from the high MSE value of 0.671 and moderate R-value of 0.90172. This could be seen from the lower MSE value of 0.1216 along with an improved R^2-value of 0.9846 using the same ANN framework and validation, but with logistic activation function at the hidden layer, a different result was obtained. From this result, the regression pattern obtained also showed a highly positive correlation relationship between the predicted and observed values with approximately 0.99 R^2-value with MSE of 0.0831. The average values of the predicted against the observed values for the sigmoid functions were 3.84 ± 0.08 versus 3.82 ± 0.08 $\log_{10}$ cfu mL^{-1}.

In summary, the best model was obtained with four hidden neurons using logistic sigmoid followed by hyperbolic tangent activation functions. The mean square errors obtained from these two functions were 0.0831 and 0.1216 respectively with R^2-Value of 0.9894 and 0.9846.



Logistics



Hyperbolic tangent

Figure 5.3: Representation of neural network showing the training patterns and epoch iteration of logistic and hyperbolic tangent activation functions on MATLAB

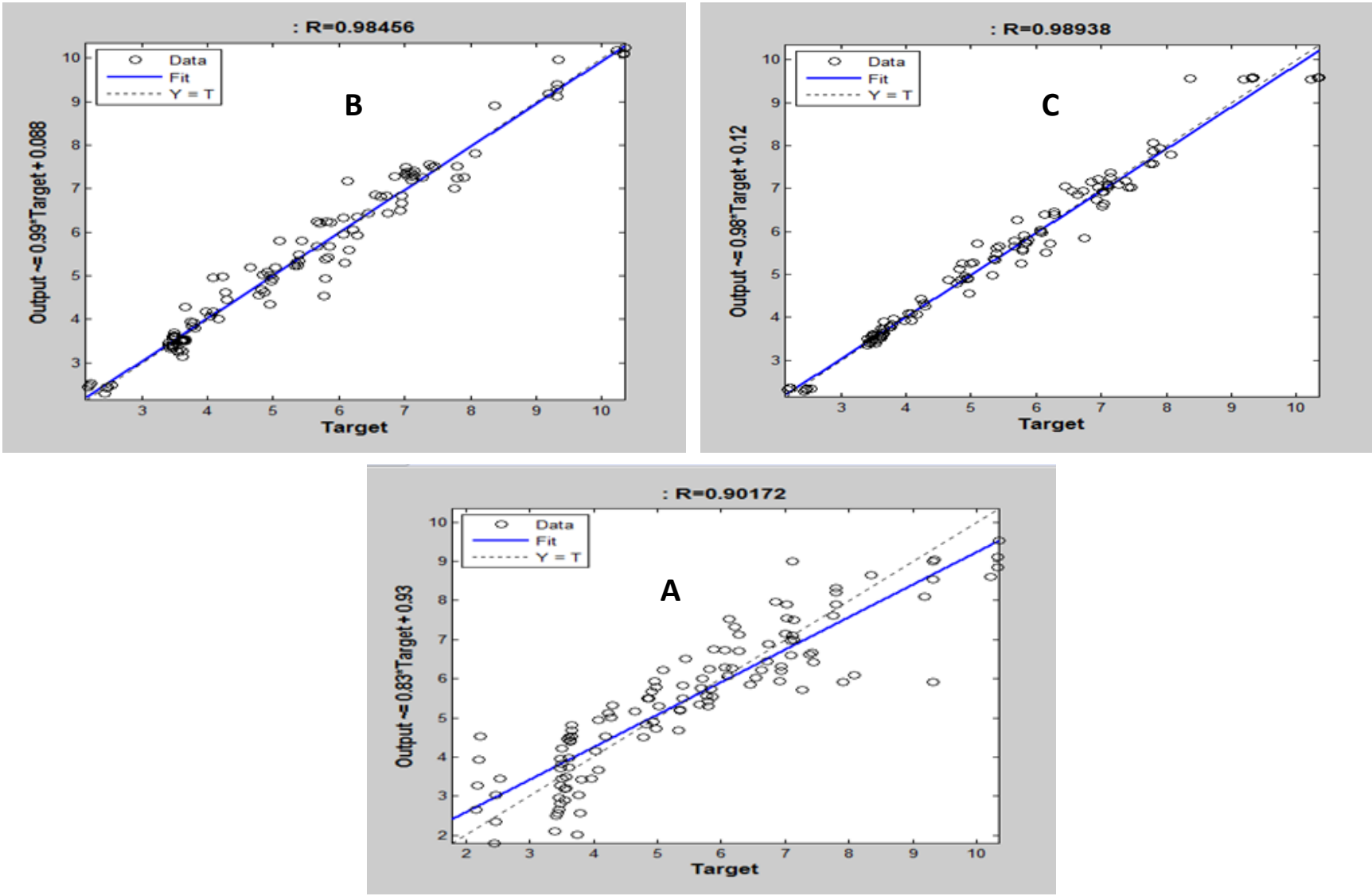


Figure 5.4: Representation of regression curves and validation performance of A: Linear, B: Hyperbolic, and C: Logistic activation models

5.4 Discussion

The use of this lytic phage at same concentration has been reported in raw catfish fillets stored at 4, 10 and 22^{o}C for 10 days, where the level of bacterial inactivation were similar in all the storage temperatures (Hald 2012). This co-evolutionary attributes enhance the possibility of phages in response to emerging mutagenic changes commonly associated with opportunistic bacteria such as *L. monocytogenes* (Lenski 1984; Koskella and Brockhurst 2014). Variation in the output of phage treatment on both fresh produce could be attributed to differences in certain inherent factors such as ionic and pH condition of the two fresh-cut samples. Phage efficacy has been reported to decline with increased acid condition of food matrix (Leverentz *et al.* 2003). From preliminary findings, tomato with a pH value of (4.3±0.1) significantly ($p<0.05$) showed a lower phage-tolerance compared to carrot with pH value of (6.0±0.1). This result is in agreement with previous findings where pH values above 5 resulted in greater inactivation by phage treatment (Oliveira *et al.* 2014; Perera *et al.* 2015). Application of bacteriophage to fresh produce with low pH can be enhanced by increasing the phage concentration bearing in mind its economic feasibility. Also, combination with other natural antimicrobials such as bacteriocin, antagonistic bacteria and essential oils has been suggested (Oliveira *et al.* 2014). Differences in SML activity could be influenced among other factors by its critical micelle concentration (CMC). This seems plausible since the SML concentration (100 ppm) used in this study is below the reported CMC range (184–210 ppm) for SML (Makino, Ogimoto and Koga 1983; Husband *et al.* 1998). Similar findings where SML at 100 ppm could not significantly ($p>0.05$) reduce bacterial population has been reported (Xiao *et al.* (2011). However, unimproved phage lysis with SML at 250 ppm could be linked to certain unexamined factors such as the time-kill assay and the overbearing survival of the phages all through the storage temperature (as more phages multiply to lyse the *L. monocytogenes* cells). Furthermore, it has been reported that sugar fatty acid esters such as SML exhibits significant antimicrobial effect only at high concentration (Marshall 1994). This assertion was confirmed in the combination of phage with SML at 400 ppm, which provides better access for lysis through the peptidoglycan cell wall, resulting in significant ($p<0.05$) reduction to 1.25-1.59 log cfu mL^{-1} folds in fresh-cut tomato and 2.17-4.00 log cfu mL^{-1} in fresh-cut carrot. Similar findings of SML lethality at 400 ppm on the broth of *L. monocytogenes* when combined with other antimicrobials have been reported (Monk, Beuchat and Hathcox 1996).

For the neural network prediction, after full training set with 10-fold cross-validation, the linear model consistently yielded a correlation coefficient of 0.9 and MSE of 0.671.

Unlike the regression model, the ANN approach could not give a prediction equation as stated earlier. However, following the 7-4-1 ANN-MLP architecture used in this work (Figure 5.2), predicted accuracy model was derived during the forward propagation operations of MLP-ANN. Choice of activation function and the number of neurons in the different layers of MLP is very crucial to the performance of the network. Results shown in figures 5.2 and 5.4 guided our choice of the ANN network topology used in this present study, while tables 5.3 and 5.4 shows the R^2-Value and MSE for both Logistic and hyperbolic tangent ANN models respectively. These outputs guided our choice of the 7-4-1 network topology (i.e. input, hidden and output layers contain 7, 4 and 1 neuron, respectively) adopted in this paper as the basis of comparison for the two ANN activation functions. Validation on the neural network was performed by using additional 25 (30%) data sets from both fresh produce (Figure 5.4). The predicted values of ANN models were positively correlated to the observed values compared to that of linear regression.

CHAPTER SIX

6.0 Carbon utilization profile of phage-treated Listeria monocytogenes using phenotypic microarray

Abstract

Carbon utilization profile of phage-treated *Listeria monocytogenes* ATCC 7644 was evaluated. 100 µl of bacteria cells harvested from phage-treated fresh produce (tomato and carrot), applied into inoculating fluid at 95% turbidity was introduced into phenotype micro array (PM1 & PM 2A) plates impregnated with tetrazolium dye containing water sample as control. All plates were incubated at 37^{o}C and color change monitored for 2 days at intervals of 4 h. Result of analysis showed that hexose and pentose sugars-ribose, glucose, fructose and sugars were maximally utilized while oligosaccharide sugars of sucrose, cellobiose and gentiobiose were similarly observed to be utilized. Notably, utilization of glucose-6-phosphate which determines *L. monocytogenes* pathogenicity was not very pronounced in the carbon profile. This result further validates that phage application can be used to control the lethality of listeriosis outbreaks associated with fresh produce consumption.

6.1 Introduction

Food borne listeriosis occasioned by *Listeria monocytogenes* remains a notable disease of public health concern in the food industry. Its associated symptoms in humans include abdominal cramps, diarrhea and vomiting (Camejo *et al.* 2011; Cossart and Lebreton 2014; Leong, Alvarez-Ordóñez and Jordan 2015). Severe cases are characterized by septicemia, encephalitis, meningitis and death among vulnerable individuals such as neonates, pregnant women, elderly and the immunosuppressed (Vázquez-Boland *et al.* 2001; Velge and Roche 2010).

The disease which is caused by consumption of foods contaminated with *L. monocytogenes* has been implicated in a large number of fresh produce (Beuchat 1996b; Hoelzer *et al.* 2014). Report of listeriosis has been quite low, but its mortality rate (20- 30 %) is comparatively high (Rocourt and Bille 1996; Ramaswamy *et al.* 2007). In the USA, about 1600 cases of foodborne listeriosis are reported yearly. Likewise in the EU, it has been rated the third leading cause of death in connection with food consumption (Scallan *et al.* 2011; EFSA 2014).

This pathogen invades and survives in host cells by utilizing certain molecular mechanism of its virulent factors such as internalin A and B, listeriolysin, phospholipases (PlcA and PlcB) to escape host macrophages and endothelial cells (Hamon *et al.* 2012). Furthermore, the pathogen exploits cellular metabolites which serve as potential nutrient medium for growth. Notably, it has been found that *L. monocytogenes* often utilizes carbon-based nutrients to enhance its virulence in host cells (Eylert *et al.* 2008; Bochner 2009; Grubmüller *et al.* 2015). Efforts to address the continuous outbreak of this disease has led to the recommendation of certain control measures (FDA 1998b). One such involves the use of bacteriophage, which has been regarded as natural antimicrobial that co-evolves with their bacterial host. It basically operates by invading host genomic make up via a lytic process (Simões, Simões and Vieira 2010; Spricigo *et al.* 2013). Bacteriophage is highly host specific, does not cause secondary infection to food biota and it can be administered in small amounts due to its high rate of replication in host genome (Guenther *et al.* 2009; Sillankorva, Oliveira and Azeredo 2012; Sulakvelidze 2013). However, from genomic studies, *L. monocytogenes* has been found to utilize pentose and hexose carbon sugar sources from glycolytic pathway as principal nutrient for growth (Glaser *et al.* 2001; Hain, Steinweg and Chakraborty 2006). Further studies on this showed that the pathogen mainly utilizes glucose-6-phosphate in intracellular environment as its carbon source. The uptake of this phosphorylated sugar is mediated

by encoded Hypoparathyroidism (*hpt*) gene under the influence of *PrfA* protein (Chico-Calero *et al.* 2002; de las Heras *et al.* 2011).

Incidentally, this genomic study has been assayed over time and found to be time-consuming. However, a simplified growth assessment of bacteria using phenotype microarrays has been reported (Bochner 2009). The mechanism of this technique operates by measuring the rate of cell metabolism using a redox dye indicator. As the cell undergoes metabolism, the rate of colorimetric reaction of dye also increases. This process is then imaged from the Omnilog instrument which utilizes a 96-well plate (1-20) (Fox and Jordan 2014). The approach thrives on the assertion that bacteria grows and survives in many ecological niches by using basic nutritional elements such as Carbon, Nitrogen, Phosphorus and Sulphur (Mchunu *et al.* 2013).

Therefore, characterizing these nutrients is paramount in understanding certain bacterial physiological processes such as water activity, internal pH, sourcing of basic elements for growth and other cellular functions (Bochner 2009). The application of this method in profiling antibacterial activity of red cabbage has been reported (Bakar, Hafidh and Abdulamir 2012). Similarly, Mchunu *et al.* (2013) reported the application of this method in carbon utilization profile of a thermophilic fungus. However, based on this growth uniqueness of *L. monocytogenes*, the present study is aimed at evaluating the carbon utilization of phage treated *L. monocytogenes* grown on some fresh produce using phenotype micro array.

6.2 Materials and Methods

6.2.1 Bacteria and preparation of inoculum

L. monocytogenes ATCC 7644 serovar 1/2c that had survived phage treatment from fresh produce (tomato and carrot) in fraser broth base (F6672 FB, Sigma-Aldrich Switzerland) containing supplement (F18038, Sigma Aldrich, Switzerland) were used in this study (Merck, South Africa). Bacteria colonies were grown on *Listeria* oxford medium slant kept at 4^{o}C (LOM, Sigma-Aldrich Inc. USA) using Oxford *Listeria* selective supplement (Fluka 75806 Sigma-Aldrich Switzerland) for 24 h at 37^{o}C prior to use (Singh, Mnyandu and Ijabadeniyi 2014).

6.2.2 Biolog Analysis

Carbon profiling of the bacteria was carried out by a modified method reported by Mchunu *et al.* (2013) using Phenotype Microarray (PM) technology (Biolog, Inc., Model ELX808BLG. Hayward CA USA).

Sterile streaking swab (Biolog Inc., USA) was used to transfer bacterial colonies from the agar plate into a sterile glass tube containing 16 ml of inoculation fluid (IF-0a GN/GP Base. Biolog, USA). Turbidity of the suspension was maintained at about 95% using Biolog turbidimeter. Bacteria suspension was later transferred into a sterile reservoir from which 100µL was inoculated into phenotype microarray (PM 1 and PM 2) 96-micro well plates (Figures 6.1 and 6.2). All plates were incubated at 37^{o}C and color change monitored at interval of 4 h using the biolog micro station. Duplicate reading was taken for 48h in both plates.

A1 Negative Control	A2 L-Arabinose	A3 N-Acetyl-D-Glucosamine	A4 D-Saccharic Acid	A5 Succinic Acid	A6 D-Galactose	A7 L-Aspartic Acid	A8 L-Proline	A9 D-Alanine	A10 D-Trehalose	A11 D-Mannose	A12 Dulcitol
B1 D-Serine	B2 D-Sorbitol	B3 Glycerol	B4 L-Fucose	B5 D-Glucuronic Acid	B6 D-Gluconic Acid	B7 D,L-α-Glycerol-Phosphate	B8 D-Xylose	B9 L-Lactic Acid	B10 Formic Acid	B11 D-Mannitol	B12 L-Glutamic Acid
C1 D-Glucose-6-Phosphate	C2 D-Galactonic Acid-γ-Lactone	C3 D,L-Malic Acid	C4 D-Ribose	C5 Tween 20	C6 L-Rhamnose	C7 D-Fructose	C8 Acetic Acid	C9 α-D-Glucose	C10 Maltose	C11 D-Melibiose	C12 Thymidine
D-1 L-Asparagine	D2 D-Aspartic Acid	D3 D-Glucosaminic Acid	D4 1,2-Propanediol	D5 Tween 40	D6 α-Keto-Glutaric Acid	D7 α-Keto-Butyric Acid	D8 α-Methyl-D-Galactoside	D9 α-D-Lactose	D10 Lactulose	D11 Sucrose	D12 Uridine
E1 L-Glutamine	E2 m-Tartaric Acid	E3 D-Glucose-1-Phosphate	E4 D-Fructose-6-Phosphate	E5 Tween 80	E6 α-Hydroxy Glutaric Acid-γ-Lactone	E7 α-Hydroxy Butyric Acid	E8 β-Methyl-D-Glucoside	E9 Adonitol	E10 Maltotriose	E11 2-Deoxy Adenosine	E12 Adenosine
F1 Glycyl-L-Aspartic Acid	F2 Citric Acid	F3 m-Inositol	F4 D-Threonine	F5 Fumaric Acid	F6 Bromo Succinic Acid	F7 Propionic Acid	F8 Mucic Acid	F9 Glycolic Acid	F10 Glyoxylic Acid	F11 D-Cellobiose	F12 Inosine
G1 Glycyl-L-Glutamic Acid	G2 Tricarballylic Acid	G3 L-Serine	G4 L-Threonine	G5 L-Alanine	G6 L-Alanyl-Glycine	G7 Acetoacetic Acid	G8 N-Acetyl-β-D-Mannosamine	G9 Mono Methyl Succinate	G10 Methyl Pyruvate	G11 D-Malic Acid	G12 L-Malic Acid
H1 Glycyl-L-Proline	H2 p-Hydroxy Phenyl Acetic Acid	H3 m-Hydroxy Phenyl Acetic Acid	H4 Tyramine	H5 D-Psicose	H6 L-Lyxose	H7 Glucuronamide	H8 Pyruvic Acid	H9 L-Galactonic Acid-γ-Lactone	H10 D-Galacturonic Acid	H11 Phenylethyl-amine	H12 2-Aminoethanol

Figure 6.1 Phenotypic micro-well (PM1) Plates of 95 Carbon Sources from Biolog Inc

A1 Negative Control	A2 Chondroitin Sulfate C	A3 α-Cyclodextrin	A4 β-Cyclodextrin	A5 γ-Cyclodextrin	A6 Dextrin	A7 Gelatin	A8 Glycogen	A9 Inulin	A10 Laminarin	A11 Mannan	A12 Pectin
B1 N-Acetyl-D-Galactosamine	B2 N-Acetyl-Neuraminic Acid	B3 β-D-Allose	B4 Amygdalin	B5 D-Arabinose	B6 D-Arabitol	B7 L-Arabitol	B8 Arbutin	B9 2-Deoxy-D-Ribose	B10 i-Erythritol	B11 D-Fucose	B12 3-0-β-D-Galacto-pyranosyl-D-Arabinose
C1 Gentiobiose	C2 L-Glucose	C3 Lactitol	C4 D-Melezitose	C5 Maltitol	C6 a-Methyl-D-Glucoside	C7 β-Methyl-D-Galactoside	C8 3-Methyl Glucose	C9 β-Methyl-D-Glucuronic Acid	C10 α-Methyl-D-Mannoside	C11 β-Methyl-D-Xyloside	C12 Palatinose
D1 D-Raffinose	D2 Salicin	D3 Sedoheptulosan	D4 L-Sorbose	D5 Stachyose	D6 D-Tagatose	D7 Turanose	D8 Xylitol	D9 N-Acetyl-D-Glucosaminitol	D10 γ-Amino Butyric Acid	D11 δ-Amino Valeric Acid	D12 Butyric Acid
E1 Capric Acid	E2 Caproic Acid	E3 Citraconic Acid	E4 Citramalic Acid	E5 D-Glucosamine	E6 2-Hydroxy Benzoic Acid	E7 4-Hydroxy Benzoic Acid	E8 β-Hydroxy Butyric Acid	E9 γ-Hydroxy Butyric Acid	E10 a-Keto-Valeric Acid	E11 Itaconic Acid	E12 5-Keto-D-Gluconic Acid
F1 D-Lactic Acid Methyl Ester	F2 Malonic Acid	F3 Melibionic Acid	F4 Oxalic Acid	F5 Oxalomalic Acid	F6 Quinic Acid	F7 D-Ribono-1,4-Lactone	F8 Sebacic Acid	F9 Sorbic Acid	F10 Succinamic Acid	F11 D-Tartaric Acid	F12 L-Tartaric Acid
G1 Acetamide	G2 L-Alaninamide	G3 N-Acetyl-L-Glutamic Acid	G4 L-Arginine	G5 Glycine	G6 L-Histidine	G7 L-Homoserine	G8 Hydroxy-L-Proline	G9 L-Isoleucine	G10 L-Leucine	G11 L-Lysine	G12 L-Methionine
H1 L-Ornithine	H2 L-Phenylalanine	H3 L-Pyroglutamic Acid	H4 L-Valine	H5 D,L-Carnitine	H6 Sec-Butylamine	H7 D.L-Octopamine	H8 Putrescine	H9 Dihydroxy Acetone	H10 2,3-Butanediol	H11 2,3-Butanone	H12 3-Hydroxy 2-Butanone

Figure 6.2 Phenotypic micro-well (PM 2A) Plates of 95 Carbon Sources from Biolog Inc

6.3 Results

6.3.1 Effect of phage treatment on carbon utilization profile of *Listeria monocytogenes*

The effect of phage treatment on the carbon utilization (oligosaccharide, pentose and hexose sugars) profile, of *L. monocytogenes* harvested from fresh-cut produce was evaluated using the hierarchical clusters analysis. From the analysis, assimilation of xylose, L-arabinose, glucose, fructose, sorbose, trehalose, palantinose and water (control) with the largest clusters was generally lower than other carbon sources while carbon sources of maltose, maltotriose, lactulose, tagatose, stachyose, raffinose and ribose were quite assimilated (Figure 6.3). However, more carbon sources were comparatively utilized by the bacteria for growth as evident in Figure 6.4. Few exceptions to this utilization were cellobiose, sucrose and to some extent lactulose and maltotriose. Further analysis of these 95 carbon sources in their respective groupings of pentose- hexose sugars, and oligosaccharides showed that ribose, glucose and fructose sugars with absorbance values of 0.86, 0.78 and 0.71 respectively were maximally assimilated, while fructose and glucose sugars with absorbance values of 0.64 and 0.52 maximally utilized respectively. Oligosaccharide sugars of sucrose, cellobiose and gentiobiose were similarly observed to be maximally assimilated and utilized (Figure 6.5-6.8).

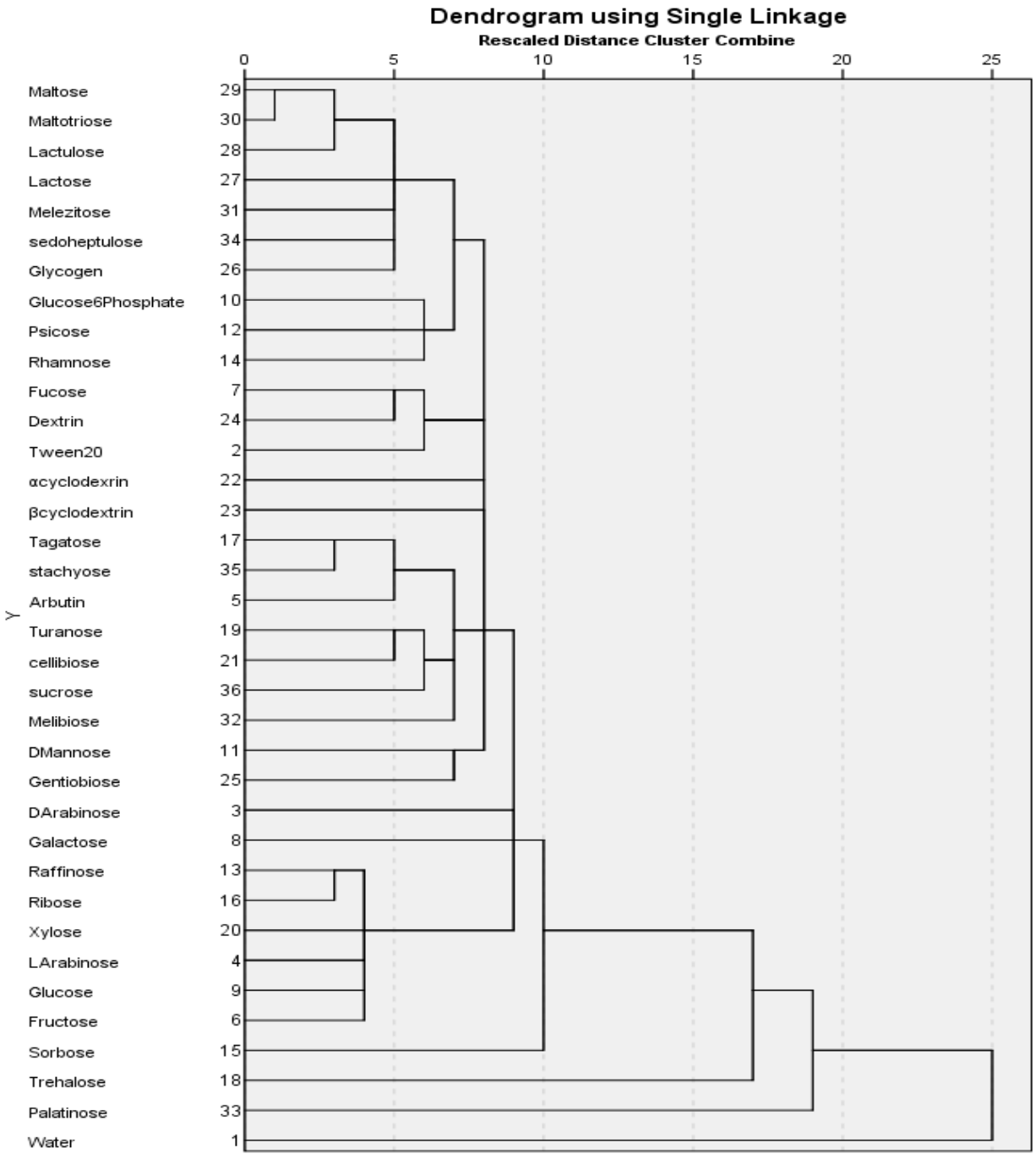


Figure 6.3 Joint clusters analysis applied to 95 carbon sources of phage-treated *Listeria monocytogenes* at 590 nm using Biolog system (Average standard deviation= 0.128).

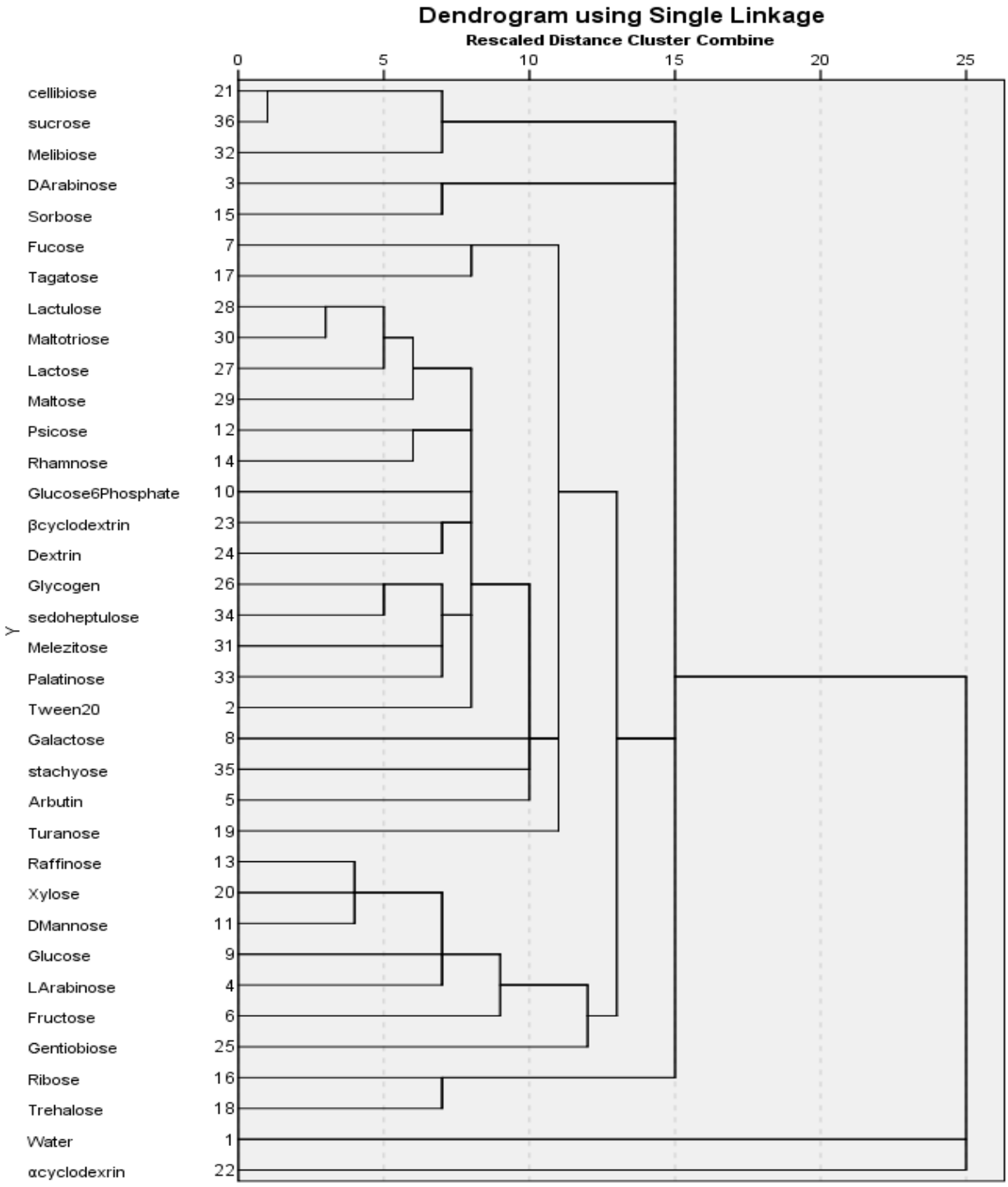


Figure 6.4 Joint clusters analysis applied to 95 carbon sources of phage-treated *Listeria monocytogenes* at 750 nm using Biolog system. (Average standard deviation = 0.095)

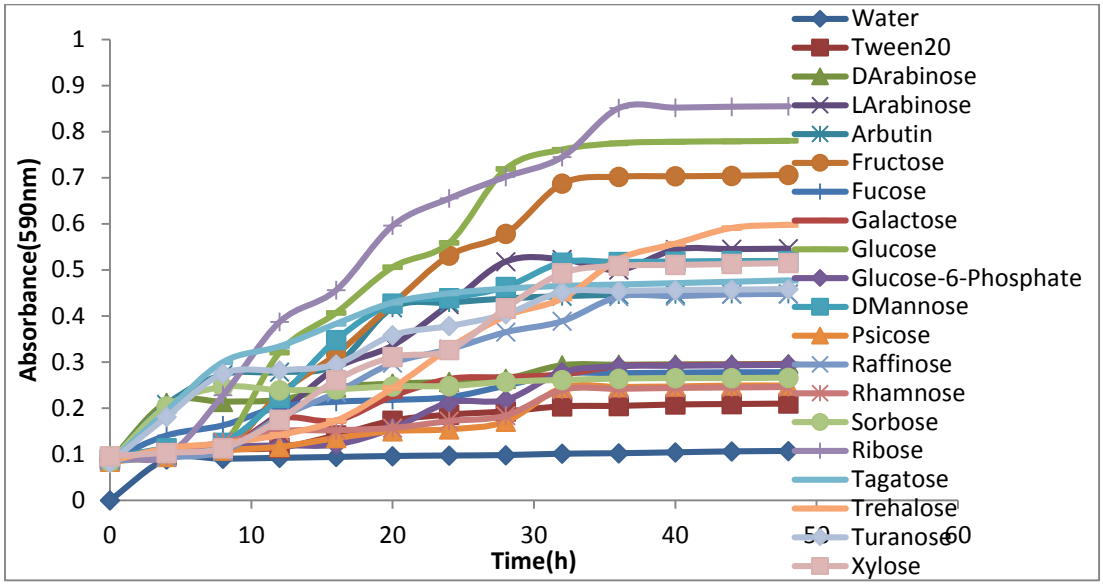


Figure 6.5 Assimilation of monomeric sugars (hexose and pentose) by *L. monocytogenes* at 490 nm absorbance

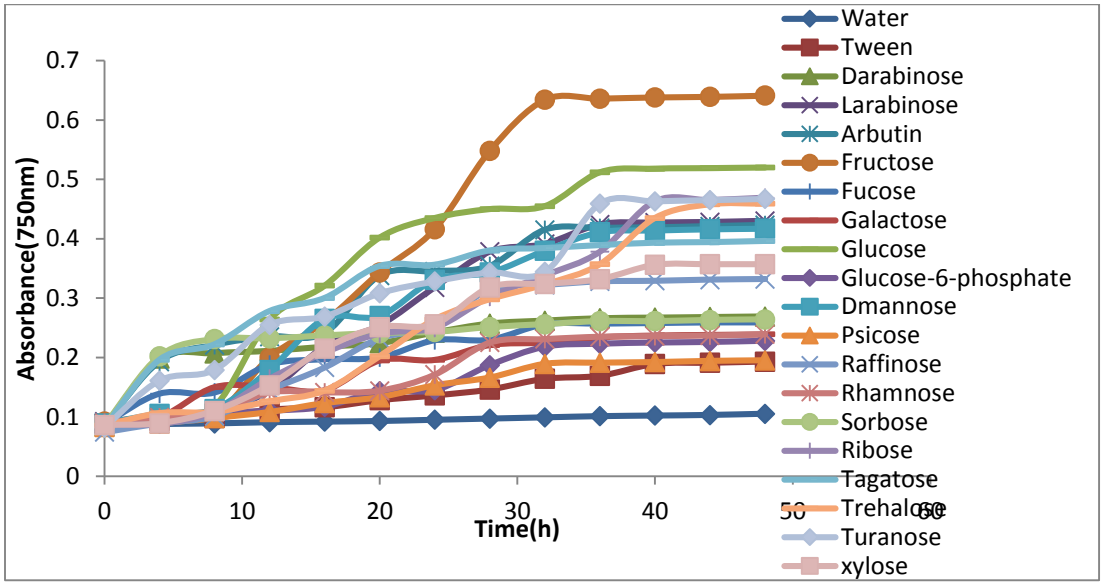


Figure 6.6 Utilization of monomeric sugars by *L. monocytogenes* at 750 nm absorbance

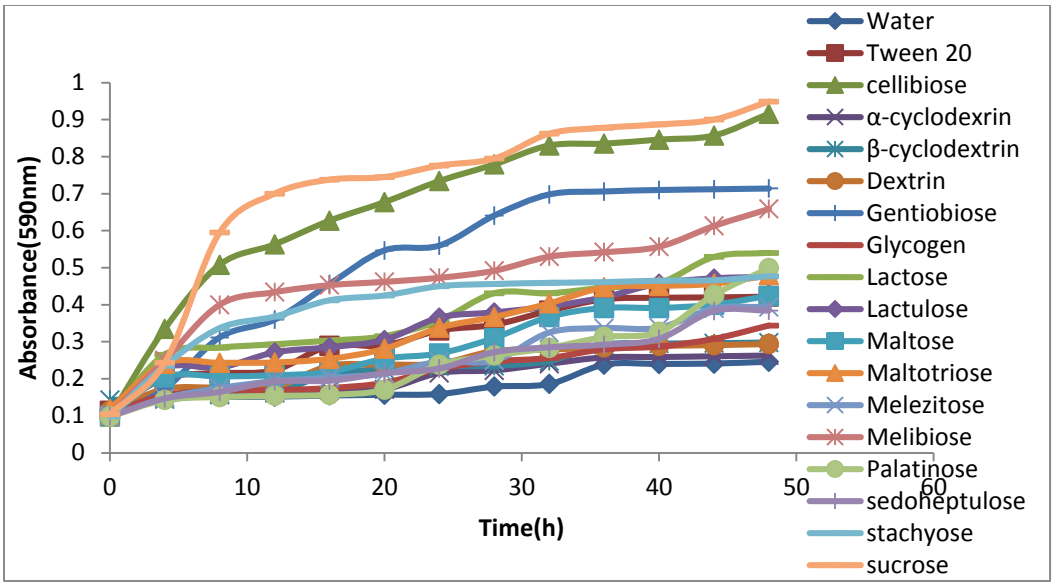


Figure 6.7 Assimilation of oligosaccharide sugars by *L.monocytogenes* at 490 nm absorbance

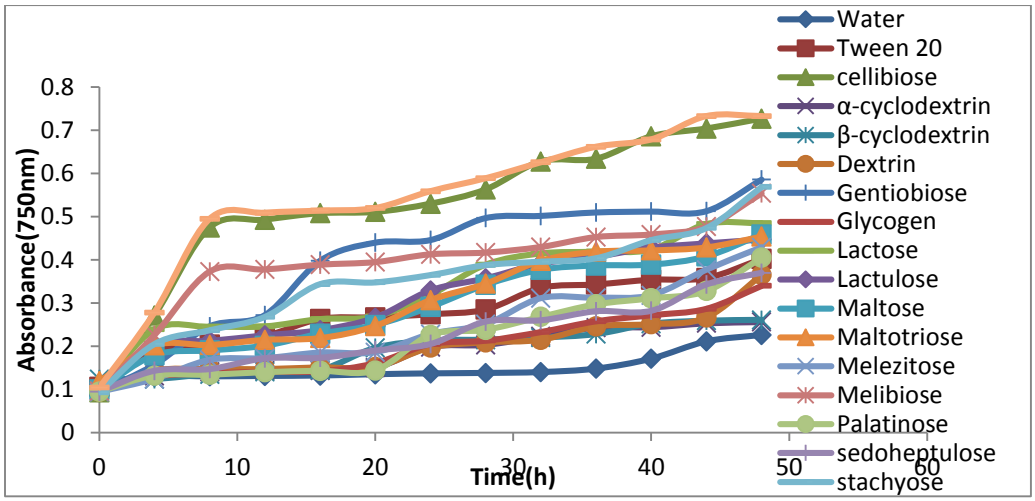


Figure 6.8 Utilization of oligosaccharide sugars by *L. monocytogenes* at 750 nm absorbance

6.4 Discussion

Microorganisms generally develop different modes of action to survive in their natural environment and when found in a supposedly harmful one. *L. monocytogenes* could adapt and proliferate itself in a host cell (often in vacuoles and cytosols) by evading defense mechanism of the cell`s macrophages (Dussurget, Pizarro-Cerda and Cossart 2004; Lecuit 2005; Hamon, Bierne and Cossart 2006). This is usually made possible by its listeriolysin and two phospholipases (PlcA and PlcB) virulent factors (Kathariou 2002). However, the survival and growth rate of this pathogen is linked to availability and optimal utilization of cellular metabolites consisting of basic nutrients such as carbon and nitrogen from the host cells (Bochner 2009).

In the present study, utilization of glucose, fructose, sucrose, cellobiose and gentiobiose sugars were observed. This result correlates to an appreciable extent in a similar investigation by Joseph and Goebel (2007). The author reported that certain sugars such as glucose, fructose, and cellobiose are the preferred carbon sources utilized by *L. monocytogenes* through a phosphotransferase (PTS) system. This could suggest the presence of specific hydrolyzing enzymes in the bacteria virulent factors helping to evade some phage virions.

From genomic analysis, it has been found that *L. monocytogenes* has a complete glycolytic and pentose-phosphate sequential pathway which is used as carbon substrates for survival and growth in intracellular medium (Glaser *et al.* 2001). Further studies revealed that the pathogen's *hpt* gene which plays an active role during intracellular survival, transports and utilizes a good number of simple and complex sugars which includes fructose, rhamnulose, rhamnose, glucose, mannose, chitin, sucrose, cellulose, pullulan, trehalose and tagatose (Nelson *et al.* 2004). Authors reported that these sugars have been identified in most niches that harbor this pathogen.

Interestingly, glucose-6-phosphate which has been fundamentally acknowledged as the main carbon source for *L. monocytogenes* virulence in intracellular cells was not found among the well utilized sugars in the present study. This development could be linked to lytic effect of phage in the genomic array of the bacteria. Bacteriophage has been found to contain peptidoglycan degrading enzymes called endolysin with specific enzymatic active domains (EAD) and cell wall-binding domain (CBD) (Loessner 2005; Borysowski, Weber-Dąbrowska and Górski 2006; Fischetti 2010). Uptake of this phosphorylated sugar by *L. monocytogenes* which has been found to be mediated by the *hpt* gene induced by PrfA protein could have been affected by the endolysin

during the process of lysis. Therefore, the pathogenicity of *L. monocytogenes* contamination can be addressed when appropriate amount of phages are present. Since oral toxicity of phages in mammals has not been reported, phage therapy can be administered to curtail the prevalence and high death rates associated with listeriosis outbreaks.

CHAPTER SEVEN

7.0 General discussion

This chapter gives a general narrative on the essence of this research. The fresh produce industry has been experiencing notable and attendant growth in recent times as the demand for a healthy lifestyle through ready-to-eat foods increases. Conversely, this growth also conveys inherent safety challenges through the outbreak of diseases by pathogenic contaminations. *L. monocytogenes* which possesses the ability to survive harsh conditions has been identified as one of such contaminants. The objective, therefore, is to control this pathogen using an environment-friendly agent called bacteriophage or phage and to predict the risk associated with the efficacy of this approach on selected fresh produce using artificial neural networks. The first part discusses the effects of bacteriophage control on artificially inoculated fresh produce. The second part discusses the use of an artificial intelligence tool to predict the efficacy of phage treatment on the fresh produce.

7.1 Bacteriophage control of *L. monocytogenes* in fresh-cut produce

Fresh-cut produce represents a good proliferating medium for microbial growth as more nutrients are leached out, hence providing easy access for microbial growth and replication (Conte *et al.* 2009; Muriel-Galet *et al.* 2013). Occurrence of *L. monocytogenes* contamination in fresh or minimally processed produce has been well documented (Sant'Ana *et al.* 2012; Botticella *et al.* 2013). However, the application of bacteriophage to inactivate *L. monocytogenes* growth in fresh produce has reported different significant levels of reductions (Leverentz *et al.* 2003; Oliveira *et al.* 2014; Hong *et al.* 2015). The pathogen exhibits certain adaptive approaches to cope with most environmental or stress conditions to facilitate survival (Botticella *et al.* 2013; Müller-Herbst *et al.* 2014). However, regardless of these stress adaptations, phage lysis has proven to be effective. This development lends credibility to phage biocontrol of pathogens of health concern such as *L. monocytogenes* in the fresh produce industry regardless of certain adaptation to post-harvest condition or treatment. This lytic interaction, as suggested, could be mediated by the ability of phage to co-habit with mutagenic changes in bacterial cells since they are both natural commensals and co-evolve under same ecological conditions (Sulakvelidze 2013; Strydom and Witthuhn

2015). This development offers the advantage of its application in the fresh produce industry as post-harvest intervention measure over other known natural antimicrobial compounds. Meanwhile, differences in nature (ionic and pH values) of the fresh produce primarily affects phage lysis against bacterial pathogens. Phage treatment on carrot with higher pH values (6.0±0.1) compared to tomato (4.3±0.1) validates this assertion. This development however could limit the efficacy of phage treatment on fresh produce with low pH values such as tomatoes, apples and picked vegetables. Similar output was reported on different fresh produce where variations in level of reduction was linked to differences in pH values (Guenther *et al.* 2009; Oliveira *et al.* 2014). This phage-host interaction in fresh produce can be improved by using a higher concentration of phage, or combined with other antimicrobials. Leverentz *et al.* (2003) and Hong *et al.* (2015) reported higher log reductions of *L. monocytogenes* of 5.7 and 6 log CFU/ml on fresh-cut melon when bacteriophage was combined with nisin and G. asaii respectively. Leverentz *et al.* (2004), further reported that a cocktail of six phages resulted in a higher reduction of *L. monocytogenes* population on honey dew melon, when applied at higher dosage. Furthermore, phage-based treatments can be made into aerosols which can be sprayed either manually or mechanically for effective distribution to cause lysis where the challenge of uneven distribution (phage immobilization) especially on solid food matrix can be addressed. From market point of view, phage-based coatings/packaging has been suggested to enhance safety of fresh produce during storage and distribution since it will not affect the organoleptic quality of the food in question. In the present study, the novel combination of bacteriophage with sucrose monolaurate at 400 ppm provided higher log reductions of *L. monocytogenes* with 1 and 3 log cycle on fresh-cut tomato and carrot respectively. This result explains the need for combination treatment as earlier stated by some researchers as a way of enhancing phage treatment on these bacterial pathogens. Higher micelle concentration of SML together with phage cocktail will further provide greater and improved log reduction to meet required regulatory and industrial standards that could prevent listeriosis during contamination. From molecular study, survival of *L. monocytogenes* in intracellular niches has been found to be linked with expression of certain virulent factors (such as listeriolysin, PlcA and PlcB) in the cytosols of host cells (Hamon *et al.* 2012). Evasion of host phagocytes is dependent on these factors while its growth and subsequent proliferation is based on its ability to resist host macrophages and phagosomal cells which is aided by effective utilization of certain nutrients of host cellular metabolites (Grubmüller *et al.* 2015). Among these nutrients,

glucose-6-phosphate has been identified as the major carbon source nutrient responsible for growth and survival (pathogenicity) of *L. monocytogenes* (Joseph and Goebel 2007; Eylert *et al.* 2008). From the present study, assimilation and utilization of this phosphorylated sugar at both absorbance wavelengths was low suggesting the influence of phage endolysin on this bacterial cell. The result further shows the therapeutic effect of bacteriophage application in reducing virulence of *L. monocytogenes* and as such, phage can be harnessed industrially and administered orally as a tablet or intravenously as a liquid injection in treating infected patients who perhaps consumed contaminated foods from a public health point of view.

7.2 Effect of prediction after phage and sucrose monolaurate control using artificial neural networks

Artificial neural networks (ANNs) are data-driven intelligence tools used in predictive microbiology for risk assessment. It basically simulates the activity of the human brain comprising input, hidden and output layers (Ross and McMeekin 2003; Huang, Kangas and Rasco 2007). In the present study, back propagation BP- ANN was used to predict the relationship between the initial bacterial load of *L. monocytogenes*, type of fresh-produce, antimicrobial concentration and residual bacteria. Mathematical models were developed using activation functions comprising linear regression and sigmoid (hyperbolic and logistic) activation functions. Data sets (120) were trained using Back propagation ANN containing one hidden layer with four hidden neurons. Data set were excellently trained showing 100 % accuracy. Prediction with logistic activation function showed the highest positive correlation relationship between predicted and observed values with ~ 0.99 R^2-value and MSE of 0.083. Similar work reported by Keeratipibul, Phewpan and Lursinsap (2011) supported this assertion where BP-ANN was used to predict inactivation of *E. coli* and coliforms on tomato and lettuce using two different sanitizers. Authors reported sum of square errors of 0.50 and 0.80 for coliforms and *E. coli* respectively, R2 values of 0.85 and 0.72 were obtained as well for both coliforms and *E.coli* respectively.

CHAPTER EIGHT

8.0 General conclusions and recommendations

Fresh produce are essential components of the human diet which offers health and nutritional importance to human consumption. Safety risk due to microbial contamination along the food value chain from pathogens such as *L. monocytogenes* can be addressed using eco-friendly antimicrobial agent of bacteriophage.

Regardless of environmental adaptive mechanism that this pathogen could assume, bacteriophage remains an excellent tool for its inactivation. It should however, be known that combination of phage with other antimicrobial will offer improved inactivation of the pathogen especially in fresh produce with high acid content.

Also, application of phages higher concentration or cocktails will also suffice as its oral toxicity at higher dosage has not been reported. Phages can also be produced and commercialized in the form of aerosols for effective diffusion and even distribution during application. This will reduce the challenge of immobilization especially on fresh produce with uneven topology and morphological structure. Also, incorporation of phage into coatings and packaging materials could further aid the safety and quality of fresh produce during storage and transportation. Combination with surface active compounds such as sucrose monolaurate at concentration above its critical micelles concentration can help reduce some draw backs that could be associated with bacteriophage biocontrol. Furthermore, the use of artificial neural network as an upcoming predictive tool in the food industry remains a viable approach in evaluating safety risk associated with control of pathogenic contaminants commonly found in fresh produce.

Due to advancement of research in biotechnology and nanotechnology, research into the consolidation of phage-based biosensors and Nano sensors incorporated with feasible artificial neural network software package should be investigated. This will go a long way to enhance rapid and on-site detection, control and risk prediction of pathogens of public health importance in fresh produce industry. However, the results obtained from this work are valid in the present conditions of fresh produce type, antimicrobial concentration, and inoculum size of bacterial pathogen. Further application of this model as a risk assessment tool to other pathogens of health concern in fresh produce safety should be encouraged. Hence, optimal experimentally conditions should be

determined in order to obtain required bacterial pathogen reduction that meets regulatory requirement in the fresh produce industry.

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APPENDIX

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Food Microbiology

Inactivation of *Listeria monocytogenes* ATCC 7644 on fresh-cut tomato using nisin in combinations with organic salts

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ABSTRACT

The inhibition of *Listeria monocytogenes* ATCC 7644 on fresh-cut tomato was investigated using nisin alone, and in combinations with organic salts. Nisin at a concentration of 5000 UI/mL was introduced alone or in combination with an organic salt (sodium citrate or sodium acetate each at 3 and 5 g/100 mL each) on fresh-cut tomato previously inoculated with 10^8 CFU/mL of *L. monocytogenes* ATCC 7644. Chlorine at 200 ppm was used as a control. The inoculated samples were incubated at different temperatures (4, 10 and 25 °C) and examined at 0, 24, 48 and 72 h. The effects of the antimicrobial treatments on quality parameters of tomato (pH, soluble solids, titratable acidity and vitamin C) were also evaluated, and colour parameters were observed at the lowest storage temperature for 10 days. Both nisin and the organic salts inhibited growth of *L. monocytogenes*, but the combinations of two compounds were more effective. The nisin–sodium citrate (5%) combination was significantly ($p \leq 0.05$) effective, while chlorine was least effective against *L. monocytogenes*. The quality parameters were substantially retained, especially at 4 °C, suggesting good shelf stability at a low temperature. These results substantiate the use of the cheap and eco-friendly approach to reducing this pathogen of health concern in common fresh produce.

Introduction

Fresh produce is an essential component of human diet. It contains micro-nutrients, vitamins and certain phyto-chemicals which contribute significantly to human diet.[1] Growing demands for exotic fresh produce in recent years have attracted laudable attention due to its health-promoting nature.[2] It has been strongly advocated by relevant global agencies, that consumption of approximately 400 g of fresh produce per day, which equals to "five servings a day" as a recommended daily intake has a prophylactic capacity to stem

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Food Science and Biotechnology

Biocontrol of *Listeria monocytogenes* ATCC 7644 on fresh-cut tomato (*Lycopersicon esculentum*) using nisin combined with organic acids

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Abstract The biocontrol of *Listeria monocytogenes* on fresh-cut tomato using nisin and organic acids was investigated. Fresh-cut samples inoculated with 10^8 CFU/mL of *L. monocytogenes*, treated with nisin (5,000 IU/mL), a combination of nisin and organic acids (acetic and citric acids at 3% and 5% each), with chlorine at 200 ppm as a control, and stored for six days at 4, 10, and 25°C were used to evaluate certain physicochemical qualities (pH, titratable acidity, soluble solid content, vitamin C content, and color). Nisin treatment significantly ($p<0.05$) reduced bacterial population by 1.91-3.07 log CFU/mL. Nisin-citric acid combination provided 2.65-3.29 log CFU/mL reduction, while nisin-acetic acid combination provided 2.93-4.15 log CFU/mL reduction. The control treatment provided <1-2 fold log reductions. Slight variations in physicochemical properties of fresh-cut tomato were observed. Nisin and organic acids can be used to improve the microbial safety of fresh-cut tomato.

Keywords: *Listeria monocytogenes*, fresh-cut tomato, nisin, organic acids

Introduction

Recently, fresh produce consumption has continued to attract consumers' interest globally because of its perceived health and nutritional benefits (1). Fresh and minimally processed tomato is an important vegetable of interest in the fresh produce industry because of its extensive utilization and composition of health-promoting constituents (2). Meanwhile, because of certain pre-harvest and post-harvest factors in the food chain, this type of fresh produce has become susceptible to microbial contaminations (3). Consequently, an increasing number of cases of diseases and product recalls due to bacterial pathogens such as *Listeria monocytogenes* has been reported (4).

The abovementioned issue has prompted the United States Food and Drug Administration and United States Department of Agriculture Food Safety Inspection Service to enact a "zero tolerance" policy on this pathogen in ready-to-eat foods. *Listeria monocytogenes*, a gram positive, psychrotrophic microorganism, has been reported as an etiologic foodborne pathogen. It has the ability to survive under a wide range of environmental conditions such as temperatures of 4-37°C and high osmotic conditions and in the presence of common sanitizers (5). This recalcitrant pathogen has also been reported as a contaminant in a broad range of fresh and minimally processed produce in the USA and other developed countries with severe clinical cases and deaths (4,6).

As a control measure, the use of chlorine as a sanitizer has been conventionally adopted to reduce microbial contamination on fresh produce prior to onward processing (7,8). However, its continuous usage has been discouraged in recent times because of toxicity concerns on the nutritional make-up of the food and health of a processor (9). This has elicited the demand for the use of less hazardous and eco-friendly intervention measures such as the use of nisin and organic acids.

Nisin is a bacteriocin that contains cationic peptides produced by some species of lactic acid bacteria, mostly *Lactococcus lactis* subsp. *lactis*. It is known to exhibit a bactericidal effect on Gram-positive bacteria (10). Nisin demonstrates this effect by creating pore channels within bacterial cell membrane via a charged potential difference between the peptide and membrane, thus disrupting the fusion of peptidoglycan cell walls (11).

This low-mass peptide is a biological and natural preservative substance that has attained a generally regarded as safe status by USA Food Drug and Administration, thus enhancing its utilization in the food industry (12). It is also commercially available, and its successful application stems from its broad range of bactericidal action against Gram-positive bacteria (13). On the other hand, organic acids exhibit an antimicrobial effect primarily by reducing the pH of the cellular environment. They also cause a disruption of

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Prediction of *Listeria monocytogenes* ATCC 7644 growth on fresh-cut produce treated with bacteriophage and sucrose monolaurate by using artificial neural network

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ABSTRACT

Combination of bacteriophage and sucrose monolaurate (SML) against Listeria monocytogenes growth on fresh-cut produce and prediction of relationship among initial bacterial load, fresh-produce type, antimicrobial concentration and residual bacteria using Artificial Neural Networks (ANNs) was investigated. Inoculated samples (tomato and carrot) containing 10^8 log cfu mL^{-1} *L. monocytogenes*, treated with bacteriophage (10^8 pfu mL^{-1}), SML (100, 250 and 400 ppm) and chlorine control (200 ppm) were stored at 4, 10 and 25 °C for 6 days. Mathematical models were developed using a linear regression and sigmoid (hyperbolic and logistic) activation functions. Data sets (120) were trained using Back propagation ANN containing one hidden layer with four hidden neurons. Phage treatment on tomato and carrot showed ($p < 0.05$) < 1 and 2 fold bacterial reductions respectively. Addition of SML at 100 and 250 ppm was ($p > 0.05$) ineffective, but showed significantly ($p < 0.05$) higher log reductions on both fresh produce at 400 ppm. Control treatment resulted in 1–2 log reductions on both fresh produce. Prediction with logistic activation function showed the highest positive correlation relationship between predicted and observed values with ~0.99 R^2-value and MSE of 0.0831. ANN offered better prediction in phage biocontrol of pathogens in fresh produce.

1. Introduction

The demand for fresh or minimally-processed produce has notably increased in the last few decades (Abadias, Usall, Anguera, Solsona, & Viñas, 2008). Ready-to-eat (RTE) fruits and vegetables contribute to a healthy lifestyle in a more convenient way, as they contain nutritional and health-stabilising constituents (James, Ngarmsak, & Rolle, 2011). As part of advocacy by relevant global organisations, consumption of "five servings per day" of fresh produce has been advised as part of measures to reduce possible incidence of certain ailments such as cancer, diabetes, and some cardiovascular disorder (Allende, McEvoy, Luo, Artes, & Wang, 2006; FSA 2006; WHO, 2003; Warriner, Huber, Namvar, Fan, & Dunfield, 2009).

Meanwhile, safety challenges caused by pathogenic contamination of fresh produce along the food chain, resulting in disease outbreaks have been well documented (Beuchat, 2002; Olaimat & Holley, 2012). *Listeria monocytogenes* which causes listeriosis has been identified as one of such prominent bacterial contaminants in fresh produce such as tomato and carrot (Ajayeoba, Atanda, Obadina, Bankole, & Adelowo, 2015). It has a high mortality rate (20–30%) and possess the ability to survive wide-range of environmental and stress conditions (Farber & Peterkin, 1991; Gandhi & Chikindas, 2007). Effort to control this pathogen in fresh produce has led to the use of certain antimicrobial agent such as bacteriophage (Meireles, Guimaris, & Simões, 2016; Olaimat & Holley, 2012). Bacteriophages or phages are viruses that invade and kill bacteria via a lytic cycle (Simões, Simões, & Vieira, 2010). They are regarded as natural anti-microbial with an approved "GRAS" (General Recognized as Safe) status (Sulakvelidze, 2013). They co-exist with bacteria in their natural habitat over time, they are host-specific, possess effective mode of action, and they do not cause any deleterious impact on food microbiota (Bueno, García, Martínez, & Rodríguez, 2012; Guenther & Loessner, 2011; Sprigiņo, Bardina, Cortés, & Llagostera, 2013). These inherent attributes make them an excellent tool for the control of recalcitrant pathogen such as

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